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Estudio biológico y técnico de sistemas aeróbicos granulares para tratar aguas residuales urbanas: Efecto de la temperatura

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Biological and technical study of aerobic granular systems for treating urban wastewaters: Effect of temperature

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Programa de Doctorado en Biología Fundamental y de Sistemas

Estudio biológico y técnico de sistemas aeróbicos granulares para tratar aguas residuales urbanas: Efecto de la temperatura

Memoria Presentada por la Lda.

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Summary

This Ph.D. thesis is based on the start-up and operation of aerobic granular sludge (AGS) bioreactors for treating urban wastewater to contribute the expansion of acknowledge about the most effective way to cultivate and operate granular sludge under different thermal conditions using activated sludge as inoculums of wastewater treatment plants located on Polar Arctic Circle and of Mediterranean region.

In this Ph.D. thesis had been described the challenges achieved in terms of stable granulation using diverse inoculum and at different temperatures in order to determine the best experimental approach for achieving stable granulation in different latitudes over the world from Polar regions (7°C) until tropical regions (28°C) at full-scale wastewater treatment plant (WWTP).

Three bioreactors were operated in a sequential batch mode feeding with synthetic medium simulating high-organic loading urban wastewater, which were operated with hydraulic retention time of 6.0 to 7.5 h. The temperatures studied were 8°C simulating northern latitudes, 15°C simulating mild temperature environments, and 26°C simulating warmer places such as tropical habitats. The activated sludges were taken from the WWTP located in Rovaniemi (Lapland, Finland) and in Granada (Andalusia, Spain). The analyses were classified as: physico-chemical performance and microbial ecology dynamics.

The start-up of the first experimentation, it was based on the using inoculum from Granada (Spain), Rovaniemi (Finland) and a mixed of both, for adapting and growing granules to mild temperature in order to understand that regardless of inoculum, the pressure of microorganisms selection was caused by the operational temperature.

Therefore, microbial ecology promote under the same conditions were similar and were not affected by the origin temperature of activated sludge.

The second experiment was carry out with activated sludge from Los Vados WWTP located in Granada (Spain). This research was done with the aim of understanding the faster mechanisms to start-up of aerobic granular sludge in regions as Granada with great seasonal and daily changes of temperature. The results showed that the faster granulation was achieved in reactor operated at native temperature, following by warm temperature, while that bioreactor operated at low temperature needed more time to get a mature granulation. The prokaryotic population was totally related to temperature, but the eukaryotic community was dominated by ciliated and fungal organisms regardless temperature. In conclusion, it is possible get stable granules with activated sludge not operational temperature adapted, but the shorter periods of start-up and higher performance removal make safer inoculated with seed operational temperature-adapted.

The third experiment was performing in bioreactors inoculated with activated sludge of WWTP located in Rovaniemi, in the Polar Arctic Circle of Finland subjected and operated to low, mild and warm temperature. It was observed that cold-adapted inoculum improve and make faster the start-up stage of AGS while that bioreactors operated at mild and warm temperature need more time to get a mature granule. The microbial community both prokaryotic and eukaryotic was drastically affected by the temperature.

Resumen

Esta tesis de doctorado se basó en la puesta en marcha y operación de biorreactores aeróbicos de fango granular para el tratamiento de aguas residuales urbanas con el objetivo de contribuir a la expansión del conocimiento sobre la forma más efectiva de cultivar y operar la tecnología de fango granular en diferentes condiciones térmicas utilizando como inóculo fango activo de plantas de tratamiento de aguas residuales ubicadas en el Círculo Polar Ártico y en la región mediterránea.

En esta tesis se describieron los desafíos alcanzados en términos de granulación estable utilizando diversos inóculos y a diferentes temperaturas para determinar el mejor enfoque experimental para lograr la granulación estable en diferentes latitudes del mundo, desde regiones polares (7 ° C) hasta regiones tropicales (28 ° C) en estaciones depuradoras a escala real.

Tres biorreactores fueron operados en reactores secuenciales discontinuos que se alimentaban con medios sintéticos que simulaban aguas residuales urbanas de alta carga orgánica, los cuales operaban con un tiempo de retención hidráulico de 7,5 a 6 h. Las temperaturas estudiadas fueron 8° C simulando latitudes del norte, 15° C simulando ambientes de temperatura templada y 26 ° C simulando lugares más cálidos como los hábitats tropicales. El fango activo fue tomado de una estación depuradora ubicada en Rovaniemi (Laponia, Finlandia) y otra en Granada (Andalucía, España). Los análisis se clasificaron en: rendimiento físico-químico y dinámica de la ecología microbiana.

La primera experimentación se basó en el uso de inóculo de una estación depuradora de Granada (España), Rovaniemi (Finlandia) y una combinación de ambos fangos activos, para adaptar y cultivar gránulos a temperatura media con el fin de comprender que

independientemente del inóculo, la selección microbiana fue causada por las condiciones operacionales de temperatura. En conclusión, la población microbiana que conformó los gránulos bajo las mismas condiciones operacionales fue similar y no se vio afectada por la temperatura de origen del fango activo.

El segundo experimento se llevó a cabo con fango activo de la planta de tratamiento de agua residual de Los Vados ubicada en Granada (España). Esta investigación se realizó con el objetivo de comprender los mecanismos más rápidos para la puesta en marcha de la tecnología aeróbica de fango granular en regiones como Granada con grandes cambios diarios y estacionales de temperatura. Los resultados mostraron que la granulación más rápida se consiguió en el reactor operado a temperatura nativa, seguido del operado a temperatura cálida, mientras que el biorreactor operado a baja temperatura necesitó más tiempo para obtener una granulación madura. La población procariota estuvo totalmente relacionada con la temperatura, sin embargo, la comunidad eucariota fue dominada por hongos y organismos ciliados y no le afectó la temperatura de operación. En conclusión, es posible obtener gránulos estables con fango activo que no esté adaptado a la temperatura operacional, pero los períodos más cortos de puesta en marcha y un mayor rendimiento de eliminación hace que sea más seguro inocular con fango activo adaptado a la temperatura operacional.

El tercer experimento se realizó en tres biorreactores, los cuales fueron inoculados con fango activo de la planta de tratamiento de aguas residuales ubicada en Rovaniemi, en el Círculo Polar Ártico de Finlandia, y fueron operados a baja, media y alta temperatura. Se observó que el inóculo adaptado al frío aceleró la etapa de puesta en marcha del

fango granular aeróbico, mientras que los reactores operados a temperatura media y alta necesitan más tiempo para obtener un gránulo maduro. La comunidad microbiana, tanto procariota como eucariota, se vio drásticamente afectada por la temperatura.

1. Introduction

The environmental problems derived from nuclei population and industrial procedures are one of the most contaminant human activities. Encompassed in these activities, the discharge of urban and industrial wastewater to natural water bodies causing negatives impacts on the ecosystems. The wastewater is defined as "used water from any combination of domestic, industrial, commercial or agricultural activities, surface runoff or storm water, and any sewer inflow or sewer infiltration" (Tilley et al., 2014). The initial problem was the depletion of dissolved oxygen in aquatic environments, followed by eutrophication issue caused by the industrialization and the population growth, as well as spreading of pathogens and toxic compounds. The goal of wastewater treatment is to remove harmful pollutants that cause damages in the water bodies receivers. Thus, the wastewater treatment through physical, biological and/or chemical processes minimizes the impact of wastewater discharges in natural habitats.

Biological processes within wastewater treatment plant (WWTP) are based on the natural biogeochemical cycles of carbon, nitrogen and phosphorous carry out by *Bacteria*, *Archaea*, *Fungi* or *Protozoa,* between others. Engineering design simulates intensified natural conditions to accelerate processes of bioconversion in terms of temperature, aeration, nutrients, pH, both electron donor and acceptor among other parameters. Thus, the aim of engineering bioprocesses is to stimulate, promote and select the microorganisms that carry out the bioconversion, optimizing the environmental conditions and operational parameters.

The most widely microorganisms studied had been *Bacteria* domain, but nowadays it is known that both virus, archaeal, fungal, algal and protozoan communities are essential and vital in the performance of biological processes in WWTPs.

The importance in the design of the bioreactor lies in promoting the optimum microbial growth due to the physic environment had a strong effect on the ecology of microbial dynamics. Thus, two big categories could be classified in biological process depending on where microorganisms grow: in suspended mixed-liquor, attached itself or on a carrier. The most widely technology implemented at full-scale around the world is a based on suspended biomass denominated conventional active sludge (CAS), which consist of the path of pollutants through aerobic/anaerobic/anoxic chamber conditions. This technology is efficient but the necessities of space for the construction and the economic cost for the exploitation are high, also it is not high effective for removing emerging and priority pollutants (Attiogbe, 2013). On the other hand, the increased of multiple sources of pollution from urban wastewater and industrial waste and wastewater has done that remove pollute be a priority for public authorities and the society (Crini et al., 2019).

The legislation is being stricter in Europe since the environmental problems can affect the human health and the normal development of natural ecosystems, as sensible water bodies. Thus, the European Water Framework Directive had implemented values of discharge more restrictive. Consequently, researches had put many efforts to development biological treatment technologies for achieving the established values in the most effective environmental way and saving-cost. Therefore, a technology based

on attached biomass was development without necessity of support material, such as plastic carriers, which is based on spontaneous formation of granular sludge under aerobic conditions with the capacity to biodegrade and bioadsorb wide spectrum of chemicals and pollutants. (Wan et al., 2015)

Aerobic granular sludge (AGS) is a promising technology that was design with the aim to treat different wastewater effluents such as organic matter, nutrients, or priority pollutants, among others. Moreover, it is a compact system with high biomass retention capacity, this has entailed improved scientific assessment such us, a lower operational cost because the aerobic-anaerobic-anoxic process can be done in a single bioreactor, and a smaller bioreactor surface than CAS (Gonzalez-Martinez et al., 2017).

1.1. Aerobic granular sludge technology

AGS was considered to be a special biofilm composing of self-immobilized cells and it was originally reported in anaerobic wastewater treatment (Moregenroth et al., 1997). However, during the last 20-year deep researches on biological wastewater treatment demonstrated that biofilm technologies are more efficient that suspended activated sludge. Few years ago, the research on AGS has got relevance due to the compact design of technology and the remove of organic matter and nutrients in the same chamber.

AGS system had a compact design due to excellent settleability of granular sludge and high biomass retention capacity with low sludge volume index. Also, AGS technology had special characteristics such as: dense and compact granular conformation with thick microbial structure, resistance to withstand high organic loading and toxic compounds,

high biomass concentration, nutrient recovery and short hydraulic retention time in comparison with CAS. All these properties facilitate solid-liquid separation, which allowed the granule cultivation without secondary clarifier.

Thus, AGS is a novel promising technology for biological wastewater treatment, becoming an advanced choice to implement in municipal and industrial wastewater treatment plant. The innovative technology requires 25-50% less of surface than CAS, as well 23-40% less electricity costs, and 20-25% less in cost of operation (Bengtsson et al., 2018; Sarma et al., 2017) besides other advantages shown in Table 1. Thus, it is a technology environmental-friendly with smaller footprint.

Table 1. Comparison AGS versus CAS.

Parameters	**Aerobic granular sludge**	**Activated sludge**
Settling velocity	10–130 m/h	2–10 m/h
Size	0.2–6 mm	<0.2 mm
Compactness and density	High	Low
Shape	Regular and spherical	Irregular and filamentous
Redox conditions	Aerobic, anoxic and anaerobic	Aerobic
Tolerability toxic compounds	High	Low
EPS production	High	Low

Gonzalez-Martinez et al., 2018; Bengtsson et al., 2018a; Nancharaiah and Reddy, 2018; Adav et al., 2008.

Granulation process is a complex mechanism that is involved in successive steps (1) initial cell-to-cell attachment, (2) conformation of microaggregates of cells, (2) production of extracellular polymeric substances (EPS) by microorganisms, and lastly (4) granular conformation and maturation by hydrodynamic shear force in the reactor generating the tridimensional matrix. This process occurs mainly due to the negative charge of cell surfaces and the hydrophobicity. In first place, cell-to cell attraction seems to be involved by the Van der Waals force and proton translocation, followed by the subsequent dehydration of cell membrane, which is another mechanism to initial granular conformation. This mechanism neutralizes the negative charges causing dehydration and make the surface hydrophobic, then with this fact begin cell to cell adhesion (Sarma et al., 2017). Also, an essential mechanisms involved in the granulation is quorum sensing (QS), that encourage diverse activities such as extracellular polymeric substances segregation (Zhang and Li,2016), so higher QS activity seems to promote the microbial attachment (Lv et al., 2014). In terms of operation, few parameters are of interest to the development of stable granules such as high hydrodynamic shear force, ratio diameter and height, sequential batch cycles, feeding strategy, feast-famine periods and wash-out of selective microorganisms during the start-up, between other. These parameters allow the granular production with high stability and density, although the main factor associated to density is the hydraulic shear force (Liu et al., 2005).

The large aggregates in a tridimensional matrix, that conform the granule, allow the presence of oxygen and nutrient gradient in several layers that consequently make possible several metabolic pathways to obtain energy. Thus, it is possible to find

different niches in the granules related to the position of microorganisms. The general model described that heterotrophic and nitrifiers are in the external outer layers, the phosphate-accumulating organisms (PAO) and glycogen-accumulating organisms (GAO) are located in the interlayers and the denitrifiers are found in the core of the granule (Figure 1). Thus, the main advantage of this system is that the nutrients removal can be done in a single chamber bioreactor, under the same oxygen operation conditions, at the same time.

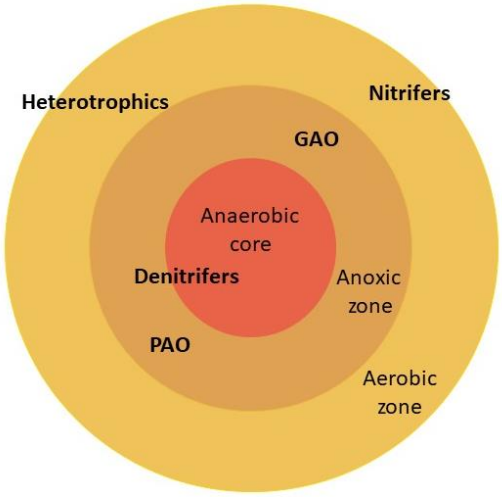

Figure 1. Distribution of microorganisms and nitrogen removal metabolic pathway in a single aerobic granular sludge.

Temperature is a key factor for the development and activity of microbial metabolisms, as well as in the selection of communities that habit in natural ecosystems. Also, temperature affects bioprocesses which involved microbial ecology for their functioning such as wastewater treatment. Of vital importance is take account the parameters that affect to mixed culture microbial ecology in the design from engineering point of view for estimating the bioprocess. Temperature changes affect to dynamics populations and

the metabolic activity. The seasonal variations had been studied to correlated environmental factor with microbial community and their activities in natural environments like lakes, soils or oceans (Barberán et al., 2012; Pajdak-Stos and Fialkowska, 2012; Venter et al., 2004). Thus, seasonal differences in performance such as nutrients removal had been reported in WWTPs (Ju et al., 2014), despite to be an artificially controlled environment, the impact of operational variables disturb significant to ecology. Cold temperature decreases the water availability, molecular motion and energetic, produce an increase of solute concentration and consequently, it provokes a dramatically affection to the microbial growth (Gonzalez-Martinez et al., 2018a). However, the adaptation of populations at cold temperature allows the microbial metabolisms and activity even at subzero temperature (Panikov and Sizova, 2007). Actually, numerous researches had detected microbial activity in permafrost and similar frozen niches with temperatures ranging 0 to -20ºC (Panikov et al., 2006). The bioconversion processes in biological treatment are usually modified by the temperature. In this way, low temperature could drive to inhibition of the microbial activity (Gonzalez-Martinez et al., 2017) and higher temperature could promote the detriment of important groups of bacteria as heterotrophic or phosphate-accumulating bacteria (Ebrahimi et al., 2010).

However, microbes growing in both warm and cold habitats had development mechanisms to persist under desiccation, very different light conditions, low abundance the nutrients under low temperature, or at high temperature such as survive with low oxygen concentration, reduction of viscosity of the water or the increasing the its ionization (Brock, 2012). Therefore, microorganisms which had development

mechanisms of adaptation at cold or warm temperature had all enzymatic mechanisms to get the energy and carbon source.

Hence, the microbial populations that conform biological wastewater treatment are the vital importance to achieve high performance in terms of organic matter and nutrients removal. So, it has been observed that WWTPs located in Nordic countries obtain high performance regardless ultra-low temperature (Gonzalez-Martinez et al., 2018b). Even, the nitrification process was reported with strong and negative correlation with the temperature (Gnida et al., 2016). However, the latest studies had reported as WWTPs at low temperature had been success in terms of performance and stability (Gonzalez-Martinez et al., 2018b).

1.2. Process configuration and operational conditions

A. Bioreactor configuration and sequential batch reactor operation

The design of reactor is of vital importance for the cultivation of granules due to the formation requires specific characteristics in the parameters, for example almost all AGS reactors are operated in cylindrical columns under sequential batch cycles with fine bubbles at the bottom as aeration. The sequential batch reactor (SBR) makes cycles that consist on aeration time, settling period, effluent discard and filling. The aeration period in SBR operation involves two stages: consumption and degradation of substrates of the water, followed by famine period in starvation phase in which no substrate is available (Adav et al., 2008). Nevertheless, in the recent years is being reported the continuous

flow for aerobic granular systems with flat geometry composed by two mixed chamber in series and a settler to promote the granular formation (Cofré et al., 2018).

In other way, the cylindrical-column design with high height diameter ratio allows that microbial aggregates are in contact continuously involved in a circular movement, which promote regular and smoother granules with minimum surface free energy (Liu et al., 2005).

One of the parameters of design more important for the right granulation process is related with the volume exchange ratio and the height/diameter ratio in the reactor. The volume exchange ratio of SBR could be management as selection pressure to get denser granules. Wang et al. (2016) demonstrated that the settle ability of granular sludge cultivated with exchange ratio of 80% is three times superior to those found at ratio of 20%.

The exchange ratio is described as the percent of volume withdrawn at the effluent discard period. For cylindrical-column bioreactor, the exchange is correlated with the height the discharging port to the water surface (Liu et al., 2005). A few research studied the affection of exchange ratio in the development of granules, observing that usually the biomass concentration was related with the volume exchange ratio. The results showed that exchange ratio between 60-80% produced exclusively granular sludge, while those smaller ratios of 40-20% presented both suspended and granular biomass. Thus, these conclusions suggest that the exchange ratio exert a pressure over the granular stability and conformation. So, this study showed that discharge time is an important criterion to selected faster floc-forming bacteria (Liu et al., 2005).

On the other hand, the ratio height/diameter (H/D) is an important design factor such as some authors reported (Kong et al., 2009; Liu and Tay, 2002). So, it was observed that higher H/D ratio could afford a longer flowing trajectory, and consequently an improvement selection pressure that promote the microbial aggregation. The ratio H/D of bioreactors described in literature ranging from 1.9 to 25, however many studies established well-controlled reactor with an H/D ratio over 10. Although, the ratio H/D values in a lab-scale is not viable at full-scale, but very different H/D ratio had been built at full-scale with ratio of 8 until below 1 (Li et al., 2014). Due to the decreased of height the shear force has to be kept for that it is necessary to increase the aeration, resulting in an increase of operational costs. Definitely, high H/D ratio increases the settle ability, although it is very flexible in the practice. Moreover, higher H/D improves the circular flow, increasing the oxygen transfer and attraction for microbial aggregates. Furthermore, reactor with higher H/D need shorter time to start up and it usually more actives that reactors with low H/D (Kong et al., 2009; Awang et al., 2016).

B. Hydrodynamic shear force

The hydrodynamic shear stress had received attention for the optimum development of granular sludge in SBR. Usually, the hydrodynamic shear force had been quantified by upflow superficial air velocity described as air flow divided by cross sectional area of aerated zone (Devlin et al., 2017). Mostly, the AGS reactor are design as airlift column where the hydrodynamic shear force is created by fine bubbles from the bottom until the top. Some studies reveal that high rate of aeration accelerate the granulation

process generating more compact and stable granules (Wilén et al., 2018; Devlin et al., 2017). In general terms, the results suggest that superficial air velocities less than 0.8 - 1.6 cm s^{-1} did not generate granules or produce unstable granules (Tay et al., 2001; Chen et al., 2007). On the contrary, the most stable and denser granules were observed at higher applied air velocity faster than 3.2 cm s^{-1} (Chen et al., 2007). However, recent studies point out that air velocity ranging 2.1 to 2.8 cm s^{-1} generate stable and denser granules too (Lochmatter and Holliger, 2014; Lochmatter et al., 2013). Finally, some studies suggest that higher hydrodynamic shear stress promote the production of EPS which favor the adhesion cell to cell.

C. Cycle and settling time

The cycle time is represented by the reaction time that comprises all stage of cycle; those stages are filling, aeration, settling and effluent discard. The cycle is directly correlated with the hydraulic retention time (HRT). Mostly, AGS technology is operated with a total cycle ranging 3 to 12 hours. Longer cycles affect though the hydrolysis of biomass causing an adverse effect in microbial aggregation, while shorter cycles could produce wash-out of biomass due to breakage of granules caused by insufficient time to metabolize compounds and consequently any change cells surface charge.

The settling time is a key factor to select the granules-forming microorganisms, especially during the set-up period. The granular sludge with short settle time (<7 min) generate denser granules and a faster granular aggregation, while that longer settling time (>20 min) create fluffy granules with poor settle abilities. The strategy of gradual decrease of time on settling period during the start-up is being widely used for

granulation, which usually is based on from 10 min to 2 min (Gonzalez-Martinez et al., 2018a, b; De Kreuk et al., 2005).

D. Feeding strategy and feed composition

The feeding strategy is an essential design parameter for the operation of AGS technology. Mostly the feeding is intermittent related sequential batch operation, although recently, there are many efforts to operate under continuous flow (Cofré et al., 2018; Adav et al., 2008).

The feeding in sequential batch reactors consist of two phase: the degradation phase when the substrate is depleted, and after that, there is not substrate. This change in substrate concentration during aeration period is called feast-famine period. The famine period promotes the hydrophobicity on superficial cells charge accelerating the aggregation and producing granules more compact and denser. Also, the famine period favor the storage of organic matter, proliferating slow-microorganisms such nitrifying bacteria (Pronk et al., 2015)

E. Inoculum

Frequently, AGS is inoculated with activated sludge from conventional biological treatments. The ecological composition of the inoculum using to seed the reactor had a crucial role in the development of granules. The origin of inoculum with specific operational parameters affect as key factor for selecting the faster microorganisms and

play an important role in the cultivation of aerobic granular sludge. The temperature-adapted of activated sludge generated larger difference in the start-up and different performance during the operational period (He et al., 2015; Mezzanotte et al., 2005), although this study pointed out that regardless of the inoculum used the microorganisms that conform the community of the granules at the end of operation were similar and were not affected by the origin on inoculum. So, the characterization of granules is given by the initial microbial ecology presented in the inoculum despite later it proliferates microorganisms well-adapted to new operational conditions. Nevertheless, the activated sludge adapted to the operational temperature of to bioreactors achieve faster startup period than an inoculum not adapted to operational temperature as reported Gonzalez-Martinez et al. (2017). The activated sludge operated at lab-scale is usually fed with synthetic wastewater using acetate, glucose, ethanol, methanol or phenol as carbon source, so the characteristics of populations of inoculums are similar, while that communities in the granules mature are given by the carbon source. However, the activated sludge proceed of different industrial WWTPs can possess completely different microbial communities, for instance the sludge proceed from beer WWTPs had a high hydrophobicity, due to those the granulation process was very fast with excellent settle ability (Song et al., 2010). On the other hand, the methods to inoculated a full-scale variate in comparison with pilot/lab scale due to takes longer time, thus some authors using as inoculum pre-cultivated granules as a promoter plus activated sludge, with the goal to reduce the start-up time for application in real-scale system (De Sousa Rollemberg et al., 2018).

F. Temperature

Temperature is a key parameter for the microbial metabolisms and therefore for the optimal performance of biological process. AGS is a robust technology able to treat high organic loading and toxic compounds and these systems had demonstrated their robustness operated in a wide range of temperature between 5°C to 30 °C. Although, some authors described that unstable cultivation of granular sludge at 8°C due to growth of filamentous microorganisms (De Kreuk et al., 2005) later some authors as Gonzalez Martinez et al. (2017, 2018a) demonstrated the success to growth granules at even lower temperature below 8°C with high performance in terms of removal of organic matter, nitrogen and phosphorous. Also, Gonzalez- Martinez et al. (2018a) demonstrated that granular sludge system is well-working even at lower temperature 7 to 5°C, although at 3°C the granules were instable showing outgrowth of filamentous microorganisms. On the other hand, it was found higher efficiency during the operation of AGS reactor at higher temperatures (20-30°C) in terms of organic matter and nitrogen (Lopez-Vazquez et al., 2009).

G. Dissolved oxygen

The dissolved oxygen (DO) is one of the most important features that affect to the main advantage of AGS technology to remove nutrient in a single reactor under aerobic conditions. The dissolved oxygen concentration affects both nitrification and denitrification processes, but the mass transfer limitation allows that exist different niches from the external layer to core of the granule. It has been reported that DO

variation can affect the substrate transfer as well as the structure and stability of granules (Yuan et al., 2010). In this sense, AGS systems had been operated positively at low DO concentration ranging to 2-6 mg O_2 L^{-1} (McSwain et al., 2008) and even at high DO until 11 mg L^{-1}. Mosquera et al. (2005) suggest that at 40% of oxygen saturation, 25°C is the optimum value to achieve the better nitrogen removal ratio, due to denitrification process could be carry out, while that Gonzalez-Martinez et al. (2018a, b) operated AGS reactors close to oxygen saturation with denitrification performances very satisfactory.

1.3. Analytical methods to characterize granular sludge

1.3.1. Physical and chemical characterization

Granular morphology

Granules show a spherical or elliptical shape, with regular and soft surface. It has been demonstrated that the morphology of AGS is directly correlated with the environmental conditions and operational parameters such as: inoculum, temperature, wastewater, feeding or organic loading among others. The size plays a crucial role for the characterization of granular biomass and in the performance ratio. With the increase of mean size of the granule, the stratification begins to form due to mass transfer. The size of the granules had been differentiated in three categories: small size (0.15 to 0.28mm), medium size (0.28 to 0.45 mm), big size (>0.45mm) (Li et al., 2019). Although average values smaller than 0.4 mm are consider granules, the diffusion limitation of oxygen and substrate could not occur, and thus the denitrification process is not successful (Gao et

al., 2011). In addition, Toh et al. (2003) reported that granules larger than 4 mm could deteriorate the density and settling ability, on the contrary, recent studies showed that even granular size higher than 10 mm are stable and kept the granular density in long-term operation (Muñoz-Palazon et al., 2019). Despite the fact the smaller granules allow major specific surface to remove pollutants, some studies with larger granules reached higher performance too. Actually, it had been linked the organic matter loading with the granular size, because operating under high organic loading it was obtained larger granules than operating at high shear conditions or with long starvation periods (Gao et al., 2011).

Settling velocity

The settle ability is one of the most important characteristics of granular sludge, due to this parameter affect to the design and size of bioreactor, and it is directly related with the capacity in biomass retention (Gonzalez-Martinez et al., 2017; De Kreuk et al., 2005). The settling velocity varied from 20 to 90 m h^{-1}, as average values, even up 130 m h^{-1}(Muñoz-Palazon et al., 2019; Gonzalez-Martinez et al., 2018a).

When the size of the granules is small enough, the model becomes the well-known Vesilind equation as follows:

$$V_S = V_o e^{-kX}$$

Where V_S is the settling velocity, V_0 is the initial settling velocity, k is the empirical settling parameter and, X is the sludge concentration (Gao et al., 2011).

Porosity

The porosity is involved in the mass transfer from external layer of both substrate and metabolites transport and oxygen diffusion. Therefore, pore size distribution and porosity have a strong influence on the fraction of bioactivity of communities that conform the granular sludge. The clog of pores by EPS could reduce the porosity in the granules, whereby the dissolved oxygen is depleted and the activity is negatively affect (Zheng et al., 2007). So it was demonstrated that the porosity will vary related to granular size as well as with the composition of microbial community. Xiao et al. (2008) indicated porosities ranging from 0.78 to 0.97 for fungal granules while that for bacterial granules ranging from 0.68 to 0.92.

Physical strength

Physical strength is a parameter which describes the capability of the granules against shear stress and abrasion. This parameter is measured indirectly through integrity coefficient, which is analyzed trough the weight of residual granules against the weight of granules after 5 min of shaking at 200 rpm, assuming that integrity coefficient is related to shaking time (Tay et al., 2001).

Surface hydrophobicity

The increase of hydrophobicity in the surface promote the contact cell to cell, those step is essential in the initial process of granulation. In this way, during the granulation the

biomass trend to get high hydrophobicity, increasing the protein/polysaccharide ratio which makes the negative surface charge increased too and this fact reduced the electrostatic repulsion between cells and augment granulation (Wilén et al., 2018; Gao et al., 2011). The changes in microbial cells induce the attach to inorganic cores of phosphate and calcium precipitates, which promote the production of EPS. On the other hand, other mechanisms that it had been reported to improve the hydrophobicity are the feast-famine periods and anaerobic feeding, acceleration the microbial aggregation (Wilén et al., 2018)

EPS content

The EPS had a strategic role in the stability and structure of the granule. Thus, the polysaccharides excreted by the microorganisms had been widely studied, because each type of polysaccharides gives to granule different properties and conformation. The hydrophobicity, as it had been mentioned before, encourages the aggregation in the tridimensional matrix. The second characteristic of EPS is the cell surface charge due to negative charge get repulsion, but the presence of EPS could increase the flexibility in the charge, making easier the co-aggregation (Gao et al., 2011). Finally, the EPS could have the function to act as unions-bridges to connect microorganisms among others or to create larger granules from small aggregates (Gao et al., 2011).

1.4. Role of extracellular polymeric substances in AGS

EPS are macromolecular compounds secreted by microorganisms under specific conditions during the growth and lysis, and it had an indispensable role in the aggregation of microorganisms in biofilms (Sheng et al., 2010), due to this product are accumulated on the microbial surface. Mainly, EPS contains two types of compounds: soluble EPS (SEPS) and bound EPS (BEPS) according to their location around the cell (Xuan et al., 2010). The composition of EPS is proteins, carbohydrates, nucleic acids, lipids, amino acid, uronic acid and inorganic compounds. The content and production of EPS are correlated with the physicochemical property of biological aggregates, being usually different EPS in flocculent activated sludge that granular sludge.

During granulation process, these metabolic products maintain the structure, stability and given to the granule the settle ability. The production of EPS by microorganisms is relevant for granular sludge due to kept the microorganisms self-immobilized into the matrix without any material. Some research demonstrated that microbial aggregation had a fluffy structure, with lipids and α-polysaccharides in the external layers, and proteins and β-polysaccharides in the internal layers and core of the granule (Lee et al., 2010; McSwain et al., 2005). Also, other study reported proteins with high molecular weight like a key component of granules, suggesting that could act as main structure. On the other hand, the content and composition of EPS seems to be related to hydrodynamic shear force, finding more amounts of polysaccharides under high shear force. In addition, if the stress is not maintaining, the granules trend to be disintegrated and to breakage when the EPS are lost (Tay et al., 2001). Due to difficulties to identify

all EPS in AGS caused by the density and the compact structure of granular biomass, big efforts must to be done to development a method to identify individually polymers and to understand the behavior and interaction of these compounds in the matrix (Wilén et al., 2015). Lin et al. (2010) detected a gel-forming exopolysaccharide called alginate with high levels of glucuronic acid, as well as by Seviour et al., (2012) identify the heteropolysaccharide called Granulan, defined it as the major gel-forming matrix component in granular biofilm in wastewater treatment. Also, other studies pointed out that addition of metal coagulant promote the segregation of EPS, helping the aggregation and attachment (Nancharaiah et al., 2018). The production and segregation of EPS seems to be done by signal of QS in the communities and the QS acquiring an important role in the granular long-term operation (Tan et al., 2014).

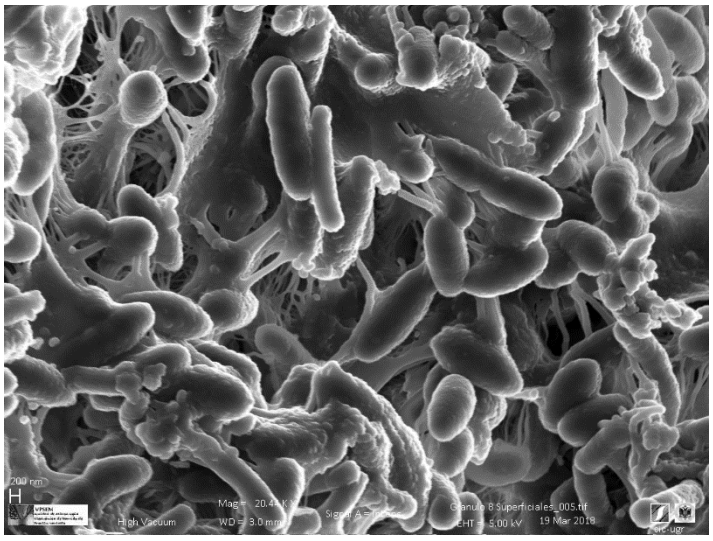

Figure 2- Scanning electron microscopy of a granule core cultivated at 8°C with high density of EPS.

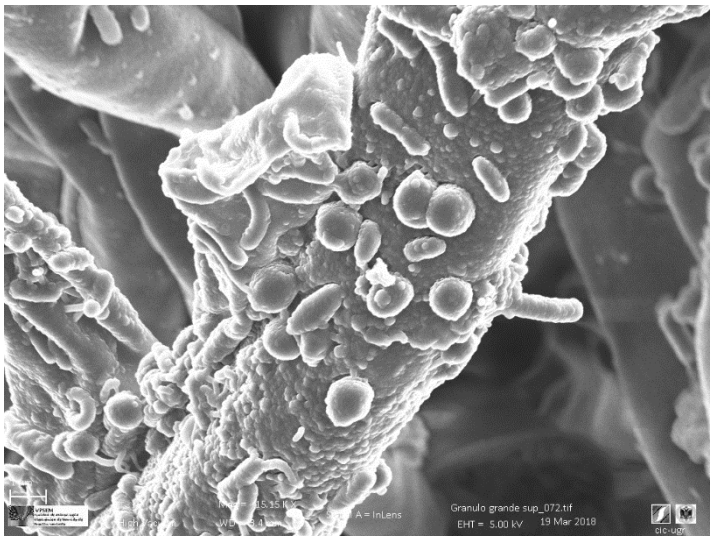

Figure 3- Scanning electron microscopy of bacterial colonization of a fungal filament that conform granules cultivated at 26°C with presence of EPS.

1.5. Loss of granular stability during long-term operation

Loss of stability during the operation had been widely reported as a limit to the implementation of AGS technology at full-scale due to granules could be disintegrated and breakage during long-term operation by different affections such as influent composition or temperature, among others.

The outgrowth of filamentous microorganisms is one of the obstacles most limiting for this technology. The granular sludge is conformed by floc-forming and filamentous microbial organisms; however, the control of populations is fundamental for the stable operation of AGS. The outgrowth of filamentous organisms could lead to fluffy and loose granules for excessive wash-out of biomass. The size of the filamentous are correlated with the mass transfer, hence the activity in the core could be limited by high

penetration of oxygen with the presence of filamentous organisms. Obviously, filamentous fungi had been identified as essential in the initial formation for the granules observing the surface of filamentous totally colonized by different morphologies of cells as cocci or bacilli (Figure 3), so the controlled growth of this kind of organisms is vital, but the outgrowth promote the loss of biomass by wash-out in each cycle. Moreover, the filamentous generated operational problems derived of block the pipelines and bulking or foaming episodes in WWTPs.

One of the causes described that had been reported as a trigger for AGS are the operation under high organic loading rate (ORL) and under low temperature (Lee et al., 2010; De Kreuk et al., 2005). On the contrary others authors had operated AGS at high ORL and at ultra-low temperature (5°C) and the granules were stable for long-term operation (Gonzalez-Martinez et al., 2018a).

Other cause of loss of stability is given by the hydrolysis of granule nuclei due to the presence of proteolytic bacteria in the internal layer that drive to deterioration of core and consequently the breakage of the granule. Also, under larges period of starvation the granules become unstably and decreased the activity because the population of core consumes the EPS situated in the nuclei, which also show more solubility. To corroborate this hypothesis studies based on fluorescent staining and CLSM demonstrate that large spaces appeared in aged granules, and the internal structure become too weak to withstand the shear stress and the granule is broken (Lee et al, 2010). A strategy for more stability is the selection of strain producers of gel-forming exopolysaccharides

through operational conditions and environmental parameter, specially the organic loading rate and carbon source.

1.6. Application of aerobic granular technology

1.6.1. Nutrients removal

Complete nitrogen removal is get in AGS in a single bioreactor carry out by nitrification and denitrification process. Ammonium is oxidized to nitrite or nitrate, and these are reduced until molecular nitrogen gas. The simultaneous nitrification-denitrification is given by the limitation by mass transfer from external layer to core of the granules. In the granule coexist heterotrophic, nitrifiers and denitrifying microorganisms carry out metabolisms under different niches. High performance in terms of nitrogen had been reported by AGS under different environmental conditions, even at full scale (Pronk et al., 2015).

Efficient removal of phosphorous had been reported by AGS technology (Bassin et al., 2012; Barr et al., 2010). Many operational parameters influence the proliferation of phosphate accumulating organisms (PAO) such as temperature, aeration, temperature, salinity or pH. PAO organisms had a strong competition against glycogen accumulating organisms (GAO), but it is possible promote the PAO over GAO proliferation fitting the optimal conditions for PAO. Candidatus *Accumulibacter phosphatis* is the main PAO genus involved in the enhanced bio-phosphorous removal capabilities in AGS (Nancharaiah et al., 2018).

1.6.2. Particulate matter

The fraction of particulate matter in real wastewater is high, which could affect to the stability of granular sludge. Particulate matter is mostly hydrolyzed in the external layer of the granules. Although, it had been described that the removal of particular matter in AGS follow two ways: during the granular formation the particulate matter is incorporated and adsorbed in the tridimensional matter become part of the granules, and if granules are mature the microbial metabolic activity, mainly protozoa activity, remove the particulate matter with higher efficiency that flocculent sludge (Muñoz-Palazon et al., 2019; Wagner et al., 2015; Schwarzenbeck et al., 2004).

1.6.3. Phenolic compounds

Phenolic compounds are chemical compounds that are found in water bodies, due to it are widely using in agri-food, pharmaceuticals and pesticides production industries, among others.

AGS technology had been extensively used to degrade nitrophenol, phenol and fluorophenol, and many studies had confirmed the robustness of system for treating high-containing phenol wastewater. Muñoz-Palazon et al. (2019) showed the stability and high efficiency of AGS technology operating with a mixture of recalcitrant phenolic compounds at more than 300 mg L^{-1} of concentration without adaption period to these contaminants, although under concentrations higher than 600 mg L^{-1} the granulation was unstable. Adav et al. (2007) reported that granules degrade phenol at 1.18 g phenol

g^{-1} VSS d^{-1}, suggested the hypothesis of high performance for these compounds is due to mass transfer barrier provided by granular matrix, which produce less concentration on individual cells.

1.6.4. Heavy metals

Water polluted by heavy metals is an environmental and human health problem. Many physic and chemical technologies had been applied to remove heavy metals from wastewater. However, the bioadsorption by biological methods is a high efficiency process which saving-cost in comparison with membrane or chemical treatments. AGS is being using to carry out bioadsorption process due to separate heavy metals from aqueous solution. Thus, AGS had been implemented to remove cations and anions such as Cu (II), Zn (II), Pb(II), which reached removal values higher than 1000 mg L^{-1} (Liu et al., 2015; Sun et al., 2011). The authors reported the fundamental role of EPS and their characteristics in the process of sorption.

1.6.5. Pharmaceuticals compounds

The presence of pharmaceuticals and personal care products (PPCPs) are consider novel pollutants, which are detected in the environment in nanograms or micrograms per liter, and it have received increasing attention since years ago. These compounds and their metabolites are spreading by human use and mainly distributed in aquatic environment. The WWTP had an important role in the spreading of this pollutant to natural bodies, so finding a highly efficiency technology to remove it, which is of vital importance. The

spectrum of these compounds is very wide: analgesic, antibiotics, antidepressant, antiepileptic, among other, considering that are very recalcitrant. Many authors had described the potential role of granular sludge for biodegrade and bioadsorbe PPCPs (Wang et al., 2019; Wang et al., 2016; Zhao et al., 2014; Amorin et al., 2014). Some of these studies demonstrated that nitrogen removal capacity of AGS were kept, the granular conformation was not affect under medium-high concentration of this compounds, defining the EPS as essential to stability under PPCPs treatment.

1.6.6. Nuclear waste

The treatment of nuclear waste with aerobically grown of granular sludge had been studied due to the characteristic of the technology. It had been reported that soluble uranium could be recovery by bioadsorption process (Nancharaiah et al., 2006; Nancharaiah et al., 2012). These studies showed the capacity of AGS technology to remove concentration of uranium ranging from 6 to 750 mg L^{-1} in acid pH ranges. During the bioadsorption of uranium it was observed that cations as sodium, potassium, calcium or magnesium were expelled out identifying the ion exchange process. Xie et al. (2015) described that the uranium recovery form aqueous solution is made in two steps, first an initial adsorption and after that a microbial reduction reaching around 99% of removal ratio. In addition, this research demonstrated that groups-amide, groups carboxyl, hydroxyl and phosphoric acid group located in external layers are involved in uranium deposition

2. Objectives

The effluent discharge of urban and industrial wastewater to natural water bodies cause environmental damages and consequently problems in human health, due to the spreading of nutrient, pathogen or toxic compounds. The remove of nutrients such as nitrogen and phosphorous was regulated for emissions of wastewater by European Framework Directive of Water. Thus, the organic matter, nitrogen and phosphorous limit values had been highly restricted, while those CAS systems fail to reach the quality standards. So, to conventional systems have to be added new stages and compartments within bioprocess, causing an increasing of building and exploitation cost. Thus, the searching of novel technologies to meet the permissible values in a saving-cost mode is being encouraged.

The AGS technology is a novel technology with high potential for treating urban and several industrial wastewaters, due to it is a system very versatile where the removal of organic matter, nitrogen, phosphorous and particulate matter are removed in a single bioreactor due to the granular conformation of biomass, that allow the coexistence of diverse of niches (aerobic/anoxic/anaerobic). The compactness and robustness of the technology as well as the cost savings and the efficiency of this system makes AGS a promise in the field of biological wastewater treatment

The AGS technology is based on biological process, so it is affected by environmental parameters such as temperature. Temperature is key parameter for the microbial metabolisms and consequently for performance of bioreactors. So, temperature changes, for instance those generated at different latitudes can pose risks in stability

and operation of AGS at full-scale reactors, strong wash-out of biomass and/or decay of nitrogen and phosphorous removal rates.

According to the aforementioned, the main goals pursued in the study to achieve the Ph.D. thesis were:

1. Understand the influence that the thermal adaptation of an inocula can exert on the AGS technology.

2. Evaluate the granulation process and the physical-chemical performance of AGS inoculated with different geographical climate sludges, operating under the same temperature condition.

3. A deep study of the bacterial, archaeal and fungal community structure responsible for the granulation process and their physicochemical performance at different temperature.

4. Know the microbial dynamics and the performance of aerobic granular sludge bioreactors at cold, mid and warm temperatures, starting from an initial microbial structure native to mild temperature environments.

5. Contribute to knowledge of the granulation process under different temperatures from cold-adapted inoculum, and the granulation process in cold environments during long-term experimentation, studying the dynamics of the microbial communities in the granulation process at native cold temperature (8 °C), mild temperature (15 °C) and warm temperature (26 °C).

6. Study of the efficiency of the AGS systems in terms of nitrogen, phosphorous and organic matter removal, as well as the emission of nitrous oxides at different temperatures.

7. Establish a correlation between qualitative and quantitative data of microbial ecology with operational parameters and physicochemical performance under different temperature operation.

3. Material and methods

3.1. Design of bioreactors

Three sequencing batch reactors were design as cylindrical air-lift with 9 cm of diameter and 45 cm height with a total volume 2.86 L and 2.5 L operational volume. The material of cylindrical reactor was pirex glass. The porous plate was implemented in the central area at the bottom, which were surrounded by a conical structure to force continuous hydrodynamic circular motion. The air was introduced by porous plate thought fine bubble with a flow ranging to 3.5 - 4.5 L min^{-1}. The volume exchange was 60% of volume and operated in batch cycles of 4.5 – 3.96 h, and the hydraulic retention time was 7.5 - 6 h. The peristaltic pump (Watson Marlow, United Kingdom) were used for feeding from the top and effluent discard at output of treat water. The temperature was controlled using a room thermostat to the following temperatures: 8 °C, 15 °C and 26 °C. The oxygen was monitoring during all experiment with a Crison probe. The pH was controlled in the range of 7.8± 0.5 with a Crison probe.

The synthetic medium was composed to simulated high-carbon loading urban wastewater with the following composition: sodium acetate 0.9 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.1 g L^{-1}; KCl 0.04 g L^{-1}; NH_4Cl 0.25 g L^{-1}, KH_2PO_4 0.03 g L^{-1}, K_2HPO_4 0.085 g L^{-1} and 0.1 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} (NH_4) $Mo7O_2 \cdot 4H_2O$; 0.03 mg L^{-1} $CuSO_4 \cdot 5H_2O$; and 0.15 mg L^{-1} $CoCl_2 \cdot 6H_2O$. (Muñoz-Palazon et al., 2019).

The cycle of SBR consisted on: 33 min of feeding, 200-230 min of aeration, 3 min of settling and 4 min for effluent discard. During the start-up phase, the time of settling

was changed from 10 min during the first three days to 5 min during the first 7 to 10 days and finally to 3 min after 8 to 11 days of operation. These changes were essential for granules formation, thus settling time controlled the wash-out of filamentous microorganisms and the selection of granule-forming microbes.

3.2. Physic-chemical analysis

Chemical oxygen demand (COD) and biological oxygen demand (BOD_5) were measured three times per week following established protocols (APHA, 2012).

The chemical oxygen demand (COD) is an estimation of content of organic matter, thought determination chemical way of amount of oxygen to oxidation. This parameter is determinate by analytic medium of closed reflux. The process is a strong oxidation of organic matter, using potassium dichromate ($K_2Cr_2O_7$) in an acid medium (H_2SO_4). The following equation reflect the oxidation for glucose.

$$C_6H_{12}O_6 + 4K_2Cr_2O_7 + 16H_2SO_4 \Leftrightarrow 6CO_2 + 4Cr_2(SO_4)_3 + 4K_2SO_4 + 22H_2O$$

The process was carry out at 150° C during two hours, using a catalyzer, avoiding interferences with halides compounds. The measurement was done by spectrophotometric method based on the comparison in absorbance after oxidation in a calibration curve expressed in mg O_2 L^{-1}. The procedure was to add 2 mL of oxidant solution, 3 mL of sample and 4 mL of acid solution. After that, the tube is kept in the thermblock at 150°C during two hours. The absorbance was measured at 600 nm. The spectrophotometer used was Helios gamma spectrophotometer (Thermospectronic).

The biological oxygen demand (BOD) is an indirect measurement of biodegradable organic matter that content a wastewater sample. The biological oxygen demand is the amount of oxygen needed to oxidize by biological way the biodegradable organic compound, which is based on the consumption by aerobic and heterotrophic microorganisms involved in the oxidation. The DBO_5 was analyzed following the manometric method described by manufactures carried out at 20°C in darkness during five days. To determinate was used the bottle and cap of Oxitop.

The inorganic nitrogen compounds (NO_3^-, NO_2^- and NH_4^+) concentrations were measured in triplicate by ionic chromatography (Metrohm Ion Chromatograph). Total suspended solids (TSS) were determined in duplicate according to standard methods (APHA, 2012). The settling velocity was measured with a chronometer in a column of 1 m and the mean size was measured by a millimeter rule using a binocular following the low cost technique for determining the granulometry described by Laguna et al., 1999. The dissolved oxygen was controlled by a Crison Oximeter and the pH was measured using a Crison and inoLab pH-meter probe.

3.3. Mass balance

Data from ion chromatography and gas chromatography was using to calculate the nitrogen conversion by the bioreactors thought mass balance over nitrogen. For the balance the only nitrogen source was ammonium in the influent, also it was assumed that all ammonium influent in the bioreactors was transformed into NH_4^+, NO_2^-, NO_3^-,

and NO, N_2O, N_2. To compute the mass balance was used the average values from period steady-stable following the equations:

$Q_{in}\ TN_{in}=Q_{out}\ TN_{out} + N_{ous\ gas}$

$Q_{in}\ (NH_4^+ - N_{in} + NO_x^- - N_{in}) = Q_{out}\ (NH_4^+ - N_{in} + NO_x^- - N_{in}) + N_2O + (NO + N_2)$

3.4. Greenhouse gases (N_2O) determination

Measurements of N_2O concentration in the off-gas were analyzed in triplicate. Therefore, a cap filter of PVC was implemented at the top of bioreactors coated with an air chamber to ensure hermetic conditions. The reactors were closed with the cap in order to capture off-gas. Based on previous experiments, off-gas (12 mL) from the reactor headspace was collected with a syringe at 0, 20 and 40 min after reactor closure, and the sample was transferred to 12 mL pre-evacuated vials. N_2O concentrations were analyzed by gas chromatography using a Perkin Elmer Clarus 500 gas chromatograph (Perkin Elmer Instruments, Beaconsfield, UK) equipped with an electron capture detector. Cumulative emissions of N_2O were calculated from the area under the curve after linear interpolation between sampling points.

3.5. Biomass collection and DNA extraction

For microbial community quantification and identification of granular biomass in AGS systems operated under different temperature and different inoculums, 50-200 mL of granular biomass was collected from inoculum in all bioreactor on operational at days 0, 30, 90, 105, 120 and 150. After collection, biomass samples were submerged in saline

solution (0.9% NaCl), centrifuged at 3000-3500 rpm at room temperature for 10-15 min and then the supernatants were discarded. Finally, the pelleted biomass was kept at −20 °C. For the DNA extraction, 0.3 g of pellet were used with the Kit FastDNA ® Spin for soil (MP Biomedicals, Solon, OH) according to the manufacturer's protocol.

3.6. Massive parallel sequencing

Extracted DNA were kept at -20°C and sent to Research & Testing Laboratory (Lubbock, TX, USA) for iTag sequencing process, which was done using Illumina MiSeq equipment and Illumina MiSeq Reagent Kit v3.

The DNA pools were subjected to amplification runs in order to investigate the community structure of *Archaea*, *Bacteria*, *Fungi* and *Prokarya* and *Eukarya*. For this purpose, the primer pairs 28F- 519R for (Gonzalez-Martinez et al., 2017), for *Archaea* 519F-1041R (Gonzalez-Martinez et al., 2016), for *Fungi* ITS1F-ITS2 (Maza-Marquez et al., 2016), Bac357-Bac806 was used for *Prokarya* (Muñoz-Palazon et al., 2019), EUK1391-EUKbr was used for the amplification of hypervariable regions of the 18S rRNA gene of *Eukarya* (Rodriguez-Sanchez et al., 2019).

3.7. Bioinformatics pipeline

The raw data from next-generation sequencing was processed using the software mothur v.1.34.4 (Schloss et al., 2009). For *Bacteria*, *Archaea*, *Fungi*, *Prokarya* and *Eukarya*, paired-end reads were merged into contigs, which were then subjected to

quality trimming, removing sequences with more than 0 ambiguous bases or more than eight homopolymers. The remaining sequences were aligned through the k-nearest neighbour method with k-mer size 8 and under Needleman criteria using SiLVA or MiDAS_S123 (McIlroy et al., 2017), which were previously aligned against SiLVA nr v128 using mothur for *Bacteria*, *Archaea*, *Prokarya* and *Eukarya*. For *Fungi*, the sequences were aligned with the UNITEv6 DATASABE (Koljalg et al., 2013), which previously aligned against itself using MUSCLE algorithm. Then, the sequences that did not align at the right position of forward and reverse primers were removed from the analysis. The chimerical sequences were detected and removed by the VSEARCH algorithm implemented in mothur (Rognes et al., 2016), and the remaining sequences were taxonomically affiliated to obtain the specific domain using MiDAS_S123 release 2.1.3 and SiLVA SEED v132. For *Fungi,* the sequences that were not affiliated with the domain analysis were discarded. All contigs were then clustered into operational taxonomy units (OTUs) with 97% similarity for *Prokarya* and *Bacteria* and 95% for *Eukarya* domains, *Fungi* and *Archaea* using the abundance-based greedy clustering method of VSEARCH implemented in mothur. After singleton OTUs were removed from the samples, and the persistent sequences were used to create a consensus for each OTU.

3.8. Quantification absolute of target genes

The number of copies of bacterial and archaeal 16S rRNA genes and 18 rRNA gene were quantified by the means of quantitative real time PCR in each DNA pool, using extracted DNA as templates. qPCR was performed using an Mx3000P QPCR system (Agilent

Technologies) and the primers and annealing conditions were reported previously for *Archaea* (Yu et al., 2008), for *Bacteria* (Muyzer et al., 1993) and for *Fungi* (Liu et al., 2012), for bacterial *amoA* gene (Rotthauwe et al., 1997), for archaeal *amoA* gene (Tourna et al., 2008), for denitrifying *norB* and *nosZ I (*Braker and Tiedje 2003 and Henry et al., 2006, respectively) as is shown in the Table 2 and Table 3. qPCR calibration curves were constructed with the aid of plasmid standards harboring inserts of the targeted genes. The calibration curves for the absolute quantification in the DNA samples were generated using ten-fold dilutions (10^2 - 10^8) of plasmid standards. The reaction mixture for was made in a total volume of 25 mL, which contained 0.125 mL of SYBR Green PCR, 2.5 mL of Buffer, 1.5 mL of $MgCl_2$, 0.5 mL of dNTPs,0.125 mL of Taq polymerase, 0.15 mL of each primer (10 mM), 0.0625 mL of BSA and template DNA diluted 1:10 and 1:50. Melting curves were used at the end of each qPCR to check amplification specificity and purity of negative controls. Negative controls gave null or negligible values and PCR efficiency for the different assays ranged from 90 to 99%.

Table 2.- Pair of primers for quantification absolute the genes

Group	**Gene marker**	**Primers**	**Sequence (5'-3')**	**References**
Total Bacteria	V3-16S rRNA	P1 (341F)	CCTACGGGAGGCAGCAG	Muyzer et al. (1993)
		P2 (534R)	ATTACCGCGGCTGCTGG	
Total Archaea	16S rRNA	ARCH915 (F)	AGGAATTGGCGGGGGAGCAC	Yu et al. (2008)
		UNI-b-rev (R)	GACGGGCGGTGTGTRCAA	
Bacteria	*amoA*	AmoA1 F	GGGGTTTCTACTGGTGGT	Rotthauwe et al. (1997)
		AmoA1 R	CCCCTCKGSAAAGCCTTCTTC	
Archaea	*amoA*	Crenamo A23 F	ATGGTCTGGCTWAGACG	Tourna et al. (2008)
		Crenamo A616 R	GCCATCCATCTGTATGTCCA	
Fungi	18S rRNA	FungiQuantF	GGRAAACTCACCAGGAGGTCCAG	Liu et al., (2012)
		FungiQuantR	GSWCTATCCCCAKCACGA	
Denitrifiers	*norB*	cnorB2F	GACAAGNNNTACTGGTGGT	Braker and Tiedje (2003)
		CnorB6R	GAANCCCCANACNCCNGC	
Denitrifiers	*nosZ*	nosZ1840F	CGCRACGGCAASAAGGTSMSSGT	Henry et al. (2006)
		nosZ2090R	CAKRTGCAKSGCRTGGCAGAA	

Table 3.- Conditions of PCR for genes studied

		Total Bacteria	**Total Archaea**	**AOB (*amoA*)**	**AOA (*amoA*)**	**Total Fungi**	**Denitrifiers (*norB*)**	**Denitrifiers (*nosZ I*)**
	Initial denaturalization	95°C, 7 minutes	95°C, 7 minutes	95°C, 7 minutes	95°C, 7 minutes	95°C, 3 minutes	95°C, 7 minutes	95°C, 10 minutes
Amplification (×40 cycles)	Denaturalization	95°C, 30 seconds	95°C, 30 seconds	95°C, 30 seconds	95°C, 30 seconds	94°C, 30 seconds	94°C, 30 seconds	95°C, 15 seconds
	Primers annealing	60°C, 30 seconds	60°C, 30 seconds	65°C, 30 seconds	65°C, 30 seconds	62°C, 30 seconds	56°C, 60 seconds	65°C, 30 seconds
	Elongation	72°C, 30 seconds	72°C, 30 seconds	72°C, 30 seconds	72°C, 30 seconds	62°C, 45 seconds	72°C, 60 seconds	72°C, 30 seconds
	Melting curve	60°C-95°C + 2°C/minutes. Fluorescence measured each 15 seconds						

3.9. Conventional statistical analysis

The ecological coverage was calculated for the samples using the redundancy abundance-weighted method (Rodriguez-r and Konstantinidis, 2014a). The redundancy abundance-weighted method coverage was done with rarified subsamples using Nonpareil software (Rodriguez-r and Konstantinidis, 2014b), with parameters of 95% of identity, 50% overlap, and query set size of 1000 sequences.

The study of α-diversity was calculated using PAST software v3.06 (Hammer et al., 2006) through the calculation of Chao-1, Shannon–Wiener, Simpson, Berger-Parker and Pielou's evenness diversity indices, computed in the 95% confidence range for 1000 bootstrap replications.

To analyze the β-diversity of biological samples, Morisita–Horn and symmetric indices were calculated among pairs of next-generation sequencing samples. These indices have been reported as the most robust indices to capture the variability of dominant and rare phylotypes presented in microbial communities (Barwell et al., 2015). These indices were calculated using the packages vegan v2.0 and vegetarian implemented in R-Project software. Whittaker and Williams were calculated as β-diversity measurement for comparison between pair of sample with PAST v3.14 software (Hammer et al., 2006).

To calculate the contribution to dissimilarity of OTUs between groups of samples, the OTU tables for *Bacteria*, *Archaea*, *Fungi*, *Prokarya* and *Eukarya* were used for calculation of SIMPER analysis through Bray–Curtis similarity using PAST software v3.4.

3.10. Compositional statistical analysis

Similarity analyses of samples were done in accordance with protocols used previously (Bian et al., 2017). For analysis of sample similarity, the OTU tables for *Bacteria*, *Archaea*, *Fungi*, *Prokary*a and *Eukarya* were used. These were first corrected to avoid zero counts by Bayesian multiplicative replacement of zero values using the zCompositions package implemented in R. The zero count-corrected OTU tables were then transformed with centered log-ratio using CoDaPack software. Corrected OTU tables were then used for singular value decomposition calculations, the results of which were represented through a principal components plot. For differential abundance analysis of OTUs between different temperatures and inoculum, the OTU table of each gene was corrected for zero values and transformed through a centered log-ratio by generation of 128 Monte-Carlo Dirichlet simulations, which was then used for the computation of expected effect size utilizing the ALDEx2 package implemented in R. To calculate linkages between OTUs, the OTU tables of each domain were corrected for zero values and transformed through a centered log-ratio by generation of 128 Monte-Carlo Dirichlet simulations, which was used for calculation of ρ metric proportionality analysis between OTUs of each domain, using ALDEx2 and propr packages implemented in R.

3.11. Multivariate analysis and PERMANOVA

Identification of statistically significant effects of temperature and operational day on the performance of the systems, the number of copies of genes of interest, and the most

abundant OTUs of Prokarya and Eukarya was done using one-way PERMANOVA. PERMANOVA was done to suggest if the number of copies, the physicochemical performance and the dominant OTUs were affected by the time of operation under low, mild and warm temperature. PERMANOVA was computed using PAST v3 with Bray–Curtis distance and under 9999 permutations.

Multivariate RDA was done to observe the relationships between dominant OTUs belonging to *Bacteria*, *Archaea*, *Fungi, Prokarya* and *Eukarya*, and physicochemical performance. The Prokarya OTUs used in the calculation of RDA were the 20 most abundant phylotypes. For Eukarya, OTUs used were those with more than 0.5% of total relative abundance in at least one sample. For *Bacteria*, *Archaea* and *Fungi* were used the most dominant OTUs with more than 1.00% of relative abundance. The number of copies of marker genes was linked with the performance and the biological samples. For each microbial group, RDA was calculated with OTU tables corrected for zero values and transformed through a centred log-ratio using CoDaPack software, computed by 499 unconstrained Monte-Carlo simulation and run using the software CANOCO 4.5 for Windows. All other parameters were transformed to the LOG(X+1) when they were expressed in different parametric units.

3.12. Oligotyping analysis

Several OTUs were selected for three domains based on their high abundance or on their presence under a specific threshold of abundance relative. The selected OTUs were subjected to oligotyping analysis according to the protocol described by Eren et al.

(2015). For each OTU, a Shannon entropy analysis was computed. Then, oligotypes were calculated with the Shannon entropy results by repeated calculation with a step increase of considered high-entropy nodes until the purity score was > 0.90 with more than 100 reads (Okazaki et al., 2017). For analysis of quality and removal of noise, the oligotypes were constrained to the following criteria: each oligotype had to appear in at least one sample; each oligotype had at least 1% relative abundance in at least one sample; and each oligotype had a substantive abundance of > 30 reads.

3.13. Scanning electron microscopy (SEM)

The preparation and visualization of granular samples was done in the Center of Scientific Instrumentation at the University of Granada. First, granules were cut and fixated in a mix solution of 2.5% glutaraldehyde in a pH 7.4 cacodylate buffer 0.1 M at 4 °C for 2 h. Then, they were washed thrice with pH 7.4 cacodylate buffer for 20 minutes. After this, the samples were post-fixated with 1% osmium tetroxide for 2 h, post-fixated with 1% osmium tetroxide for 1 h in darkness and at room temperature, followed by an additional wash with distilled water. Then, granules were dehydrated by successive baths in ethanol for 15 min: one at 50, 70 and 90%, and two at 100%. After ethanol dehydration, samples 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 observation by scanning electron microscope (Carl Zeiss LEO 906E).

4. Results

Chapter I

Pollutants degradation performance and microbial community structure of aerobic granular sludge systems using inoculums adapted at mild and low temperature

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Muñoz-Palazon, B., Pesciaroli, C., Rodriguez-Sanchez, A., Gonzalez-Lopez, J., & Gonzalez-Martinez, A. (2018). Pollutants degradation performance and microbial community structure of aerobic granular sludge systems using inoculums adapted at mild and low temperature. Chemosphere, 204, 431-441. https://doi.org/10.1016/j.chemosphere.2018.04.062.

Scientific quality index: Q1, IF: 5.108.

Abstract

Three aerobic granular sequencing batch reactors were inoculated using different inocula from Finland, Spain and a mix of both in order to investigate the effect over the degradation performance and the microbial community structure. The Finnish inoculum achieved a faster granulation and a higher depollution performance within the first two month of operation. However, after 90 days of operation, similar physico-chemical values were observed. On the other hand, the Real-time PCR showed that *Archaea* diminished from inoculum to granular biomass, while *Bacteria* and *Fungi* numbers remained stable. All granular biomass massive parallel sequencing studies were similar regardless of the inocula from which they formed, as confirmed by singular value decomposition principal coordinates analysis, expected effect size of OTUs, and β-diversity analyses. *Thermoproteaceae*, *Meganema* and a *Trichosporonaceae* members were the dominant phylotypes for the three domains studied. The analysis of oligotype distribution demonstrated that a fungal oligotype was ubiquitous. The dominant OTUs of *Bacteria* were correlated with bioreactors performance. The results obtained determined that the microbial community structure of aerobic granular sludge was similar regardless of their inocula, showing that the granulation of biomass is related to several phylotypes. This will be of future importance for the implementation of aerobic granular sludge to full-scale systems.

Keywords: aerobic granular sludge; microbial community dynamics; granulation; inoculum; temperature adaptation.

1. Introduction

In the last decade, the aerobic granular sludge (AGS) technology had been promising for treatment of municipal and industrial wastewater (Pronk et al., 2015; Świątczak and Cydzik-Kwiatkowska, 2017). This technology has advantages to conventional technology of activated sludge system, mainly due to granular conformation of its biomass. Aerobic granular sludge (AGS) has superior settling time and higher biomass retention capabilities resulting in a lower cost in biomass removal and recycling (Henriet et al., 2016). AGS is a biofilm with spherical morphology, formed by aggregation of microorganisms and extracellular polymeric substances (Yuan et al., 2017). Moreover, extracellular polymeric substances have a crucial role that keep microbial aggregates together in a three-dimensional matrix for both granulation and stability properties given that they allow immobilization of microorganism in a complex matrix (Zhou et al., 2014; Cydzik-Kwiatkowska, 2015).

Temperature plays an important role in natural environments affecting directly the microbial communities, and thus it could influence biological processes. It is generally assumed that the rate of conversion processes in biological systems depends on environmental temperature (Henze et al., 2015). As an example, biological nitrogen removal processes have a negative correlation with the temperature, with complete inhibition at 10 °C or lower when the bioreactors are inoculated with 20 °C adapted activated sludges (Gnida et al., 2016). However, in cold regions (i.e., Finland), the wastewater treatment processes achieve high removal efficiencies at very low temperatures (<10 °C), caused by adaptation of microbial communities at low temperature.

Although it has been shown that low temperature can generate problems in aerobic granular sludge technology, such as destruction of the granules and the loss of biomass (De Kreuk et al., 2005), sufficient data are not available to establish general criteria on the correct functioning of these systems at different temperatures. Equally, although the responses of AGS with different microbial inocula have been studied (Song et al., 2010), no evidences are available to understand the influence that the thermal adaptation of the inocula can exert on the AGS technology. Consequently, the main objective of this study is to evaluate the granulation process and the physical-chemical performance of AGS inoculated with different geographical climate sludges, operating under the same temperature condition (15 °C). Moreover, a deep study of the bacterial, archaeal and fungal community structure has been carried out during the experimental period since it is the microbiota existing in the bioreactors responsible for the granulation process and their physicochemical performance.

2. Materials and methods

2.1. Start-up of bioreactors and operational conditions

The experiments were performed using 3 sequencing batch bioreactors (SBR) of 45 cm height, 9 cm of diameter and 2.5 L of operational volume (Figure S1). These three bioreactors were operated for 105 days. The first bioreactor was inoculated with 1.5 L of activated sludge from Granada (GAS) WWTP (37°10'41"N, 3°36'03"W), Spain. A second bioreactor was inoculated with 1.5 L of activated sludge adapted at low temperature from Rovaniemi (RAS) WWTP (66°30'00" N; 25°43'00" E) at the Finnish

Polar Arctic Circle. Finally, the third bioreactor was inoculated with 1 L of a mixture of activated sludge (MAS) from Granada (750 mL) and Rovaniemi wastewater treatment plant (WWTPs) (750 mL). Air was introduced via fine bubbles at the bottom of the bioreactors (4 L min^{-1}) and the dissolved oxygen (DO) was kept at 10.4 mg O_2 L^{-1}. The temperature of the three systems was controlled at 15 °C with an electric thermostat. The volume exchange was 60% of total volume and they were operated in successive cycles of 4.5 h. The cycles consisted of: 33 min feeding from the top of the bioreactor under anaerobic conditions, 230 min of aeration, 3 min of settling and 4 min for effluent discard to wash-out filamentous microorganisms. During the start-up the settling time was reduced from 10 min during the first three days, then 5 min until operational day 10, and 3 min after 11 days of operation.

The bioreactors were fed with synthetic wastewater medium. The composition used was sodium acetate 0.9 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.1 g L^{-1}; KCl 0.04 gL^{-1}; NH_4Cl 0.25 g L^{-1}, KH_2PO_4 0.03 g L^{-1}, K_2HPO_4 0.085 g L^{-1} and 0.1 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} $(NH_4)Mo_7O_{24} \cdot 4H_2O$; 0.03 mg L^{-1} $CuSO_4 \cdot 5H_2O$; and 0.15 mg L^{-1} $CoCl_2 \cdot 6H_2O$.

2.2. Physicochemical determinations

The Chemical Oxygen Demand (COD), Suspend Solids (SS) and Total Nitrogen (TN) were measured continuously in the influent and effluent following established protocols. The pH was measured by Crison-pH meter digital.

2.3. DNA extraction and iTag sequencing

A total of 200 mg of biomass collected from each bioreactor were taken for DNA extraction at operational days 0 (RAS0, GAS0 and MAS0), 30 (RAS1, GAS1 and MAS1) and 105 (RAS2, GAS2 and MAS2). After collection, biomass samples were submerged in saline solution (0.9% NaCl) and centrifuged at 3000 rpm at room temperature for 10 min. The supernatants were discarded and the pelleted biomass was kept at −20 °C. Then, all pellets of biomass were subjected to DNA extraction using the FastDNA SPINK Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol. Extracted DNA were kept at −20 °C and sent to Research & Testing Laboratory (Lubbock, TX, USA) for iTag sequencing process, which was done using Illumina MiSeq equipment and Illumina MiSeq Reagent Kit v3.

The DNA pools were subjected to three independent amplification runs in order to investigate the community structure of *Archaea*, *Bacteria* and *Fungi*. For this purpose, the primer pairs 28F-519R (5'-GAGTTTGATCNTGGCTCAG-3' and 5'-GTNTTACNGCGGCKGCTG-3', respectively) (Gonzalez-Martinez et al., 2017), 519F-1041R (5'-CAGCMGCCGCGGTAA-3' and 5'-GGCCATGCACCWCCTCTC-3') (Gonzalez-Martinez et al., 2016) and ITS1F-ITS2 (5'-CTTGGTCATTTAGAGGAAGTAA-3' and 5'-GCTGCGTTCTTCATCGATGC-3') (Maza-Márquez et al., 2016) were chosen for the amplification of the hypervariable regions V1-V3 of 16S rRNA gene of *Bacteria*, the hypervariable regions V4-V6 of 16S rRNA gene of *Archaea*, and ITS region of *Fungi*, respectively.

2.4. Quantitative real time PCR of the bacterial 16S rRNA archaeal 16S rRNA and fungal 18S rRNA

The number of copies of bacterial and archaeal 16S rRNA genes and fungal 18 rRNA gene of each of the extracted DNA pools was measured by the means of quantitative real time PCR (qPCR). The number of copies of bacterial and archaeal 16S rRNA genes and 18 rRNA gene were quantified in each DNA pool, using extracted DNA as templates. qPCR was performed using an Mx3000P QPCR system (Agilent Technologies) and the primers and annealing conditions were reported previously for *Archaea* (Yu et al., 2008), for *Bacteria* (Muyzer et al., 1993) and for *Fungi* (Liu et al., 2012). qPCR calibration curves were constructed with the aid of plasmid standards harboring inserts of the targeted genes. The calibration curves for the absolute quantification in the DNA samples were generated using serial ten-fold dilutions (10^{-2} - 10^{-8}) of linearized plasmid standards. The reaction mixture for was made in a total volume of 25 µL, which contained 0.125 µL of SYBR Green PCR, 2.5 µL of Buffer, 1.5 µL of $MgCl_2$, 0.5 µL of dNTPs, 0.125 µL of Taq polymerase, 0.15 µL of each primer (10 µM), 0.0625 µL of BSA and template DNA diluted 1:10, 1:20 or 1:50. Melting curves were used at the end of each qPCR to check amplification specificity and purity of negative controls. Negative controls gave null or negligible values and PCR efficiency for the different assays ranged from 90 to 99%. The threshold cycle (CT) values determined were plotted against the logarithm of the initial template copy concentrations. The numbers of copies of the targeted genes were expressed per grams of granules in the samples against the corresponding standard curves within the linear range ($r^2 > 0.990$). All the calibration curves had a correlation coefficient $r^2 > 0.95$ in all the assays. The efficiency of PCR amplification was between 90

and 100%. Real-time PCR data was analyzed using a MxPro QPCR software version 3.0 (Stratagene, USA).

2.5. iTag sequencing post-process

The raw data from iTag sequencing process was treated using the software mothur (Schloss et al., 2009). Paired-end reads were merged into contigs avoiding the generation of ambiguous bases due to different quality score of nucleotides in the same position at the overlap (Unno, 2015). Contigs were then screened to eliminate those with >0 ambiguous bases and >8 homopolymers. The remaining were then aligned against a database of choice. For Archaea and Bacteria, this database was MiDAS 2.0 (McIloy et al., 2017), previously aligned against the SiLVA nr v128 release using mothur and the kmer searching method of kmer size 8 bp. For Fungi, the UNITEv6 database (Kõljalg et al., 2013) was used after being aligned against itself using MUSCLE algorithm (Rognes et al., 2016). The aligned sequences were then eliminated if they did not align at the forward and reverse primers positions. The remanent contigs were later checked for chimeras using VSEARCH (Edgar, 2004) implemented in mothur. Non-chimeric sequences were affiliated taxonomically against the database of choice, MiDAS 2.0 or UNITEv6, through a kmer searching method of kmer size of 8 bp, and the sequences that could not affiliated with the domain of analysis were discarded. The remaining contigs were then clustered into OTUs through the abundance-based greedy clustering method (Westcott and Schloss, 2015; Schloss, 2016) in VSEARCH implemented in mothur. After clustering, singleton OTUs were removed from the samples, and the persistent contigs

within each OTU were used to create a taxonomic consensus for each OTU. The raw data from iTag sequencing process can be accessed in GenBank through SRP125026 accession number.

2.6. Study of sequencing coverage

Analysis of ecological coverage was conducted for the iTag sequencing samples using a redundancy abundance-weighted method (Rodriguez-r and Konstantinidis, 2014a). The redundancy abundance-weighted coverage analysis was calculated with rarified subsamples using Nonpareil software (Rodriguez-r and Konstantinidis, 2014b) through calculation parameters of 50% overlap, 95% identity, and query set size of 1000 sequences.

2.7. α-diversity of rarified subsamples

The calculation of α-diversity indices included the species richness (S), Shannon (H), Simpson (1-D), and equitability (J) indices. Whittaker and Williams indices were taken as β-diversity measurement for pairwise comparison of samples. The calculations of α- and β-diversity was done using the PAST v3.14 software using the information of the OTU community structure for each domain.

2.8. Similarity analyses of samples

Similarity analyses of samples were done in accordance with protocol done previously (Bian et al., 2017). For exploration of samples similarity, the OTU maps of the three

domains were used. These were first corrected to avoid zero-count problems utilizing the zero-count multiplicative method for a Bayesian multiplicative replacement of zero values through zCompositions package implemented in R. The zero-count corrected OTU maps were then transformed with a centered log-ratio using CoDaPack software. The transformed, corrected OTU maps were used for a singular value decomposition calculation, which results were represented through principal components plot. For a quantification of similarity in terms of OTUs abundance between inoculum and aerobic granular sludge samples, the OTU maps of the three domains were corrected for zero values and transformed through a centered log-ratio by generation of 128 Monte-Carlo Dirichlet simulations, which was then used for the computation of effect size differences, utilizing ALDEx2 package implemented in R.

2.9. Multivariate redundancy analyses (RDA)

Multivariate redundancy analyses were done to observe the linkage between bacterial, archaeal and fungal dominant OTUs with physicochemical parameters (COD removal, ammonium oxidation, total nitrogen removal, suspend solids), and the relationships between domains. The calculations of the RDAs were made by 499 unconstrained Monte-Carlo simulations under a full permutation model and run using the software CANOCO 4.5 for Windows.

3. Results and discussion

3.1. Operation and physicochemical parameters of SBRs

The start-up of the three aerobic granular sludge bioreactors (GAS, RAS and MAS) were done under the same operational conditions but with different geographical activated sludge as inoculum. Thus, differences between the active sludge granulation processes were studied. The performance of the three bioreactors during the operation time is shown in Figure 1.

The GAS reactor after 3 days of operation, showed an irregular sludge granular shape, and the wash-out of filamentous biomass was successful. After 15 days, regular and small granules were visible. The granules changed to a configuration with high density. MAS reactor after 1 day of operation showed visible granules inside activated sludge, although the wash-out of the biomass was high, so biomass was removed quickly. After 15 days of operation, a sharp decrease in the suspended solids concentration was observed. Thus, differences in conformation between GAS and MAS granules were observed in a short period of time. The surfaces of the granules in the GAS reactor were softness and more regular, however MAS granules had rougher and more filamentous surfaces. Finally, RAS reactor showed granules after 1 day of operation, although during the experimentation the granules became much smaller and fluffy, with a high amount of filaments in their surfaces.

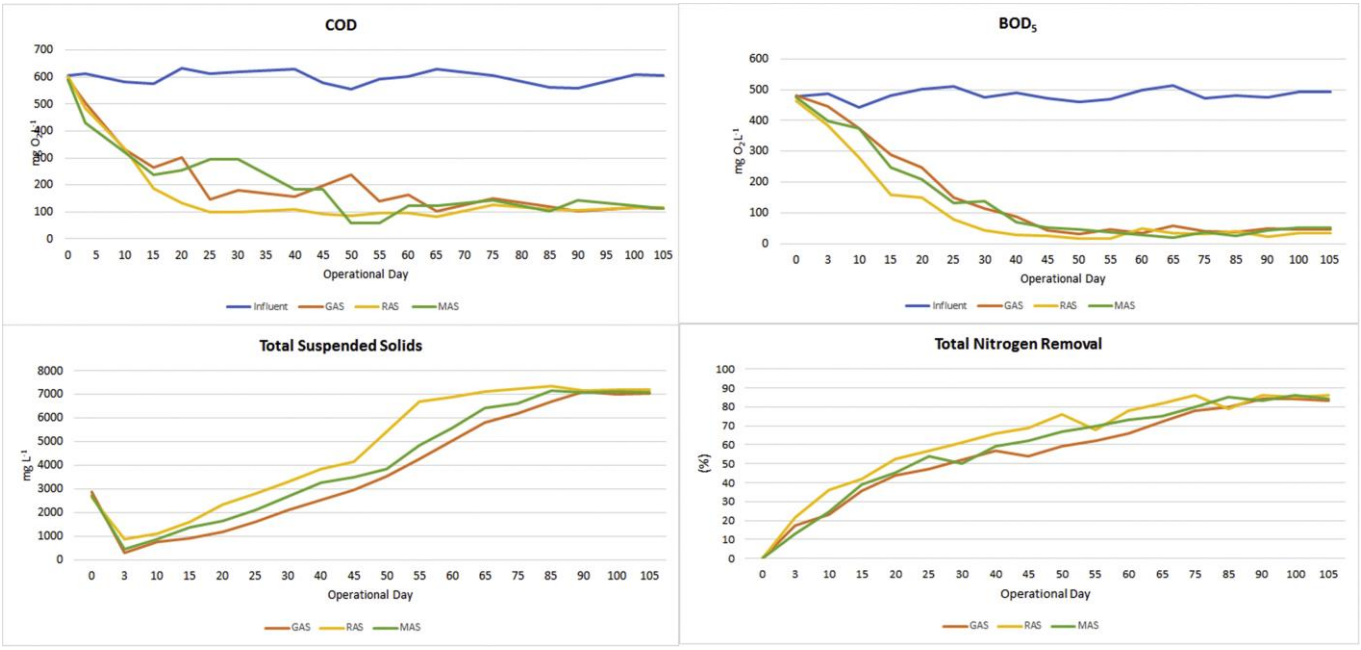

Figure 1. Physico-chemical performance of the AGS bioreactors during the operation time.

The three systems showed similar trends in terms of organic matter and total nitrogen removal, and in the evolution of suspended solids. The granulation process selected for biomass with high settle ability, generating a decrease of suspended solids concentration after 3 days of operation. From that day the three systems experienced an increase in biomass concentration nearly constant over operational time, which was related to the formation of granular biomass. The removal of organic matter and nitrogen increased during the whole operational time for all inocula, reaching a stability period with similar removal ratio for all bioreactors. In this sense, the mean removal performance of BOD_5 and COD were higher than 90% in all the bioreactors. The effluent total nitrogen concentration also appeared unaffected by the inoculum, showing similar mean values during all experimental periods. In this way, regardless of the inocula used, the performance of the systems was similar after 105 days of operation.

During the operation of the three systems, RAS showed the lowest loss of biomass in terms of suspended solids. This could be caused by adaptation of RAS inoculum to low temperatures, which would increase the density of its biomass and its settle ability, hence diminishing the loss of solids with respect to the mild adapted inoculum. The adaptation to low temperature could also explain the superior capacity of the RAS for organic matter and nitrogen removal during granulation process. RAS inoculum experienced operational temperature below 8 °C during winter and around 12 °C during summer (Gonzalez-Martinez et al., 2017). Thus, operation of cold temperature-adapted inoculum at 15 °C would increase its performance, while the opposite would occur when operating mild temperature-adapted inoculum at 15 °C. Nevertheless, initial differences in performance of the bioreactors disappeared over operation time around day 75, showing that all biomass could be used to form granules with high performance in organic matter and nitrogen removal. Despite of the inoculum used, the degradation performance and the granulation process selected for biomass was similar in the three bioreactors.

3.2. Quantitative real time PCR of bacterial and archaeal 16S rRNA and fungal 18S rRNA genes

The quantification of the bacterial and archaeal 16S rRNA and fungal 18S rRNA genes in the DNA pools subjected to qPCR is shown in Figure 2 and Table S1. A higher number of copies of bacterial 16S rRNA genes with respect to fungal 18S rRNA were found in the three bioreactors, especially in bioreactors GAS and MAS. Interestingly, the bioreactor

RAS showed two orders of magnitude higher fungal 18S rRNA gene copies and two orders of magnitude higher archaeal 16S rRNA gene copies than the other two bioreactors. This could be due to promotion of growth of *Fungi* and *Archaea* in cold environments, which has been reported in other studies involving aerobic granular sludge at low temperature (De Kreuk et al., 2005; Sarma and Tay, 2018). During the experimentation, the number of archaeal 16S rRNA gene copies decreased, possibly due to being displaced by other domain presented in the formation of the granules, while the number of fungal 18S rRNA copies was stable from start-up to stable conditions in the RAS bioreactor.

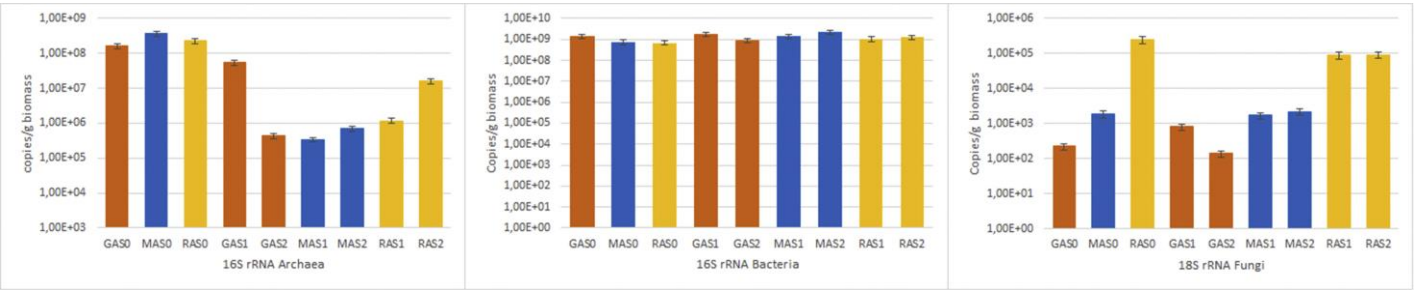

Figure 2. Quantitative real-time PCR measurements of 16S rRNA gene of *Archaea*, 16S rRNA gene of *Bacteria* and 18S rRNA gene of *Fungi* in the inocula and granular biomass.

The results of qPCR of Fungi in the DNA pools showed a variation between $2.23 \cdot 10^2$ and $2.44 \cdot 10^5$ copies per grams of biomass. The abundance of *Fungi* showed a strong differentiation between inoculum adapted at different temperatures, being evident a greater number of fungal 18S rRNA gene copies in RAS bioreactor, which was inoculated with activated sludge from Rovaniemi previously adapted to low temperature, suggesting that the fungal community has an essential role in cold environment as

corroborate Gunde-Cimerman et al., 2003 reported the abundant presence of Fungi at ultra-low temperature such as in permafrost, water or glacier ice.

The results of the qPCR measurements suggested that the process of granular biomass formation reduced severely the archaeal population of the systems. On the other hand, *Bacteria* and *Fungi* representatives could adapt to biomass washout and their numbers remained in the system. In this sense, the performance of the aerobic granular SBR would be related to bacterial and fungal populations mainly. The loss of *Archaea* might be caused, among others, by inability to compete with fast-kinetics bacterial phylotypes, incapacity to growth in the presence of competitors within granular biomass, or inadequacy of archaeal phylotypes to bind to granular biomass by EPS production.

3.3. Study of sequencing coverage of iTag sequencing subsamples

The values for the analysis of redundancy abundance-weighted coverage are shown in Table S2. The lowest expected coverage was higher than 98%. Also, the mean effort ratio for complete coverage was less than 5% of total coverage, about 5.6 Mbp mean value for a total of around 69 Mbp mean value. In this sense, the results showed that the massive parallel sequencing captured successfully the archaeal, bacterial and fungal community structure of the biomass samples analyzed. *Fungi* was the best captured domain, followed by *Archaea*.

3.4. α- and β-diversity of iTag sequencing samples

The values of the α-diversity indices for biomass samples showed higher diversity and evenness (Table S3). The values of the Shannon index and species richness were higher in GAS0 and MAS0 than in all other samples. Their values decreased when the inocula granulated as can be seen in the samples in the day 30 (GAS1, MAS1) and in the day 105 (GAS2, and MAS2), indicating a loss of microbial diversity caused by the adaptation of inocula biomass to the aerobic granular SBR operation. The evenness also followed the same trend, as stated by the dynamics of the Simpson and equitability indices, getting lower values as the biomass granulated. Thus, microbial adaptation to granular biomass from inocula showed that only a few phylotypes could adapt, therefore suggesting that granules had lower diversity and stronger dominance of certain phylotypes in comparison with the inoculum.

The β-diversity estimates based on Whittaker and Williams reported higher dissimilarities between the inocula and the granular biomass than among granular biomass themselves (Figure S2). This highlighted that the OTU distribution was more similar for granular biomass formed from different inocula than for granular biomass and its inoculum. Thus, microbial adaptation from inocula to granule was accompanied by the proliferation of several phylotypes that were present in the mature granular biomass regardless of the inoculum used.

The α- and β-diversity analyses demonstrated that the granulation of biomass selected for a few phylotypes, which accounted for a high representation in the granules, and that were similar among them regardless of the inocula used for its formation. This

showed the strong selection of biomass of the aerobic granular SBR and suggested that the microbial composition of the granular biomass depends more on technological configuration than on inoculum community structure.

3.5. Similarity analysis of Archaea, Bacteria and Fungi in the aerobic granular biomass formed from different inocula

The ordination plots showing the similarities of *Archaea*, *Bacteria* and *Fungi* in the bioreactors demonstrated that, for the three domains, the community structure was similar at the stages of granular biomass (Figure 3). This trend was more evident for *Archaea* and *Bacteria*, which differed greatly from the inoculum to the granule communities. The clusterization of *Archaea* and *Bacteria* samples at granular biomass stages, and the distance encountered with the three inocula, suggested that communities of *Archaea* and *Bacteria* were very similar for granular biomass regardless of the origin of the inoculum used. This was less clear for *Fungi*, in which almost all samples reflected a high similarity, even in the inoculum.

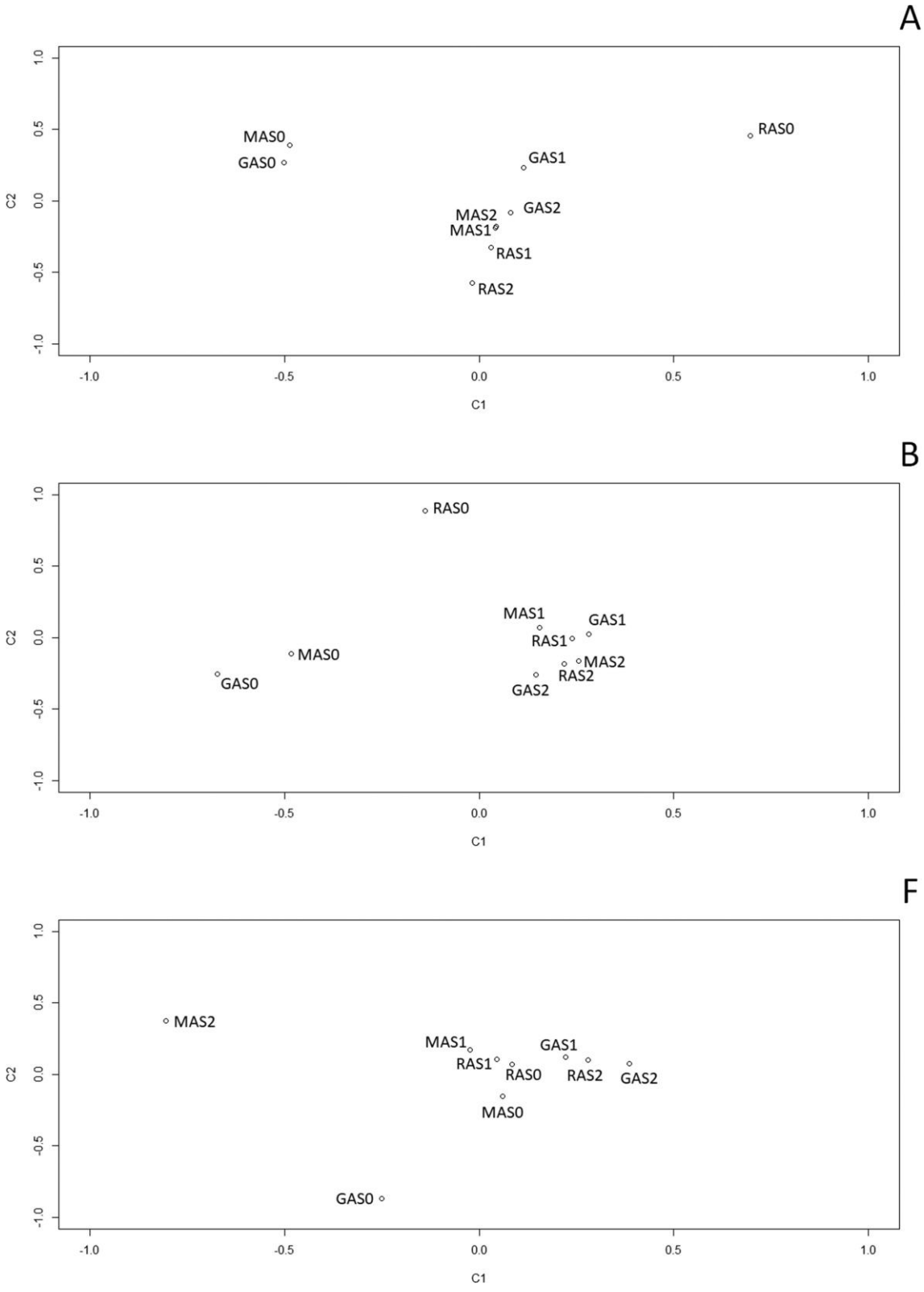

Figure 3. Principal components analysis plot of the singular value decomposition values from the centered log-ratio transformation of the OTU map of *Archaea* (A), *Bacteria* (B) and *Fungi* (F) domain.

The differences among the dominant OTUs in the archaeal, bacterial and fungal assemblages also highlighted the differences and similarities between inocula and granular biomass (Figure 4). The expected effect size difference for *Fungi* showed that only one dominant OTU was statistically different between the inocula and the granules, remarking the similarity in the different biomass in terms of dominant fungal phylotypes. On the other hand, 4 dominant OTUs for *Archaea* and 18 dominant OTUs for *Bacteria* had statistically significant differences in relative abundance between the inocula and the granular biomass.

In this way, the analyses of samples similarity reported that *Fungi* from the inocula, which were relatively similar, mainly remained when the biomass granulated, and that granulation of biomass selected for similar phylotypes for *Archaea* and *Bacteria* regardless of the inocula used. This fact may indicate that granulation of biomass is related to a certain presence of archaeal and bacterial phylotypes, which could improve our understanding on the granulation process.

Granulation of biomass in aerobic granular SBR under different organic loading rates also showed a similar bacterial community structure in the granules developed from very different bacterial communities in the inocula used (Szabó et al., 2017a). In this sense, there are evidences that the stable granular biomass communities are defined by the aerobic granular SBR operational conditions regardless of the diversity of the inoculum.

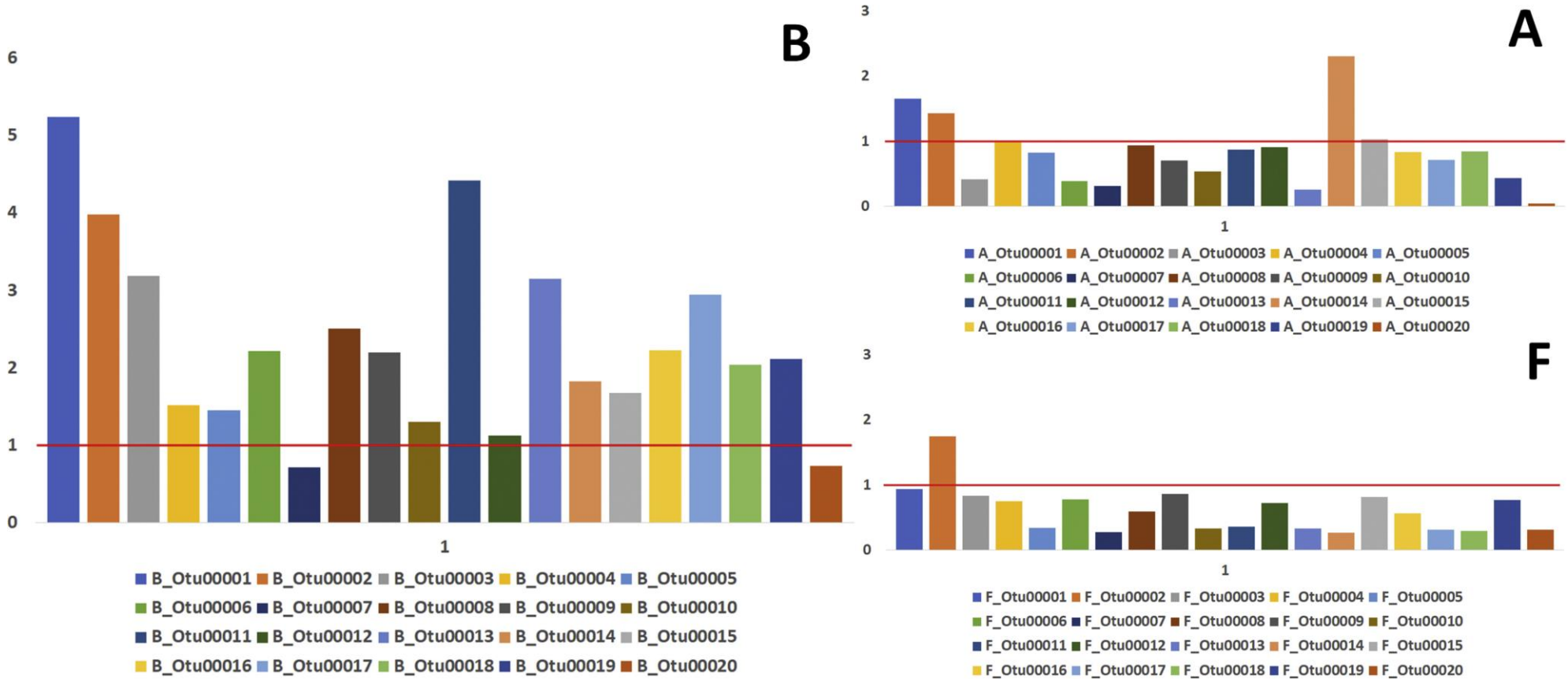

Figure 4. Expected effect size of the dominant OTUs of *Archaea* (A), *Bacteria* (B) and *Fungi* (F) domains.

3.6. Community structure of dominant archaeal, bacterial and fungal OTUs at the inocula and granular biomass

3.6.1. *Archaea* domain

Among archaeal OTUs, those with greater importance of the granular biomass were A_Otu00001, A_Otu00002 and A_Otu00004, affiliated with *Thermoproteaceae* crenarchaeal family, and additionally A_Otu00003, an unclassified *Euryarchaeaota* especially dominant in GAS1 (Figure 5A). On the other hand, the most represented archaeal OTUs in the inocula were affiliated to *Methanospirillum* and *Methanosarcina* form the RAS0, and a *Methanococcales* member, some unclassified *Euryarchaeota* and NRP-Q representative for the GAS0. The samples of granular biomass were similar regardless of the difference of the inocula, which is in accordance with the results obtained from similarity analyses.

The disappearance of methanogenic archaea from inoculum to granular sludge has been previously reported (Liu et al., 2017). However, this is the first time that members of *Thermoproteaceae* family have been found as dominant in aerobic granular sludge. The sulfur metabolism associated with *Thermoproteaceae* could promote its growth in the aerobic granular sludge. Thus, possibly *Thermoproteaceae* could find syntrophic relations with other bacterial phylotypes, as it has been reported in subsea floor mats (Itoh, 2014).

3.6.2. *Bacteria* domain

The dominant bacterial OTUs in the inocula were affiliated with different *Comamonadaceae* members, *Thauera* and *Thiothrix* (Figure 5B). Nevertheless, these phylotypes disappeared, with dominant OTUs B_Otu00001 through B_Otu00004, related to *Meganema*, *Sphingobacteriales* env.OPS17, *Thauera* and an α-Proteobacteria DB1-14 member, becoming of importance in the granular biomass. Among these, *Meganema* and the *Sphingobacteriales* env. OPS17 were present at both granular biomass samples, being the most important bacterial OTUs in granular biomass. The similarity of bacterial communities at granular biomass stages, independently of the differences found in the inocula found, were correlated with the results from similarity analyses.

Dominant bacterial OTU in granular biomass was related to *Meganema*, which has been reported in aerobic granular bioreactors and related with an increase of the granule size and their further breakage (Figueroa et al., 2015). The results obtained seemed to support the hypothesis that filamentous phylotype *Meganema* was of extreme importance for the formation of aerobic granular sludge, regardless of the origin of the inocula used. In addition to its filamentous nature, *Meganema* has been reported to complete organic carbon storage and denitrification in aerobic granular sludge SBR systems (Szabó et al., 2017a). The relative presence of *Meganema* and *Thauera* has been linked to organic loading rate, suggesting that these microorganisms compete with each other for organic carbon and that therefore are involved in organic matter degradation in these systems (Szabó et al., 2017a). In this context, *Meganema* would compete with

Thauera for the same substrates, as *Thauera* has been reported for similar metabolisms in aerobic granular sludge system (Szabó et al., 2017a). Their high relative abundance has been found by other authors, suggesting that these bacterial genera had a very fast growth rate in granular biomass systems (Szabó et al., 2017b).

3.6.3. *Fungi* domain

The dominant fungal OTU at all biomass samples was F_Otu00001, which was classified as a *Trichosporonaceae* member (Figure 5F). This was in accordance with the results from the similarity analyses. Its presence at that high relative abundance in GAS0 and RAS0 suggested that *Trichosporonaceae* members could be ubiquitous in wastewater treatment systems. The presence of this OTU at very high relative abundance in all granular biomass samples also showed that *Trichosporonaceae* could adapt to granular biomass conditions and even becoming important for granular biomass formation due to the network of fungal community provides an increase of area available for bacterial colonization.

Fungal populations improve the settling of activated sludge biomass by formation filaments that trap flocs and by generation of EPS that binds flocs among them (Avella et al., 2014), also the production of EPS by fungi in aerobic granular sludge was reported by Weber et al. (2007).They also have been considered to aid in the formation of granular biomass (Gonzalez-Martinez et al., 2017), so fungal was data as the core in the granule and as precursors of the structure of the biofilm in order to immobilize the bacterial matrix (Weber et al., 2007). Considering the qPCR measurement results that

showed fungal populations remaining stable from the inoculation to stable granular biomass, the persistence of Fungi members may be attributed to the inability of the system to remove them in terms of discard of suspended biomass. Therefore, it is possible that the persistence of the *Trichosporonaceae* OTU F_Otu00001 was more related to the operation of the system that to the ecological role of this fungal phylotype in the bioreactor.

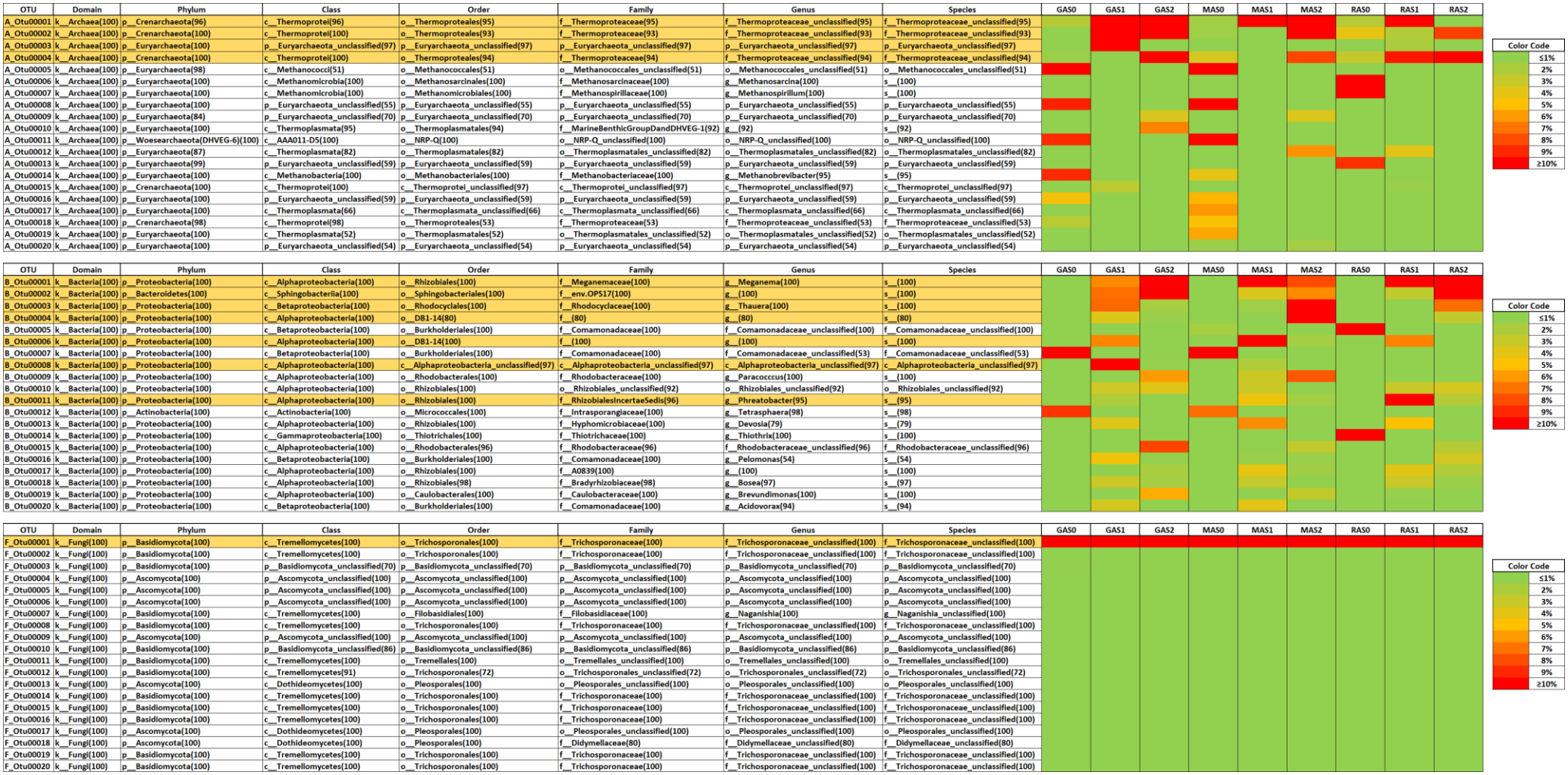

Figure 5. OTU heat map of *Archaeal*, *Bacterial* and *Fungal* populations in the AGS bioreactors.

3.7. Oligotype structure of dominant OTUs in granular biomass

The oligotype structure of dominant archaeal OTUs A_Otu00001, A_Otu00002, A_Otu00003 and A_Otu00004 differed significantly (Figure 6). Nevertheless, in all cases, certain oligotypes predominated within the OTUs structure at the stage of granular biomass. This finding supported the hypothesis that there are certain evolutional changes within the OTUs, promoting the proliferation of certain oligotypes that either would help granulation of biomass or would be benefitted from it.

Differently as with *Archaea* domain, the oligotype structure of dominant bacterial OTUs showed a high oligotype diversity and evenness at granular biomass (Figure 6). Thus, it is possible that dominant OTUs of *Bacteria* domain could successfully proliferate to granular biomass configuration and therefore no certain oligotypes were related with granular biomass configuration.

The dominant fungal OTU consisted on a single, massively represented oligotype that was found at all stages during the experiment, regardless of the origin of the inocula used (Figure 6), which showed to be ubiquitous, highlighting its importance in granular biomass. A search of the oligotype representative sequence in the GenBank nr database yielding an uncultured *Tremellomycetes* FM178264 found in aerobic sewage granular sludge (Weber et al., 2009) as the highest-ranked match (max score and total score 335, 100% query cover, 98% identity and $3e^{-88}$ e-value). This fact supported the influence of *Tremellomycetes* as important phylotypes for the ecology of granular biomass, possibly due to its good settling properties.

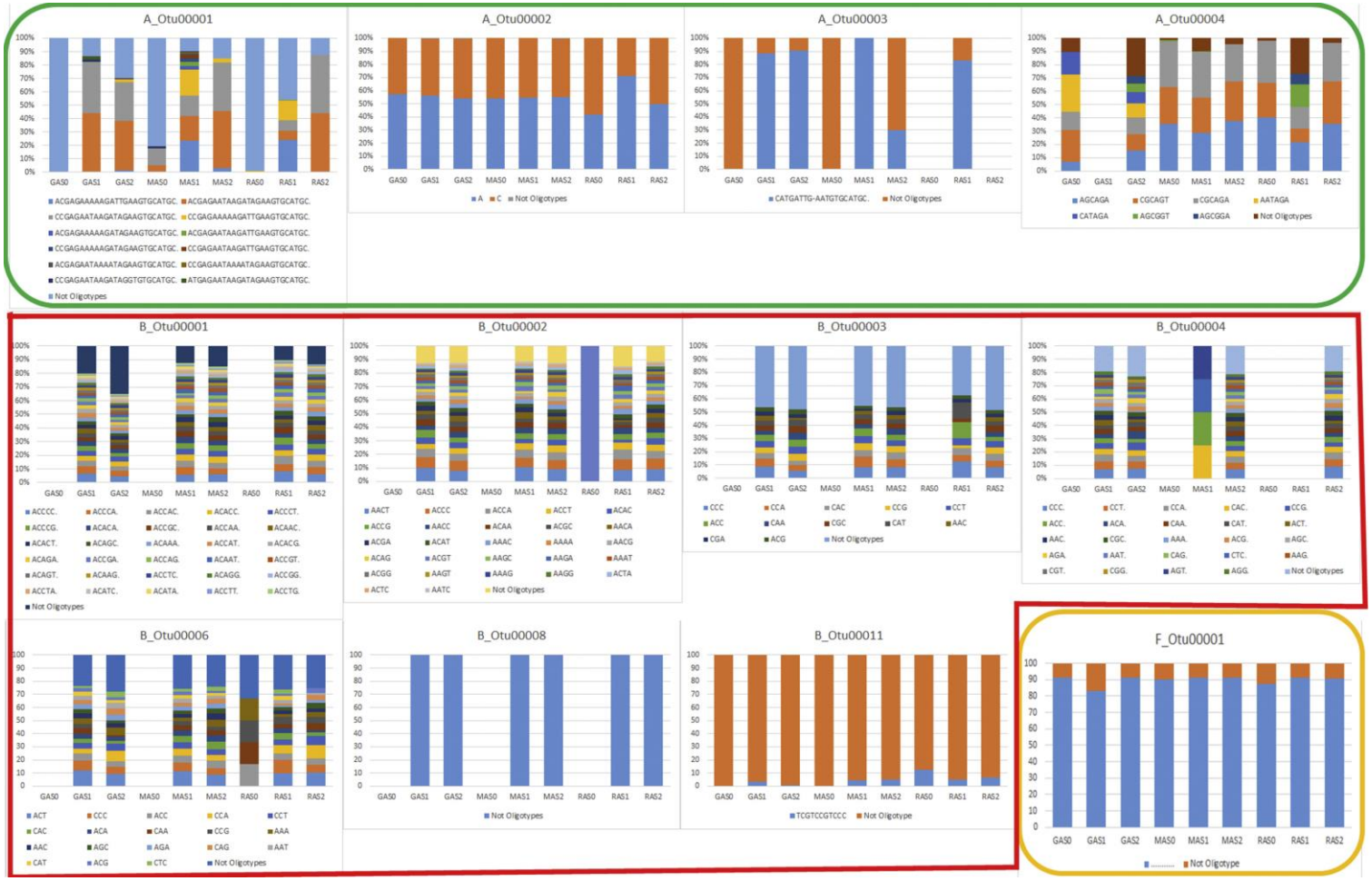

Figure 6. Oligotypes distribution for the dominant OTUs of Archaea, Bacteria and Fungi for the *Archaea* (green globe), *Bacteria* (Red globe) and *Fungi* (yellow globe). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

3.8. Multivariate redundancy analyses

3.8.1. Linkage of dominant archaeal, bacterial and fungal OTUs, and bioreactors performance

The high dominance of fungal OTUs at any given instant in the experimentation supported that no syntrophies would be set between F_Otu00001 and dominant archaeal and/or bacterial OTUs, as shown by multivariate redundancy analyses (Figure 7A and B). On the other hand, effective linkages were observed between dominant archaeal and bacterial OTUs, indicating possible inter-domain relationships (Figure 7C). B_Otu00001, related to *Meganema*, showed positive correlation with all archaeal dominant OTUs. On the other hand, the three *Thermoproteaceae*-belonging OTUs showed different affiliation with bacterial OTUs. A_Otu00001 was strongly and positively correlated with the α-*Proteobacteria* members DB1-14 and *Phraetobacter*; on the other hand, A_Otu00002 and A_Otu00003 were strongly and positively correlated with the *Sphingobacteriales* env. OPS17, *Thauera* and another α-*Proteobacteria* DB1-14 phylotype. The uncultured *Euryarchaeaota* representative was linked positively with the unclassified α-*Proteobacteria* OTU. It is relevant to notice that correlation among dominant *Thermoproteaceae* dominant OTUs was rather independent, suggesting that the prevalence of one over another was not driven by competition among them but by the differential presence of the dominant bacterial OTUs.

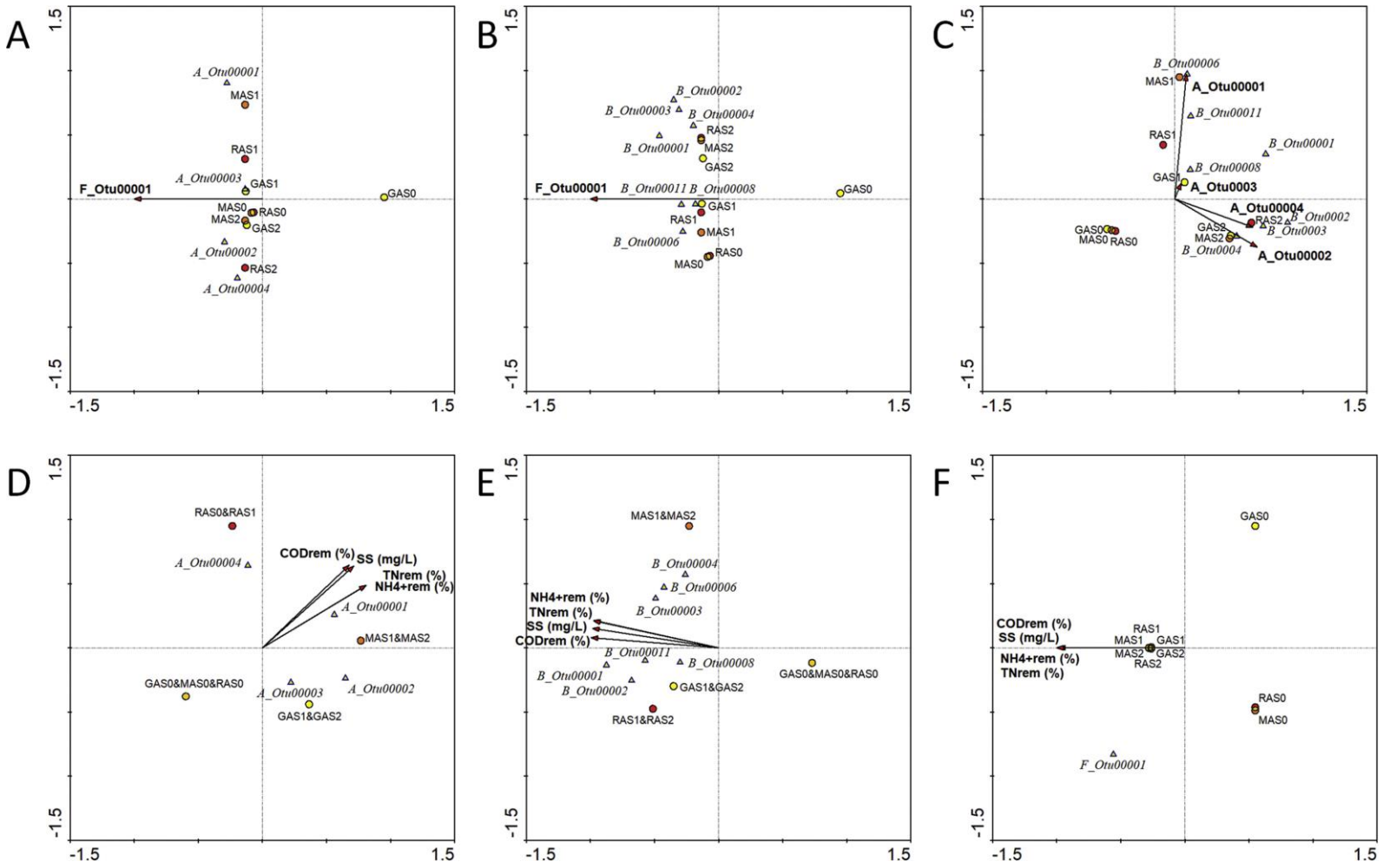

Figure 7. Multivariate redundancy analyses of syntrophic relationships between *Archaea* and *Fungi* (A), *Bacteria* and *Fungi* (B), *Archaea* and *Bacteria* (C), as well as linkages between *Archaea*, *Bacteria* and *Fungi* with operational conditions of the bioreactors (D, E and F), respectively.

Again, the massive presence of F_Otu00001 in all samples supported that its presence was not linked to the removal capacity of the systems (Figure 7F). On the other hand, the dominant archaeal and bacterial OTUs were positively correlated with COD, NH_4^+ and TN removal efficiencies, showing that these phylotypes have the metabolic capabilities that either make them able to remove organic matter and nitrogen effectively or make them work syntrophically with other phylotypes that could (Figure 6D and E). *Meganema* and the most represented *Thermoproteaceae* OTU were the bacterial and archaeal phylotypes with highest positive correlation with systems' efficiency, but overall all other OTUs, except A_Otu0003, were strongly correlated with performance. Nevertheless, qPCR measurements suggested that *Bacteria* was the domain that most contributed to the performance of the system given their higher representation in comparison with *Archaea*.

4. Conclusions

Three AGS were startup with different inocula adapted to mild and cold temperature. The degradation process in terms of organic matter and total nitrogen removal was high regardless inocula used. The massive parallel sequencing analysis showed a similar dominant phylotypes in all the aerobic granular bioreactors despite of the inoculums location, which suggest that the operational conditions determine the phylotypes diversity of the bacterial, archaeal and fungal communities. Mature granules showed higher number of copies of bacterial and fungal target rRNA genes than in the inocula, suggesting an important role of these domains in the stability and conformation of

granule. However, the archaeal community didn't show an essential role in the granule due to decrease in the number of copies from inocula samples. Dominant archaeal OTUs in the granular biomass were related to *Thermoproteaceae* family, and dominant bacterial OTUs was related to *Meganema*. For *Fungi*, a *Tremellomycetes* related OTU clearly dominated all samples. Oligotyping analyses of the dominant archaeal, bacterial and fungal OTUs suggested that a fungal oligotype was dominant in all cases, while archaeal OTUs oligotype structure showed an adaptation from inocula to granular biomass and bacterial OTUs oligotype distribution showed high diversity and evenness in oligotypes. Furthermore, multivariate redundancy analyses showed that dominant bacterial and archaeal OTUs were positively correlated with bioreactor performance and that inter-domain syntropies could be hinted. These results suggested that, regardless of the inocula used, the microorganisms that developed in AGS systems operated under the same conditions were similar and were not affected by the temperature of origin. Therefore, the observed *Meganema* and *Trichosporonaceae* phylotypes could be of crucial importance for the formation and stabilization of granular biomass. The results of our study could be useful for the implementation of AGS system at full-scale in different latitude increasing the knowledge of the granulation process in AGS started up with inoculants previously acclimated at different temperatures.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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Supplementary data

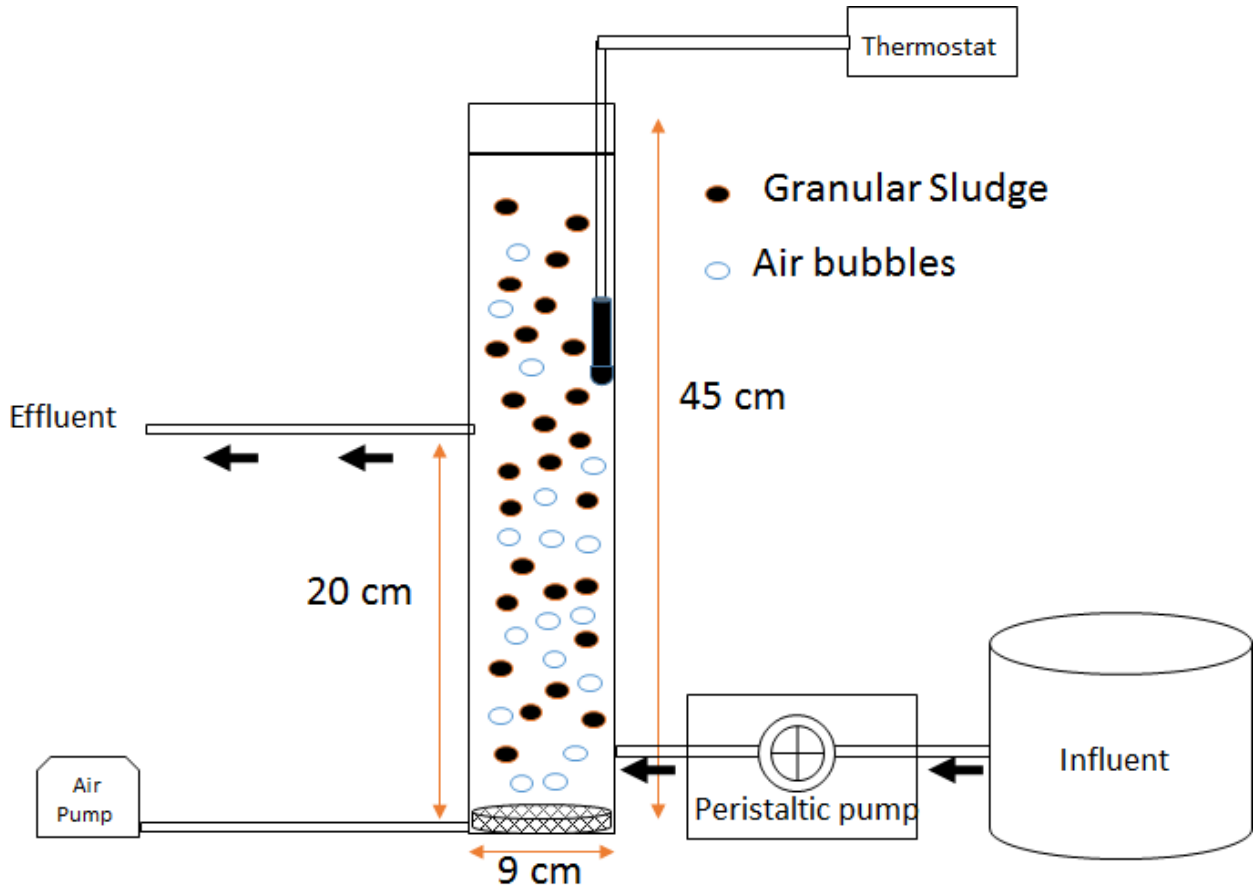

Figure S1. Sequential bath AGS bioreactors scheme.

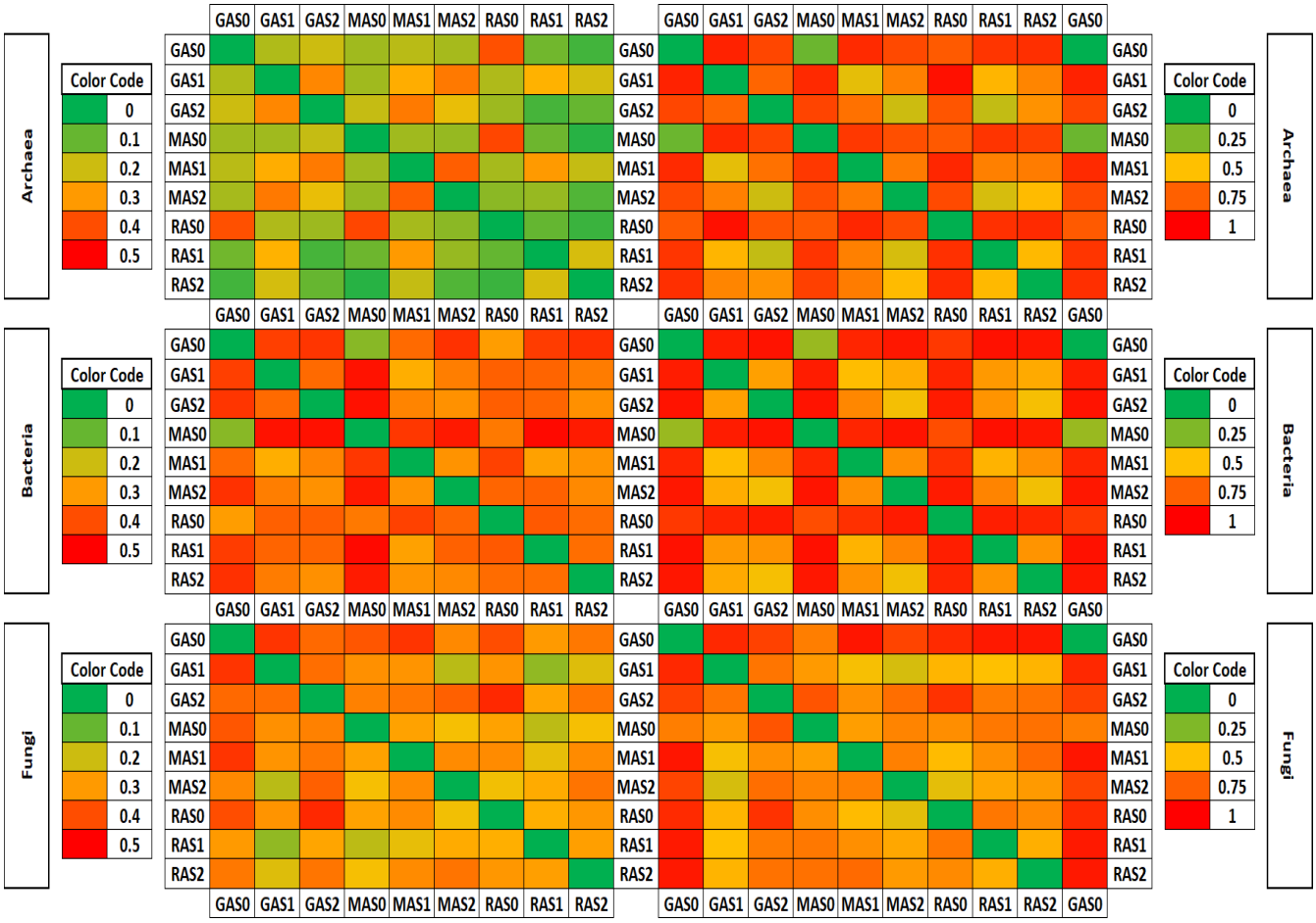

Figure S2– Heat map of Williams (left column) and Whittaker (right column) β-diversity indices.

Table S1 -Number of copies of bacterial and archaeal 16S rRNA and fungal 18S rRNA in DNA pools from the bioreactors analyzed based on quantitative PCR and expressed as number of gene copies per grams of granules.

	Bacterial 16S rRNA	Archaeal 16S rRNA	Fungal 18S rRNA
GAS0	1.44E+09	1.61E+08	2.23E+02
MAS0	7.52E+08	3.62E+08	1.86E+03
RAS0	6.86E+08	2.25E+08	2.44E+05
GAS1	1.74E+09	5.52E+07	8.10E+02
GAS2	8.76E+08	4.37E+05	1.39E+02
MAS1	1.39E+09	3.39E+05	1.69E+03
MAS2	2.22E+09	6.94E+05	2.15E+03
RAS1	1.07E+09	1.18E+06	8.85E+04
RAS2	1.29E+09	2.E+07	9.09E+04

Table S2. Results of the redundancy abundance-weighted coverage analysis of massive parallel sequencing samples at operational days 0 (RAS0, GAS0 and MAS0), 30 (RAS1, GAS1 and MAS1) and 105 (RAS2, GAS2 and MAS2).

Domain	Sample	Estimated Coverage (%)	Actual effort (Mbp)	Required effort (Mbp)	Effort ratio (%)
Archaea	**GAS0**	99.03	4.844	-	-
	GAS1	100	29.82	-	-
	GAS2	99.99	21.6	-	-
	MAS0	99.62	8.77	-	-
	MAS1	100	113.9	4.953	4.348551361
	MAS2	99.87	18.62	-	-
	RAS0	98.46	9.957	-	-
	RAS1	99.1	1.389	-	-
	RAS2	99.55	3.881	-	-
Bacteria	**GAS0**	99.24	52.34	2.917	5.573175392
	GAS1	99.55	48.74	-	-
	GAS2	99.36	34.86	-	-
	MAS0	99.29	31.79	1.949	6.130858761
	MAS1	99.49	30.33	-	-
	MAS2	99.27	46.47	-	-
	RAS0	98.56	33.12	1.824	5.507246377
	RAS1	99.59	19.74	-	-
	RAS2	99.23	56.77	-	-
Fungi	**GAS0**	100	26.53	-	-
	GAS1	100	120.5	5.348	4.438174274
	GAS2	100	180.4	7.85	4.351441242
	MAS0	100	105	4.41	4.2
	MAS1	100	181.7	7.745	4.262520638
	MAS2	99.84	188.9	8.131	4.304393859
	RAS0	100	146.2	6.425	4.394664843
	RAS1	99.84	108.6	5.048	4.64825046
	RAS2	99.94	242.3	10.41	4.296326868

Table S3. - α-diversity of *Bacteria*, *Archaea* and *Fungi* domain

Bacteria					
Sample	Simpson	Shannon-	Pielou´s	Berger-Parker	Chao-1
RAS 0	0.712	2.188	0,029	0,5	426,3
RAS 1	0,7347	2,369	0,026	0,491	573,6
RAS 2	0,735	2,38	0,026	0,491	624,8
GAS 0	0,961	4,273	0,191	0,115	428,7
GAS 1	0,777	2,349	0,025	0,329	602,9
GAS 2	0,769	2,15	0,021	0,329	661,1
MAS 0	0,965	4,307	0,243	0,107	307,3
MAS 1	0,776	2,329	0,025	0,329	609,8
MAS 2	0,773	2,218	0,022	0,329	609,4

Archaea					
Sample	Simpson	Shannon-	Pielou´s	Berger-Parker	Chao-1
RAS 0	0,273	0,6765	0,151	0,849	20,5
RAS 1	0,73	1,625	0,211	0,413	42,33
RAS 2	0,651	1,482	0,118	0,457	56,43
GAS 0	0,684	1,364	0,156	0,415	43,2
GAS 1	0,522	1,219	0,120	0,662	34
GAS 2	0,720	1,899	0,128	0,485	79,27
MAS 0	0,819	2,166	0,218	0,328	48,25
MAS 1	0,298	0,880	0,068	0,835	54,43
MAS 2	0,724	1,756	0,148	0,416	66,2

Fungi					
Sample	Simpson	Shannon-	Pielou´s	Berger-Parker	Chao-1
RAS 0	0,516	0,960	0,074	0,648	103
RAS 1	0,032	0,1	0,065	0,983	35
RAS 2	0,047	0,145	0,041	0,975	67
GAS 0	0,816	1,999	0,069	0,251	153,4
GAS 1	0,054	0,172	0,038	0,972	42
GAS 2	0,086	0,262	0,039	0,955	44,25
MAS 0	0,582	1,172	0,047	0,580	118,8
MAS 1	0,058	0,174	0,037	0,97	37,5
MAS 2	0,073	0,207	0,041	0,962	46,5

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Chapter II

Performance and microbial community structure of aerobic granular bioreactors at different operational temperature

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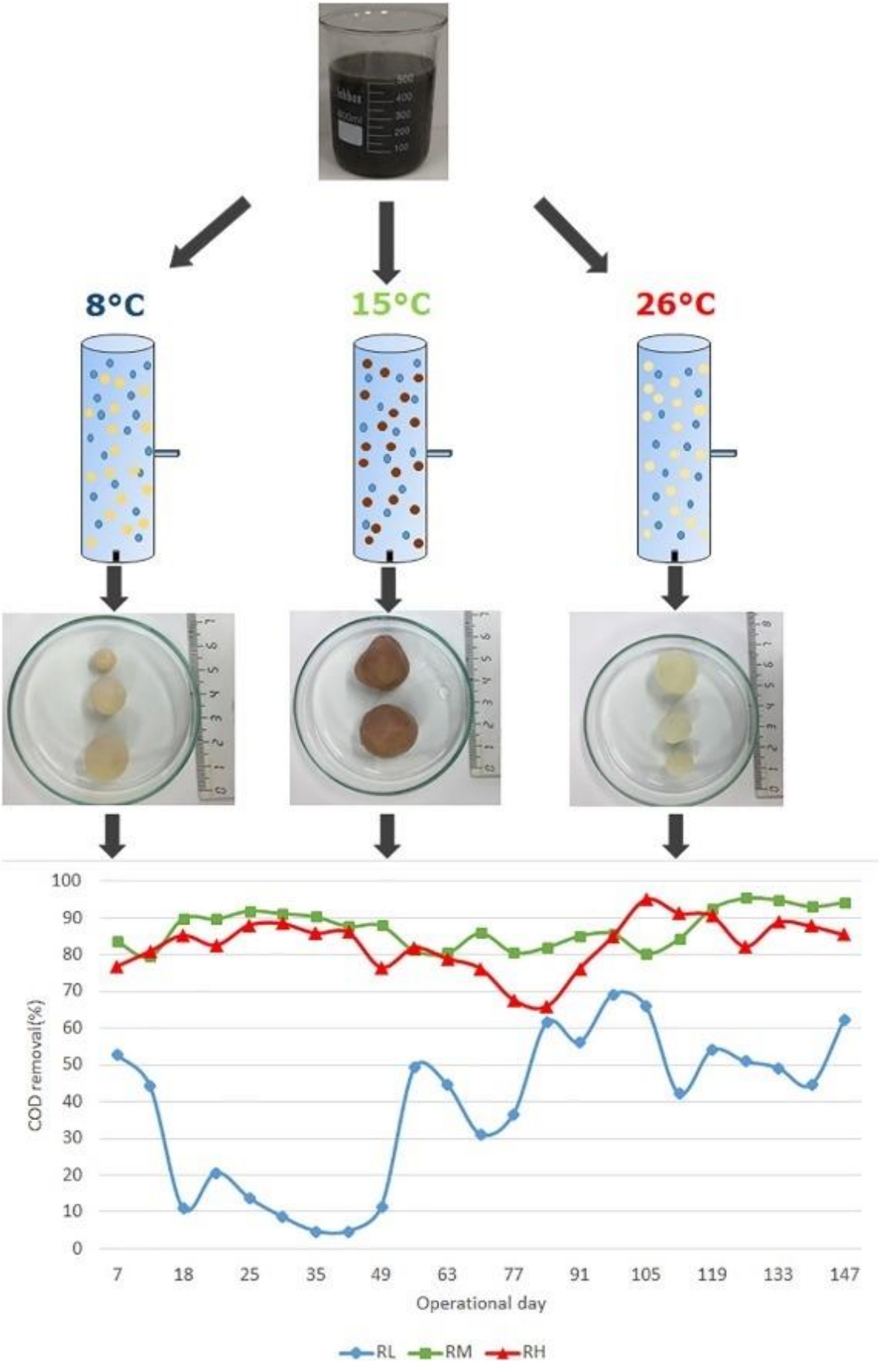
Abstract

Three lab-scale sequential batch reactors were operated under aerobic granular sludge technology for 150 days. The reactors were inoculated with mild temperature-adapted activated sludge from Granada (WWTP) in order to evaluate the microbial dynamics, the granulation process and the performance of the systems under low, mid and high temperature. The faster start-up was achieved at mid temperature at operational day 6, followed by warm temperature at day 10, with nitrogen and organic matter removal ratio higher than 90%. Conversely, the reactor under cold temperature produced granular biomass but the nitrogen and carbon removal ratios did not achieve good performance, close to 80%. The dynamics of Prokarya communities showed that *Corynebacterium* phylotype was present in all bioreactor despite temperature, although the microbial diversity at mid and warm temperature was higher than at cold temperature. The Eukarya communities in mature granules were represented by *Pezizomycotina* fungi and *Oligohymenophorea* ciliate.

Keywords: AGS; wastewater treatment; prokaryotics and eukaryotic community; qPCR; massive parallel sequencing; inoculum adaptation.


8°C
15°C
26°C
100
90
80
70
60
50
40
30
20
10
0
COD removal(%)
7
18
25
35
49
63
77
91
105
119
133
147
Operational day
RL
RM
RH

1. Introduction

Increasing wastewater discharge events are a global problem that concerns human health and ecosystems such as freshwater bodies. Wastewater is formed by a variety of contaminants such as nitrogen, phosphorous and diverse emergent contaminants such as pharmaceuticals or endocrine disruptors (Pal et al., 2010). The treatment of wastewater is currently based on conventional activated sludge (CAS) technology, which does not have the potential to remove the increasing concentrations of nutrients (N and P) derived from the human population or pollutants of emerging concern (Ndulini et al., 2018). CAS technology was developed for enhanced nutrients removal performance, but the related high costs of a building site, operation and aeration makes this option not environmentally sustainable (Bajracharya et al., 2016), thus, the development of new wastewater treatment technologies is increasing. In the recent years, the aerobic granular sludge systems (AGS) have produced an improvement for urban and industrial wastewater treatment plants (WWTPs), mainly caused by saving costs. This technology has some advantages over CAS technology due to higher biomass concentration and retention capacity, more efficient nutrient removal, lower sludge production, resistance against harmful pollutants, lower operational volume and no need of secondary sedimentation (Henriet et al., 2016; Muñoz-Palazon et al., 2018). Granular sludge is an aggregation of microorganisms wrapped in extracellular polymeric substances that have a crucial role to conform to a tridimensional matrix that will form the granule and permit the growth of microbiota (Wang et al., 2018).

The estimation of the temperature range in which microorganisms are able to grow is very important for the start-up and successful exploitation of full-scale WWTPs given that temperature has a key role in the activation or inhibition of microbe metabolisms (Henze et al., 2000). Thus, the weather is an important factor to consider because it affects wastewater temperature and with it the performance of full-scale WWTPs. Seasonal changes should be considered in the start-up period because they cause strong alteration in operational temperature and, consequently, in dissolved oxygen concentration. The high range of temperature variation between seasons, latitudes or regions forced the microorganisms to find mechanisms of adaptation. Accordingly, even though biological nitrogen removal of mild-temperature (20 °C) adapted biomass becomes inhibited when the temperature drops to cold levels (~10 °C) (Gnida et al., 2016), although biological processes reach high removal efficiencies in cold regions (Gonzalez-Martinez et al., 2018a). In agreement with this, some authors have reported that microbes can adapt to a changing environment, including extreme temperatures, but on the contrary, other highly specialised species may be vulnerable to environmental fluctuations (Hawkes et al., 2015). Thus, it is important to know how species present in aerobic granular sludge systems respond to the alteration of temperature in order to understand the ecological role and versatility of granule-forming microbial communities and, as a consequence, how they affect the performance of this technology.

It is of special interest to analyse the microbial dynamics and the performance of aerobic granular sludge bioreactors at cold, mid and warm temperatures, starting from an initial microbial structure native to mid temperature environments (14−16 °C). This knowledge

will help in the implementation of full-scale aerobic granular sludge reactors in regions or latitudes with high temperature variation due to seasonal changes.

2. Materials and methods

2.1. Description of sequential batch bioreactors and operational conditions

The three systems were designed as cylindrical sequential batch reactors (SBR) with a 9 cm diameter and 45 cm height (Figure S1). The bioreactors were operated for 150 days. Each bioreactor was inoculated with 500 mL of activated sludge collected from Los Vados WWTP (37°10'41"N, 3°36'03"W), located in Granada, Spain. The air was controlled and introduced via fine bubbles at the bottom of the bioreactors. The volume exchange ratio was 60%, and the bioreactor operated in a 4 h cycle with 6.6 h of hydraulic retention time. The temperature was set at 8 °C (RL), 15 °C (RM) and 26 °C (RH) by operating inside a temperature-controlled chamber. The dissolved oxygen was controlled at 7.4 ± 0.5 mg L^{-1} for all bioreactors. The cycle consisted of: 33 min of feeding, 200 min of aeration, 3 min of settling and 4 min for effluent discard. During the start-up stage, the time of settling was changed from 10 min during the first three days to 5 min during the first week, and finally to 3 min after eight days of operation (Muñoz-Palazon et al., 2018). Changes in settling time were controlled for the wash-out of filamentous microorganisms and the selection of granule-forming microbes. The procedure of wash-out of biomass during the startup thought settling velocity has high relevance for the formation and stability of granular biomass during the operation of bioreactors. The bioreactors were fed with synthetic wastewater simulating urban

sewage composed by sodium acetate 0.9 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.1 g L^{-1}; KCl 0.04 g L^{-1}; NH_4Cl 0.25 g L^{-1}, KH_2PO_4 0.03 g L^{-1}, K_2HPO_4 0.085 g L^{-1} and 0.1 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} (NH_4) $Mo_7O_2 \cdot 4H_2O$; 0.03 mg L^{-1} $CuSO_4 \cdot 5H_2O$; and 0.15 mg L^{-1} $CoCl_2 \cdot 6H_2O$ (Gonzalez-Martinez et al., 2018a)

2.2. Determination of physicochemical parameters

The chemical oxygen demand (COD) and biological oxygen demand at day 5 (BOD_5) were monitored three times a week following established protocols (APHA, 2012). The settling velocity and the mean size of granules were measured according to the protocol developed by Gonzalez-Martinez et al. 2018B. The concentrations of the inorganic nitrogenous compounds (NH_4^+, NO_2^-, NO_3^-) were measured in duplicate for the influent and effluent using ionic chromatography. The pH, dissolved oxygen and temperature were measured using probes and a Crison digital meter.

2.3. Collection of biological samples, nucleic acids extraction and next-generation sequencing

Samples of mixed liquor and the granular biomass of each bioreactor were taken on operational days 15, 30, 45, 90 and 150, following sampling protocols described in Gonzalez-Martinez et al. (2017). Thus, biological samples were treated to extract nucleic acids using the FastDNA SPIN Kit for Soil. Then DNA pools were kept at −20 °C and 90 µL

was sent to RTL Genomics Laboratory (Lubbock, TX, USA) for next-generation sequencing procedure.

The primer pair Bac357-Bac806 was used for the amplification of the hypervariable regions of the 16S rRNA gene of prokaryotes. The primer pair EUK1391-EUKbr was used for the amplification of the hypervariable regions of the 18S rRNA gene of eukaryotes (Rodriguez-Sanchez et al., 2018)

2.4. Bioinformatics pipeline of next-generation sequencing data

The raw data obtained from sequencing the 16S rRNA gene of prokaryotes and 18S rRNA gene of eukaryotes was analyzed using the software mothur v1.39.5 (Schloss et al., 2009). First, paired-end reads were merged into contigs (Unno et al., 2005). Then the sequences underwent a quality-trimming process based on the removal of sequences with any ambiguous bases or more than eight homopolymers. The remaining sequences were aligned against the SILVA SEED v132 database using the k-nearest neighbour algorithm with k-mer search using a k-mer size of 8 bp under Needleman conditions. After this, the sequences that failed to start and finish at the position of forward and reverse primers were eliminated. The sequences were later checked for chimeras using the VSEARCH algorithm (Rognes et al., 2016). The remaining non-chimeric sequences were classified against SILVA nr v132 database under a taxonomic cutoff of 80%. After this step, the remaining sequences were used to construct operational taxonomy units (OTUs) using a cutoff of 97% for prokaryotes and 95% for eukaryotes (Westcott and

Schloss, 2015). After the clustering of sequences into OTUs, the persistent reads were used to create a taxonomic consensus.

2.5. qPCR assays

The number of copies of bacterial and archaeal 16S rRNA gene, fungal 18S rRNA gene, archaeal and bacterial *amoA* genes and bacterial *norB*, *nosZ* and *nos Z* Clade II genes were quantified in all extracted DNA pools by the means of quantitative real-time PCR (qPCR). qPCR was conducted using a Mx3000 P qPCR system (Agilent Technologies) and data was analyzed using a MxPro qPCR software (Stratagene, USA). The primers and cycling conditions were set as reported previously for each marker gene (Muyzer et al., 1993; Rotthauwe et al., 1997; Braker et al., 2003; Henry et al., 2006; Tourna et al., 2008; Yu et al., 2008; Liu et al., 2012; Jones et al., 2013). qPCR calibration curves were constructed with the plasmid standards of the targeted genes. The calibration curves were done using serial ten-fold dilutions of plasmid standards. The reaction mixture was made how reported Muñoz-Palazon et al., 2019.

2.6. Study of α and β diversity of all samples

The study of α-diversity was done using PAST v3.06 software (Hammer and Harper, 2008) through the calculation of diversity indices of Chao-1, Shannon-Wiener, Simpson and Pielou's evenness. The Whittaker analysis was used to estimate the β-diversity among pairs of samples of prokaryotes and eukaryotes calculated using PAST v3.06.

2.7. Similarity analysis of prokaryote and eukaryote community structure

The Similarity Percentages analysis (SIMPER) was calculated to observe the contribution of dominant OTUs to dissimilarity between groups of samples (RL vs RM; RL vs RH; RM vs RH). The OTU tables for prokaryotes and eukaryotes were used for calculation of analysis through Bray–Curtis similarity using PAST software v3.4. Further analysis of samples was conducted using a compositional statistics approach. First, OTU tables were corrected to avoid 0 values by a Bayesian multiplicative replacement algorithm using the zCompositions package implemented in R. The corrected OTU maps were then transformed to a centred-log ratio. Then, transformed OTU distributions were taken for the calculation of singular value decompositions Bian et al., 2017.

To investigate the differential abundance of OTUs between different temperatures, their expected effect size was calculated. The zero-corrected and transformed OTU tables were generated by 128 Monte-Carlo Dirichlet distributions through the ALDEx2 package implemented in R software.

2.8. Permutations and multivariate redundancy analysis

Multivariate Redundancy Analysis (RDA) were done to observe the relationship between dominant OTUs of prokaryotes and eukaryotes with more than 1.00% of total relative abundance in at least one sample and performance parameters corrected to a centred-logarithm in a reactor operated under cold, mid and warm temperatures. Also, RDA was calculated to link the logarithm of number of target genes, physico-chemical

performance and biological samples. The RDA was computed through 499 Monte Carlo simulations under a full permutation model using CANOCO 4.5 for Windows.

The PERMANOVA analyses were done to suggest if the number of copies, the physicochemical performance and the dominant OTUs were affected by the time of operation and by the different temperatures. The PERMANOVA analyses were computed using PASTv3 with Bray-Curtis distance and under 9999 permutations.

2.9. Oligotyping analysis of OTUs of interest

Shannon entropy was calculated for each OTU of interest, which were selected by their relevant presence in terms of total relative abundance. Based on the Shannon entropy results, oligotypes were constructed for each OTU by repeated calculation until the purity score of the oligotypes with > 100 reads was 0.90 (Eren et al., 2014). The noise removal step during the process was done as reported (Muñoz-Palazon et al., 2008). The selections of OTUs were based on their high abundance (≥30.00% of relative abundance in at least on sample) or on their presence (≥5.00% of total relative abundance in one sample, and presence in the rest samples).

3. Results and discussion

3.1. Physic-chemical performance

The performance of the bioreactors was different when comparing low (8 °C), mid (15 °C) and high (26 °C) temperatures. For the start-up of the reactor at a low temperature (RL), the granular conformation began to be observed around operational day 30. However, for the mid temperature bioreactor (RM), the granules were observed at operational day 6 and for the high temperature bioreactor (RH) at day 10, as shown in Figure 1. These results suggest that the granular formation was faster when the inoculum was adapted to a similar temperature as that of start-up for the granulation process. Moreover, the mean size of the granules was smaller in bioreactors RL and RH, possibly due to a temperature change from inoculum despite being operated in a steady-stable stage. The reactor at mid temperature reached a mean size of 2.1 cm at operational day 120. At the end of experimentation, the values of granule diameters were in the range of 1.1–1.4 cm (Figure 1), larger than other results reported by De Kreuk et al. 2005; Gonzalez-Martinez et al. 2018a and Muñoz-Palazon et al. 2018. The settling velocity did not follow the same trend as the granule size. For this parameter, RH reached faster velocities up to 138 m h^{-1} along with a generation of denser granules. The slower velocity was related to low operational temperature. During the operation of the bioreactors, the granular conformation showed different stages of maturation, which affected the colour of the granules, their density and their external shapes. For the monitoring of organic matter removal, an analysis of chemical oxygen demand and biological oxygen demand were done (Figure 2). For all bioreactors, the trend was similar, with a high removal ratio during the first days of operation when the granules

were not observable yet. During the granule formation process, there was a drop in removal performance, although this trend was weaker in RM. Last, in the steady-stage, an increased removal of organic matter was reached, ranging 50–95% for COD and 83–98% for BOD_5 at operational day 80 for RL and at operational day 100 for RH.

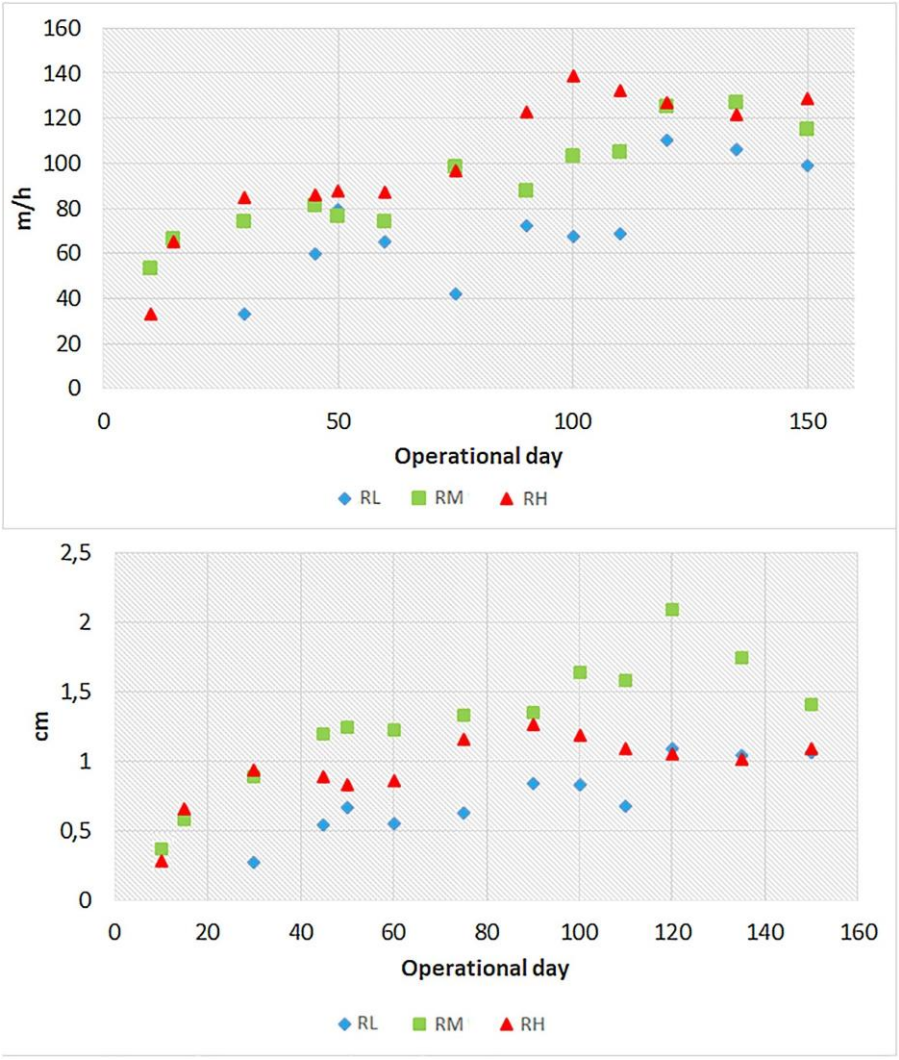


Figure 1. Granular properties of sludge for each bioreactor: (A) settling velocity expressed as m h.1 and (B) mean size expressed as cm. The sludge of reactor at 8 °C granulated later than the rest reactors.

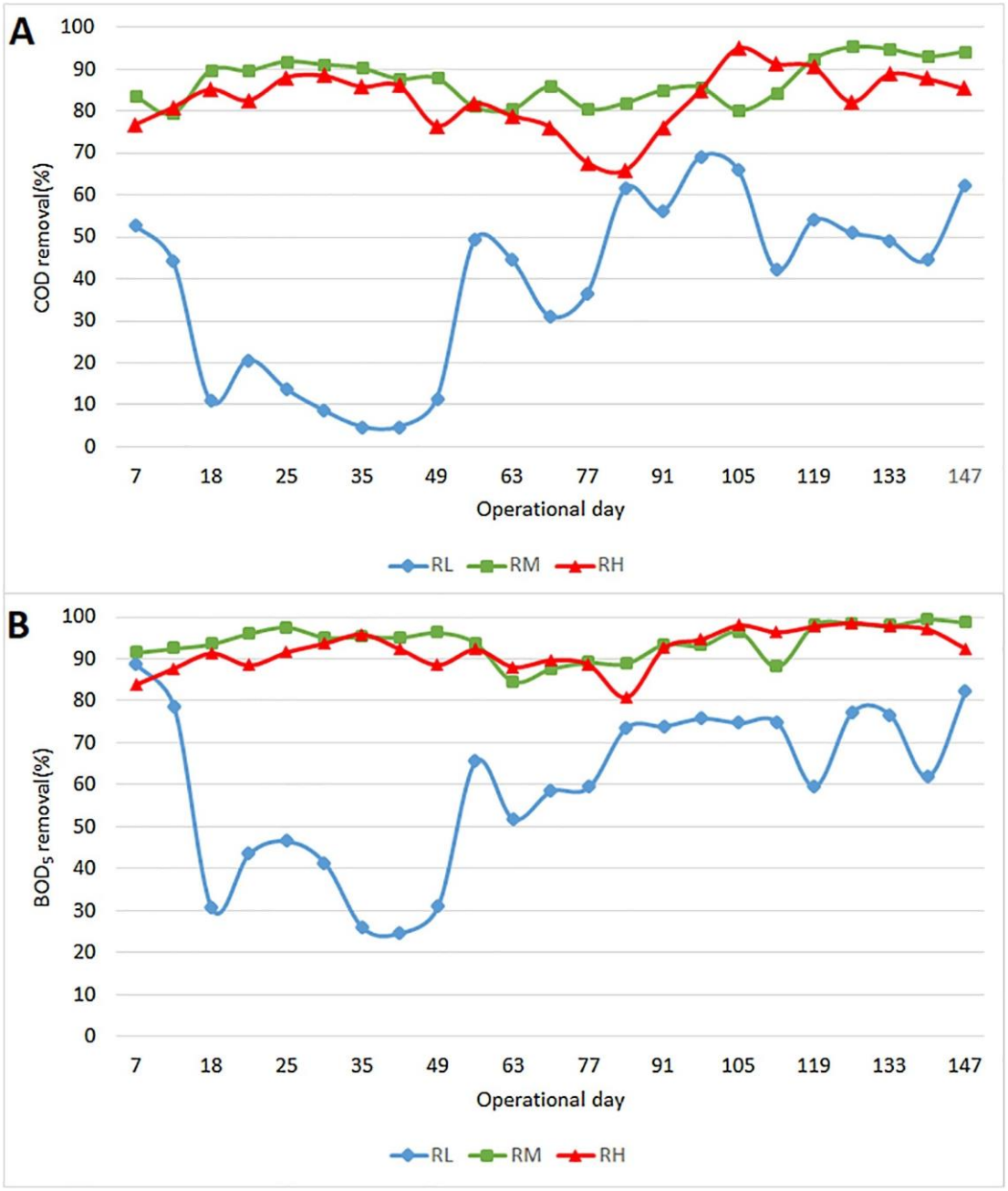


Figure 2. Ratio of degradation of (A) chemical oxygen demand (COD) and (B) biological oxygen demand (BOD5) for reactors at low temperature(RL), at mid temperature (RM) and high temperature (RH) over operational time

For nitrogen, Figure 3 shows that the effluent nitrite concentration had a decreasing trend with stable values for bioreactor RM and RH; however, the concentration of nitrite in the RL increased, reaching higher values, possibly due to the accumulation of this kind

of compound in the water, corroborating the negative relation between low operational temperatures and the elimination of nitrogen (Gnida et al., 2016). A similar fact was reported in lab-scale aerobic granular sludge reactors operated at an ultra-low temperature (Gonzalez-Martinez et al., 2018a). Also, the accumulation of nitrite in biological systems has been reported previously due to inactivity of NOB bacteria (Kim et al., 2006; Laureni et al., 2016). The NO_3^- concentration in the effluent was lower in RM, although RL and RH had higher concentrations that were stable over experimental time. The ammonium oxidation ratio was increasing generating effluents with low concentration (>20 mg NH_4^+ L^{-1}), which occurred first in reactors RM and RH at operational day 60, followed by RL at operational day 90. The total nitrogen removal increased in all bioreactors, reaching more than 80% total nitrogen removal at day 80. In conclusion, these results suggest that, despite the acclimatisation of microorganisms from mid, low or high temperatures, the performance of the systems was better when the bioreactor was adapted to the same temperature as that of the inoculum.

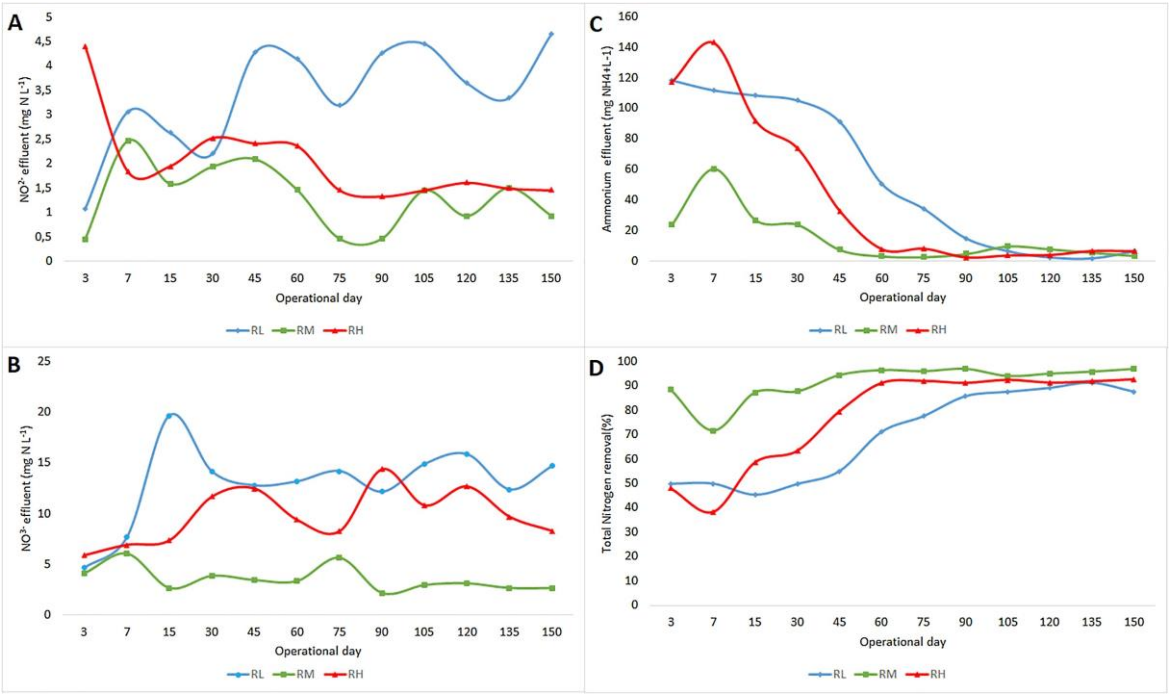


Figure 3. Concentration in the effluent of nitrite (A), nitrate (B) and ammonium (C) expressed as mg L^{-1}. The removal ratio of total nitrogen(D) expressed in percent.

3.2. Number of target genes in the bioreactors over experimental time

The absolute quantification of target genes was done to observe the trends of bacteria, archaea, fungi, ammonia-oxidising microorganisms and denitrifying bacteria under the effect of temperatures over the granulation process and steady-state stage of aerobic granular sludge technologies as it is shown in Figure 4. For *Bacteria*, the number of copies of 16S rRNA gene showed an increase from activated sludge inoculum to the rest of biological samples in at least one order of magnitude. Thus, the numbers of copies of 16S rRNA were stables with values of 10^{11} copies per gram of granule. For *Archaea*, the copies of the 16S rRNA gene in RL did not suffer variations with respect to the initial samples. However, a slight decrease in samples from Day 30 that had a rising trend until the final operation was observed in RM and RH.

The number of gene copies of fungal 18S rRNA in bioreactors RL and RH showed a trend previously described by Muñoz-Palazon et al. 2018, which explains that the increase in the number of copies of this gene during the start-up of operation from activated sludge to granular biomass is caused because fungal microorganisms comprise the core of the granules that will be colonised afterwards by bacteria and exopolysaccharides (EPS). This trend was observed in all bioreactors. In RM, the copies of fungal 18S rRNA gene copies remained in steady-state. However, in RL and RH the number of copies decreased at the end of experimentation. It has been reported that the role of *Fungi* is essential at the first step of granular conformation, but when the granule is completely formed, the number of Fungi members decrease (Muñoz-Palazon et al.,2018; Gonzalez-Martinez et al., 2018a). De Kreuk et al., 2005 described how aerobic granular sludge systems had problems at a low temperature caused by the growth of filamentous fungi. However, this did not occur during the operation of the bioreactor at 8 °C.

The ammonia-oxidizing bacteria were observed by *amoA* gene copies. The target gene for this microbial group showed an increase from activated sludge inoculum to granular conformation in RL and RM; however, in RH it was kept stable during the operation. The *norB* gene was studied to quantify the number of copies of nitrous oxide-producing microorganisms and decreased with oscillations in bioreactors RM and RL contrary to RL in which it had the opposite trend. These results suggest that *norB* genes were reduced at warm temperatures as corroborated by Jung et al., 2011. The effect of temperature on copies of *nosZ* gene was detrimental for high temperatures in reactors RM and RH on the first days of experimentation, although after 90 days the number of copies was

recovered. The *nosZ Clade* II gene was not detected by absolute quantification using the primers and following the thermal profile of Jones et al., 2013.

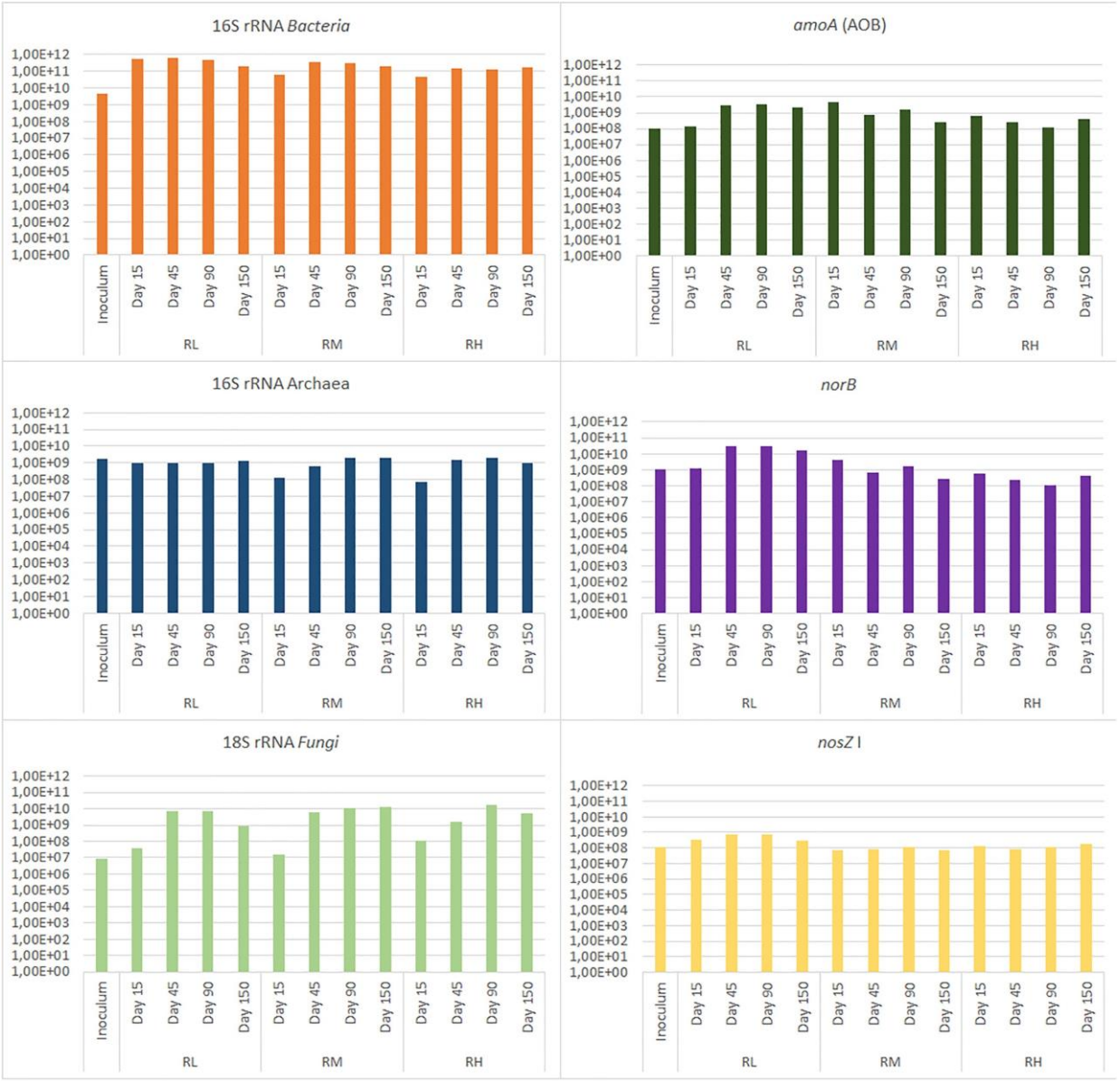


Figure 4. Quantification absolute of genes of interest thought RT-qPCR methods for operational days 15, 45, 90 and 150 for each bioreactor and inoculum.

3.3. Diversity indices for biological samples

The values of the Simpson and Shannon-Wiener indices showed similar values in diversity and evenness for the prokaryotes community from the inoculum sample to the end of experimentation in bioreactors RM and RH as is displayed in Table 1. On the other hand, the results of diversity in RL reflected lower diversity and evenness, showing effects from cold temperature. The Chao index species richness showed higher values in the inoculum sample. At steady-state stage the lower value was for RL, followed by RM. For the eukaryotic community, the diversity indices had weak oscillations in comparison with the prokaryotic community. With respect to the inoculum, the diversity and evenness were weakly lower in RL and RM and slightly higher in RH. The species richness had a decreasing trend over operational days, more accused at mid and high temperature.

Table 1. Analysis of α-diversity: diversity, evenness and species richness of all biological samples for Prokarya and Eukarya.

	Inoculum	RL			RM			RH		
		Day 30	Day 90	Day 150	Day 30	Day 90	Day 150	Day 30	Day 90	Day 150
					Prokarya					
Simpson	0,73	0,63	0,67	0,66	0,73	0,72	0,74	0,73	0,71	0,73
Shannon-Wienner	2,69	1,34	1,87	1,83	2,55	2,11	2,54	2,67	2,07	2,58
Pielous Evenness	0,03	0,02	0,05	0,02	0,03	0,03	0,04	0,04	0,03	0,03
Chao-1	595,40	241,50	173,90	331,10	552,60	281,00	352,60	502,90	324,40	428,80
					Eukarya					
Simpson	0,6823	0,6083	0,6738	0,6094	0,6584	0,575	0,6293	0,6942	0,5823	0,697
Shannon-Wienner	1,611	1,239	1,452	1,205	1,385	1,002	1,271	1,64	1,098	1,62
Pielous Evenness	0,08634	0,08849	0,1295	0,1112	0,09507	0,1184	0,1371	0,1097	0,1199	0,1943
Chao-1	61,46	50,14	40	37,5	66	41	27,5	48,67	25,17	29

The β-diversity study is shown in Figure S3. The Whittaker index showed greater dissimilarities for the prokaryotic community over the operational time in RM and RH. On the contrary, the phylotypes of bioreactor RL trended with higher similarities from start-up until the end of the operation. At operational day 150, microbial communities studied in the bioreactors trended to resemble every other. For the eukaryotic community, the results of β-diversity showed a similar trend in greater dissimilarities over operational time, including in RL. These results suggest that the initial community in the inoculum changed deeply when the biomass was granular. Moreover, it could be observed that the community in steady-state stage for each bioreactor showed great differences, reflecting the effect of temperature in the community of stable granules.

3.4. Contribution of sensitive OTUs and similarity analysis by compositional data

The contribution of dominant OTUs between reactors RL, RM and RH is shown in Figure S4 for prokaryote and eukaryote communities. SIMPER analysis results showed that the proliferation of P_Otu001, P_Otu002 and P_Otu009, taxonomically affiliated with *Acinetobacter*, *Corynebacterium* and *Pseudomonas*, respectively, was the major driving factor for the differences for low temperature against mid and warm temperature (>10.00%). The SIMPER analysis showed P_Otu003, taxonomically affiliated with *Arenimonas* genus, as the most sensitive prokaryotic OTU that contributed to dissimilarities between RL (7.60%) and RM (8.80%) versus the RH reactor. The contribution of sensitive OTUs between RM and RH showed a minor value for each phylotype (8.00-5.00%); however, the number of OTUs involved were higher than in the rest of the relationships. For the eukaryotic community, the SIMPER analysis showed that E_Otu001, E_Otu002, E_Otu003 and E_Otu004 were those contributing to greater dissimilarities in all bioreactors regardless of operational temperature. These phylotypes were affiliated with *Pezizomycotina* fungi, *Oligohymenophorea* ciliate and an unclassified eukaryote, which were of essential importance in the global community within the structure of the granules.

For prokaryotes, the ordination plots illustrated in Figure S5 reflect significant grouping in the samples including the inoculum, but show higher distances in samples from RH during the start-up stage and at the end of the operation. For eukaryotes, the results of

principal component analysis showed the inoculum sample as mostly different than the rest of the biological samples. The ordination plots illustrate that the samples belonging to operational day 30 had a large distance with respect to the samples at operational day 90 and 150, regardless of temperature.

The expected effect size for prokaryotes for the different reactors is illustrated in Figure S6. Major numbers of OTUs with significantly different relative abundance were found in the reactors RL and RH. Minor numbers of significantly different OTUs were registered between RL and RM; these results were corroborated by β-diversity. Within the eukaryotic communities, the greatest number of significantly different OTUs was found between RL and RH, following the same trend as the prokaryote population with five OTUs. Conversely, there was one significantly different OTU between RL and RM and two between RM and RH. These results could be a complement of the community study to observe which phylotypes were involved in the significant differences between reactors operated at cold, mid and warm temperature.

3.5. Microbial ecology of granulation process under different temperatures

3.5.1. Prokaryote population dynamics

For the prokaryote community it could be observed that the effect of temperature generated a deep change in the conformation of populations (Figure 5). The initial sludge inoculum had high diversity and evenness, with the *Comamonadaceae* and

Intrasporangiaceae families and *Dietzia* genus as dominant phylotypes. The *Intrasporangiaceae* family is common and widely found in activated sludge (Hanada et al., 2002).

The community dynamics were completely different during the operation due to the effect of temperature in each bioreactor and the granulation process. Bioreactor RL had deep changes in all stages driven by the cold temperature. At day 30, the prokaryotic community was mainly composed by P_Otu001 and P_Otu004, both taxonomically affiliated to *Acinetobacter*. *Acinetobacter* has been found in diverse environments, among which were aerobic granular sludge systems. These phylotypes have been reported as responsible for auto-aggregation activity and consequently secreting proteins to strengthen their aggregation (Weber et al., 2017), but under high organic loading ratio *Acinetobacter* contributes to granule disintegration (Adav et al., 2010). These results suggest that this phylotype could be essential for the right development during the first stages of granulation. After 90 days of operation, the populations changed with a proliferation of P_Otu009, classified as Pseudomonas. At the end of operation, *Corynebacterium* proliferated as the dominant phylotype related to P_Otu002. These dynamics of populations suggest that there were not phylotypes in the activated sludge inoculum that could adapt to low temperatures and/or the granulation process.

For the RM, in the first stage of operation there was shown a proliferation of P_Otu005, P_Otu010 and P_Otu001, belonging to the *Rhizobium* and *Acinetobacter* genera. After

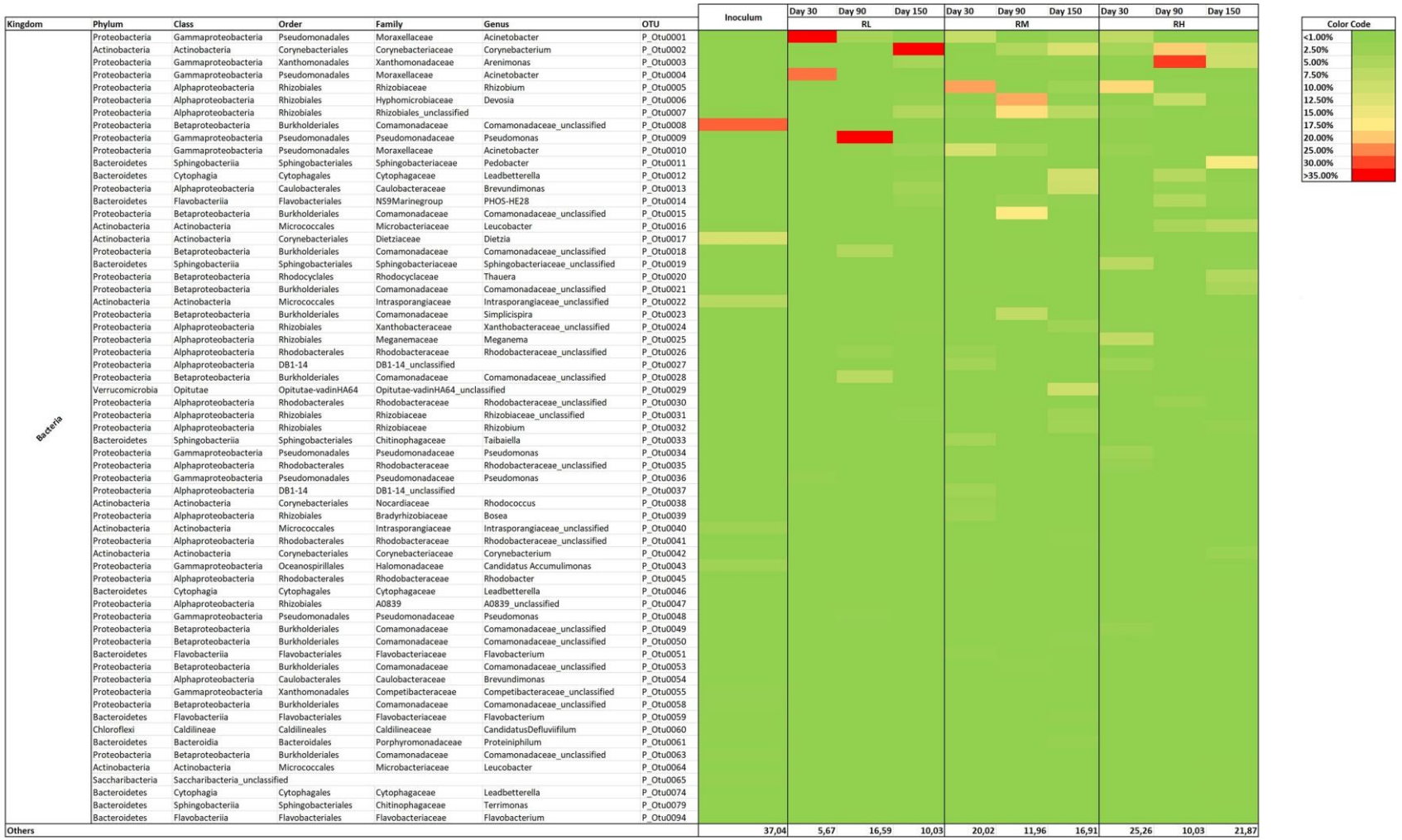
start-up, the OTUs found as relevant were P_Otu006, P_Otu007 and P_Otu015, taxonomically affiliated to the *Hyphomicrobiaceae* family, *Rhizobiales* order and *Devosia* genus, respectively. Last, for biological samples taken at operational day 150, the populations changed, acquiring high diversity represented by a wide number of OTUs. The OTUs with higher representation (>5.00%) were P_Otu002, P_Otu007, P_Otu012, P_Otu013 and P_Otu0029, classified in order as *Corynebacterium* genus, *Rhizobiales* order, *Leadbetterella* genus, *Brevundimonas* genus *and Opitutae-vadinHA64* order, which were related to varied metabolisms.

For RH, the first stage was similar to RM with the populations mainly dominated by P_Otu005, P_Otu001 and P_Otu025 belonging to *Rhizobiales*, *Acinetobacter* and *Meganema*, respectively. *Meganema* has been described as related with an increase of the granule size and their further breakage (Muñoz-Palazon et al., 2018; Figueroa et al., 2015). These results seem to be in relation with the largest mean size of the granular biomass in reactor RH. *Meganema* has been linked to organic loading rate, suggesting that these microorganisms could compete with each other for organic carbon and could therefore be involved in organic matter degradation in these systems (Szabó et al., 2017). At day 90 the diversity increased despite the decrease in evenness, driven by the strong proliferation of the *Arenimonas* genus. The OTUs with more relative abundance were P_Otu002, P_Otu003 and P_Otu006 affiliated with *Corynebacterium*, *Arenimonas* and *Devosia*. At the last stage of operation, the bioreactor acquired more evenness, with P_Otu0011 achieving a high representation. The *Arenimonas* genus was present in all

bioreactors at operational day 150. This genus has been described as a heterotroph responsible for the generation of extracellular polymeric substances (EPS) (Kwon et al.,2007; Huy et al., 2013), which play a crucial role in the formation and structural stability of bioaggregates (Zhu et al., 2015). This genus was most representative in RH. In the same way, the *Devosia* genus has been widely found in water environments and has been linked with metabolisms for facultative denitrification associated with incomplete denitrification (Falk et al., 2010). Also, it has been related with COD/N ratios higher than 3–4, improving the stability in the granules (Luo et al., 2014).

On the other hand, the *Corynebacterium* genus was present in all reactors at operational day 150. It is an important genus in wastewater, which possesses nitrate reductase and organic nitrogen hydrolase. Also, Liu et al., 2018 demonstrated that *Corynebacterium* improved the performance of nitrogen removal in biological wastewater treatment systems. On the other hand, this phylotype has been described in relation to phosphate metabolism. Some species are pathogens that cause diphtheria and are catalogued as an emerging pathogen (Nayak et al., 2017).

A



Kingdom	Phylum	Class	Order	Family	Genus	OTU	Inoculum	Day 30	Day 90	Day 150	Day 30	Day 90	Day 150	Day 30	Day 90	Day 150
								RL			RM			RH		
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	P_Otu0001										
	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium	P_Otu0002										
	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Arenimonas	P_Otu0003										
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	P_Otu0004										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	P_Otu0005										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	P_Otu0006										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_unclassified		P_Otu0007										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0008										
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P_Otu0009										
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	P_Otu0010										
	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	P_Otu0011										
	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella	P_Otu0012										
	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	P_Otu0013										
	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9Marinegroup	PHOS-HE28	P_Otu0014										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0015										
	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Leucobacter	P_Otu0016										
	Actinobacteria	Actinobacteria	Corynebacteriales	Dietziaceae	Dietzia	P_Otu0017										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0018										
	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacteriaceae_unclassified	P_Otu0019										
	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera	P_Otu0020										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0021										
	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Intrasporangiaceae_unclassified	P_Otu0022										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Simplicispira	P_Otu0023										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Xanthobacteraceae_unclassified	P_Otu0024										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Meganemaceae	Meganema	P_Otu0025										
	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_unclassified	P_Otu0026										
	Proteobacteria	Alphaproteobacteria	DB1-14	DB1-14_unclassified		P_Otu0027										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0028										
	Verrucomicrobia	Opitutae	Opitutae-vadinHA64	Opitutae-vadinHA64_unclassified		P_Otu0029										
	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_unclassified	P_Otu0030										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobiaceae_unclassified	P_Otu0031										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	P_Otu0032										
	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Taibaiella	P_Otu0033										
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P_Otu0034										
	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_unclassified	P_Otu0035										
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P_Otu0036										
	Proteobacteria	Alphaproteobacteria	DB1-14	DB1-14_unclassified		P_Otu0037										
	Actinobacteria	Actinobacteria	Corynebacteriales	Nocardiaceae	Rhodococcus	P_Otu0038										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea	P_Otu0039										
	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Intrasporangiaceae_unclassified	P_Otu0040										
	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_unclassified	P_Otu0041										
	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium	P_Otu0042										
	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Candidatus Accumulimonas	P_Otu0043										
	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	P_Otu0045										
	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella	P_Otu0046										
	Proteobacteria	Alphaproteobacteria	Rhizobiales	A0839	A0839_unclassified	P_Otu0047										
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P_Otu0048										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0049										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0050										
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	P_Otu0051										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0053										
	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	P_Otu0054										
	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Competibacteraceae	Competibacteraceae_unclassified	P_Otu0055										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0058										
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	P_Otu0059										
	Chloroflexi	Caldilineae	Caldilineales	Caldilineaceae	CandidatusDefluviifilum	P_Otu0060										
	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Proteiniphilum	P_Otu0061										
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_unclassified	P_Otu0063										
	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Leucobacter	P_Otu0064										
	Saccharibacteria	Saccharibacteria_unclassified				P_Otu0065										
	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella	P_Otu0074										
	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Terrimonas	P_Otu0079										
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	P_Otu0094										
Others							37,04	5,67	16,59	10,03	20,02	11,96	16,91	25,26	10,03	21,87

Figure 5. Heat map of prokaryotic community presented in the granules with more than 1.00% of relative abundance, respectively, for inoculum and reactors RL, RM and RH.

3.5.2. Eukaryote population dynamics

The eukaryotic dynamics were defined by the presence of numbers of unclassified eukaryote OTUs by the MiDAS_S123 database. Among them, one had high diversity in the first stages of granular biomass development (Figure 6). The inoculum had high diversity, with the Protista clade as representative eukaryotic microorganisms in E_Otu002 and E_Otu005, both affiliated to the *Oligohymenophorea* class of ciliate microorganisms, and without a representative abundance of the Fungi kingdom. Protozoa organisms play an essential ecological role since they control the suspended bacteria and peripheral cells of flocs and support a healthy trophic chain in the sludge environmental dynamics (Papadimitriou et al., 2007). Also, the ciliate protozoa improved sludge settling and effluent quality by reducing particulate matter, reducing the biodegradable organic matter and, specially, acting as a control of pathogenic microorganisms that cause diseases (Ntougias et al., 2011).

For RL, the selection of organisms by settling time promoted the development of one OTU belonging to *Pezizomycotina* fungi. The promotion of filamentous fungi under a low temperature has been reported previously by De Kreuk et al., 2005. After 90 days of operation, this OTU affiliated with *Pezizomycotina* proliferated, causing the loss of representation of ciliate protozoa OTUs in the granule. This trend increased with the operational time, reaching 75.00% of relative abundance at the end of experimentation, suggesting that the fungi play an essential role in the formation and structure of the granular core. For RH, the trend was similar; however, the dominance of

Oligohymenophorea was lost to a complete dominance of one OTU belonging to *Pezizomycotina* fungi.

The dynamics of RM were completely different to RL and RH. The initial populations remained during the granulation process, not affected by the massive selection of microorganisms during the start-up of the aerobic granular sludge systems. However, after 90 days of operation the populations suffered deep changes with a rise of the proliferation of OTUs E_Otu004 and E_Otu005 related to the *Pezizomycotina* and *Oligohymenophorea* class, respectively. In the last stage, these OTUs were lost in the community by the proliferation of *Pezizomycotina* fungi, which was dominating at the end of the operation in RL and RH. In general, these results seem that the strong change of temperature, both cold and warm, allows for a faster selection of eukaryotic communities essential in the granule. However, if the reactor operated at a temperature similar to that of the inoculum, the selection would be slower. Thus, the results suggest that the eukaryotic communities are more conditioned by strong changes of temperature than by the selection pressure exerted during the granulation process.

B

	Clado	Supergroup	Kingdom	Subkingdom	Clado	Phylum	Subphylum	OTU
Fungi and Animalia	Opisthokonta	Holozoa	Metazoa	Eumetazoa	Bilateria	Arthropoda		E_Otu006
	Opisthokonta	Holozoa	Metazoa	Eumetazoa	Bilateria	Nematoda		E_Otu016
	Opisthokonta	Nucletmycea	Fungi	Dikarya		Ascomycota	Pezizomycotina	E_Otu001
	Opisthokonta	Nucletmycea	Fungi	Dikarya		Ascomycota	Pezizomycotina	E_Otu011
	Opisthokonta	Nucletmycea	Fungi	Dikarya		Ascomycota	Saccharomycotina	E_Otu031
	Opisthokonta	Nucletmycea	Fungi	Dikarya		Ascomycota	Pezizomycotina	E_Otu004
	Opisthokonta	Nucletmycea	Fungi	Dikarya		Basidiomycota	Agaricomycotina	E_Otu019
	Opisthokonta	Nucletmycea	Fungi	Fungi_unclassified				E_Otu036
	Supergroup	**Superphylum**	**Phylum**	**Subphylum**	**Infraphylum**	**Class**		**OTU**
Protista	SAR	Alveolata	Ciliophora	Intramacronucleata	Conthreep	Oligohymenophorea		E_Otu005
	SAR	Alveolata	Ciliophora	Intramacronucleata	Conthreep	Oligohymenophorea		E_Otu007
	SAR	Alveolata	Ciliophora	Intramacronucleata	Conthreep	Oligohymenophorea		E_Otu029
	SAR	Alveolata	Ciliophora	Intramacronucleata	Conthreep	Oligohymenophorea		E_Otu013
	SAR	Rhizaria	Cercozoa	Thecofilosea	Thecofilosea_unclassified	Thecofilosea unclassified		E_Otu026
	SAR	Stramenopiles	Ochrophyta	Chrysophyceae	Chrysophyceae_unclassified	Chrysophyceae unclassified		E_Otu008
	SAR	Stramenopiles	Ochrophyta	Chrysophyceae	Chrysophyceae_unclassified	Chrysophyceae unclassified		E_Otu025
	SAR	SAR unclassified						E_Otu021
	SAR	Alveolata	Ciliophora	Intramacronucleata	Conthreep	Conthreep unclassified		E_Otu033
	SAR	Alveolata	Ciliophora	Intramacronucleata	Litostomatea	Haptoria		E_Otu023
	SAR	Stramenopiles	Ochrophyta	Ochrophyta unclassified	Ochrophyta unclassified			E_Otu010
	SAR	Alveolata	Ciliophora	Intramacronucleata	Intramacronucleata unclassified			E_Otu009
	SAR	Alveolata	Ciliophora	Intramacronucleata	Conthreep	Oligohymenophorea		E_Otu002
	Subkingdom	**Superphylum**	**Clado**	**Phylum**	**Subphylum**	**Class**	**Subclass**	OTU
	Excavata	Discoba	Discicristata	Euglenozoa		Kinetoplastea	Metakinetoplastina	E_Otu020
	Excavata	Discoba	Discicristata	Heterolobosea	Tetramitia	Tetramitia_unclassified		E_Otu015
								OTU
Eukaryota	Eukaryota unclassified							E_Otu018
	Eukaryota unclassified							E_Otu027
	Eukaryota unclassified							E_Otu028
	Eukaryota unclassified							E_Otu045
	Eukaryota unclassified							E_Otu035
	Eukaryota unclassified							E_Otu012
	Eukaryota unclassified							E_Otu017
	Eukaryota unclassified							E_Otu024
	Eukaryota unclassified							E_Otu003
	Eukaryota unclassified							E_Otu022
Others								

Inoculum	Day 30	Day 90	Day 150	Day 30	Day 90	Day 150	Day 30	Day 90	Day 150
	RL			RM			RH		
3,09	13,11	0,95	0,98	1,63	0,60	1,02	2,11	0,77	0,45

Color code
<0.50%
2.50%
5.00%
10.00%
20.00%
30.00%
40.00%
50.00%
60.00%
>70.00%

Figure 6. Heat map of eukaryotic population presented in the granules with more than 0.50% of relative abundance, respectively, for inoculum and reactors RL, RM and RH.

3.6. Multivariate redundancy analysis (RDA) and PERMANOVA

The multivariate redundancy analysis of prokaryotic and eukaryotic communities with the performance of RL is shown in Figure 7A. The analysis reflected the positive correlation between nitrogen, COD and BOD_5 removal, settling velocity and mean size along with a weak relation with the final stage of operation at day 150. Great relevance in terms of relative abundance had E_Otu001, related to *Pezizomycotina* fungi, verify the hypothesis of the essential role of filamentous fungi in the structure and formation of granular biomass. Besides that, P_Otu031 and P_Otu010, related to the *Rhizobiaceae* family and the *Acinetobacter* genus, respectively, were positively correlated with high performance in RL. On the other hand, the relation of P_Otu001, P_Otu004, P_Otu036, E_Otu002 and E_Otu036, which comprised an important part of the community during the granulation process, were correlated negatively with performance. The linkage of performance and phylotypes represented in RM is shown in Figure 7B. A high number of OTUs, mainly presented in day 30, were positively correlated with nitrate and nitrite in the effluent. COD removal was strongly linked with P_Otu034 and P_Otu41, affiliated with the *Pseudomonas* and *Rhodobacteraceae*, which are heterotrophic bacteria. Also, the BOD5 was weak and positively correlated with E_Otu001, E_Otu010, E_Otu011, P_Otu012, P_Otu013, P_Otu024 and P_Otu029. The granular biomass parameters were correlated favorably with P_Otu002 and P_Otu003, affiliated with *Corynebacterium* and *Arenimonas*, respectively.

Figure 7C shows the links of samples from RH with performance parameters. It shows the favorable relation of mean size, settling velocity and total nitrogen removal with the

Brevundimonas genus represented by P_Otu013. The size of the granule was positively correlated with several OTUs of interest, such as P_Otu002, P_Otu003, P_Otu014 and E_Otu001, classified as *Corynebacterium*, *Arenimonas* and PHOS-HE28 genera and *Pezizomycotina* subphylum, respectively. The opposite cluster in respect to size, TN removal and velocity was composed by the nitrite concentration in the effluent and the BOD5 removal, mostly linked with sensitive OTUs at operational day 30 when the system was not yet under steady-state operation. Many OTUs of interest were correlated with the biological sample at operational day 150, but no strong positive correlation was found with any performance parameter.

The multivariate redundancy analysis illustrated in the Figure 7D shows the relationship between the number of copies of target genes, the physicochemical performances and biological samples of each bioreactor. The main linkage was observed between a higher number of copies of fungal 18S rRNA gene and archaeal 16S rRNA gene with the granular biomass properties, settling velocity and mean size, corroborating a previous hypothesis described by Gonzalez-Martinez et al. 2018a. On the other hand, the RDA showed a strong positive correlation of number of *norB* and *amoA* genes with higher concentrations of nitrate and nitrite in the effluent caused by microorganisms involved in nitrogen metabolisms. In addition, the biological samples belonging to RM and RH were strongly correlated with the granular biomass properties and organic matter removal, which were deeply negatively correlated with the samples of RL.

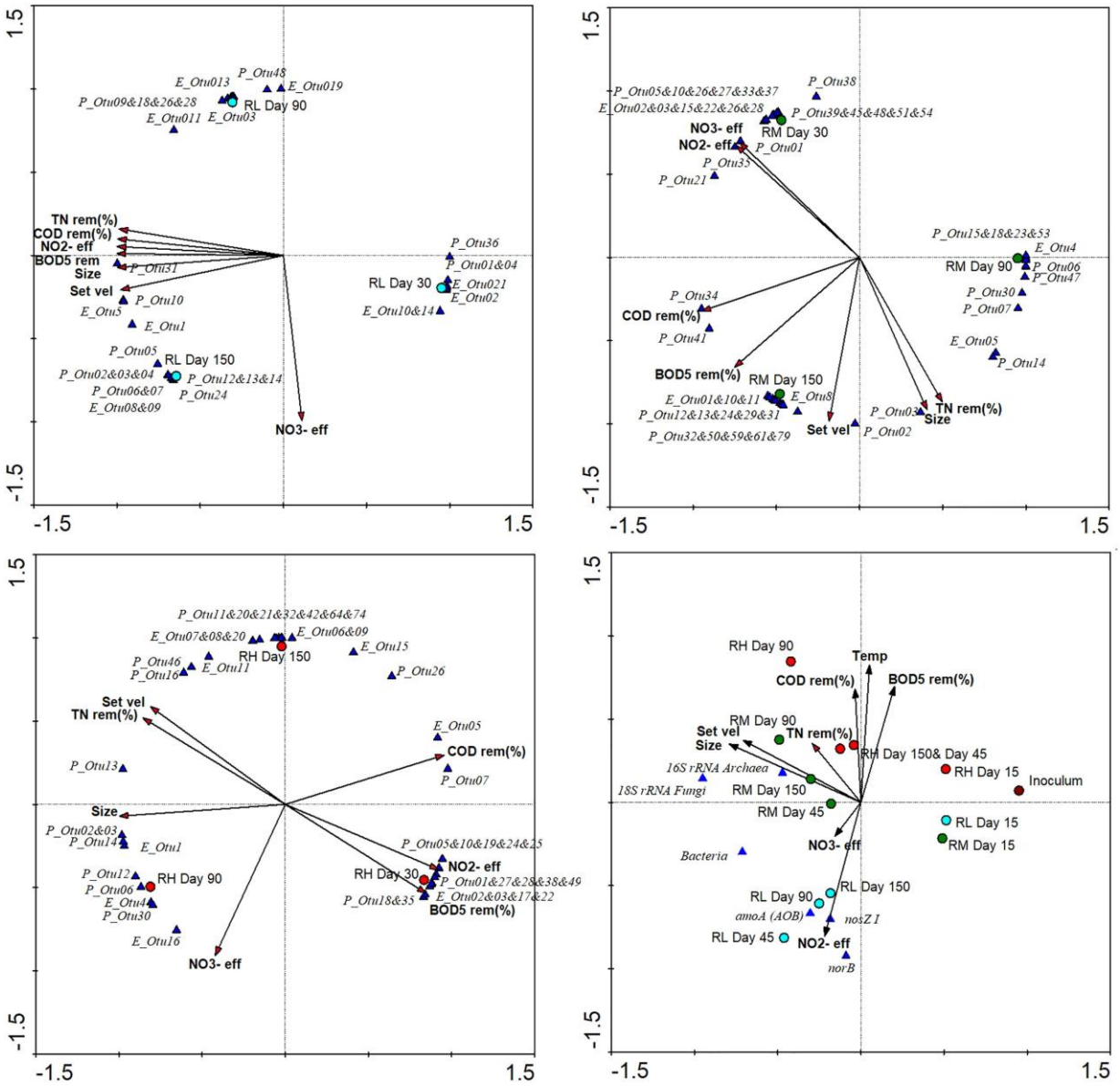


Figure 7. Multivariate redundancy analysis of prokaryotic and eukaryotic OTUs of interest, the physic-chemical parameters and biological samples for reactor at low temperature RL (A); reactor at mid temperature RM (B); and reactor at high temperature RH (H). The multivariate redundancy analysis was calculated for the copies of target samples, all biological samples of the bioreactor and performance (D).

The PERMANOVA analysis was calculated to observe the statistically significant influence of temperature on all dominant prokaryote OTUs ($p < 0.05$) since these OTUs were not affected by operational time (Table S1). In the same way, a high number of eukaryote dominant OTUs was significantly affected by the temperature, although

E_Otu002, classified as a ciliate microorganism, was significantly affected by operational time, suggesting that the role of this OTU was relevant at the first stage of operation but irrelevant in the mature granular community. On the other hand, all physico-chemical parameters were affected significantly by operational temperature except for settling velocity. The relationship between the performance of aerobic granular sludge technology and temperature has been described by some authors who also described its direct link with the granular biomass properties and nitrogen and carbon removal (Bao et al., 2009; Ebrahimi et al., 2010). Finally, the number of copies of archaeal 16S rRNA gene and fungal 18S rRNA gene were affected by the operational time, and the proliferation of fungal members that formed the essential structural unit in the granules. The number of copies of the nosZ gene was affected by the operational temperature ($p > 0.05$). Also, the temperature played a crucial role in influencing denitrifiers growth and metabolism, denitrification gene expression and, subsequently, denitrification rate (Saleh et al., 2009).

3.7. Oligotypes of interest OTUs

The oligotyping analysis for the prokaryotic community was calculated for dominant phylotypes. All oligotypes belonging to P_Otu001 were discarded during the noise removal step. With respect to the P_Otu002, the oligotypes changed from the inoculum to the rest of the samples, given that the sequence CA-TTTGTCT was the only oligotype in the inoculum and a high number of oligotypes presented in the granular biomass regardless of operational temperature, although in all granular samples the phylotypes

of the OTUs without oligotypes were the most common (Figure S7A). P_Otu003 showed a wide and even presence of oligotypes, except for the sample from operational day 30 of RL. The selection of these two phylotypes could be due to the strong change of temperature at the start-up of the operation. This change was observed on day 30 in RH, supporting the hypothesis that the drastic change of temperature affected this OTU. The P_Otu008 showed a clear trend that was marked by a decrease of relative abundance of diverse oligotypes and a strong increase of no-oligotype over the operational time. Last, the P_Otu009 showed a high diversity and evenness of the 7 oligotypes belonging to it. The deepest changes were found in RM on operational days 30 and 150 with the proliferation of ACT and AAT oligotypes, respectively.

The oligotypes analysed for the eukaryotic communities were calculated for OTUs of interest as shown in Figure S7B. No trends were observable for the E_Otu001 and E_Otu003. For E_Otu002 it was found that the oligotype disappeared over operational time, proliferating the no-oligotype. E_Otu004 showed that the inoculum was dominated by an oligotype, but in the bioreactor, this oligotype lost relevance. Finally, the oligotypes belonging to E_Otu005 showed 3 oligotypes in the inoculum that disappeared in RH. On the contrary, in RL and RM they were kept at day 30 and then were dominated by the oligotype with the representative sequence C. These results suggested that the oligotype with sequence C of E_Otu005 had advantages competitive in granular biomass independent of temperature.

4. Conclusions

The startup of three aerobic granular sludge reactors inoculated with mid-adapted activated sludge and operated under a cold (8 °C), mid (15 °C) and warm (26 °C) temperature was done. The highest performance in terms of nitrogen removal and carbon degradation was achieved in the reactor operated at the same temperature as the initial inoculum (RM), followed by the reactor at the warm temperature (RH), which followed a similar trend. The bioreactor operated at a cold temperature (RL) achieved a good performance in terms of nutrients and organic matter removal despite not having reached steady-state conditions. For the granulation process, all bioreactors generated dense and regular granules, although the biggest and smallest granules were registered in RH and RL, respectively. The eukaryotic community in mature granules was dominated by *Pezizomycotina* fungi and *Oligohymenophorea* ciliates regardless of operational temperature, and the prokaryotic community was more diverse and diverged between temperatures, although *Corynebacterium* had an essential role in the steady-state stage. Consequently, this technology implemented in mid environment could have faster startup periods if the bioreactor is inoculated with mid-adapted inoculum. Also, this study suggest the stability of aerobic granular sludge systems operated under different temperature regardless the temperature during seasonal or daily changes.

5. Statement of novelty

The aerobic granular sludge system is a novel biotechnology for the treatment of conventional pollutants of urban wastewater, such as organic matter, nitrogen and phosphorous in the same chamber. However, this technology is a promising to remove and adsorb a wide variety of pollutant from industrial wastewater for instance phenolic compounds, particulate material, pharmaceuticals compounds, pesticides, nuclear waste. Etc. Thus, our investigation is based on the development of this technology to be easier, shorter and more stable biologically for the startup of aerobic granular sludge system at full scale, avoiding the discharges of hazardous material in natural water bodies.

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Supplementary data

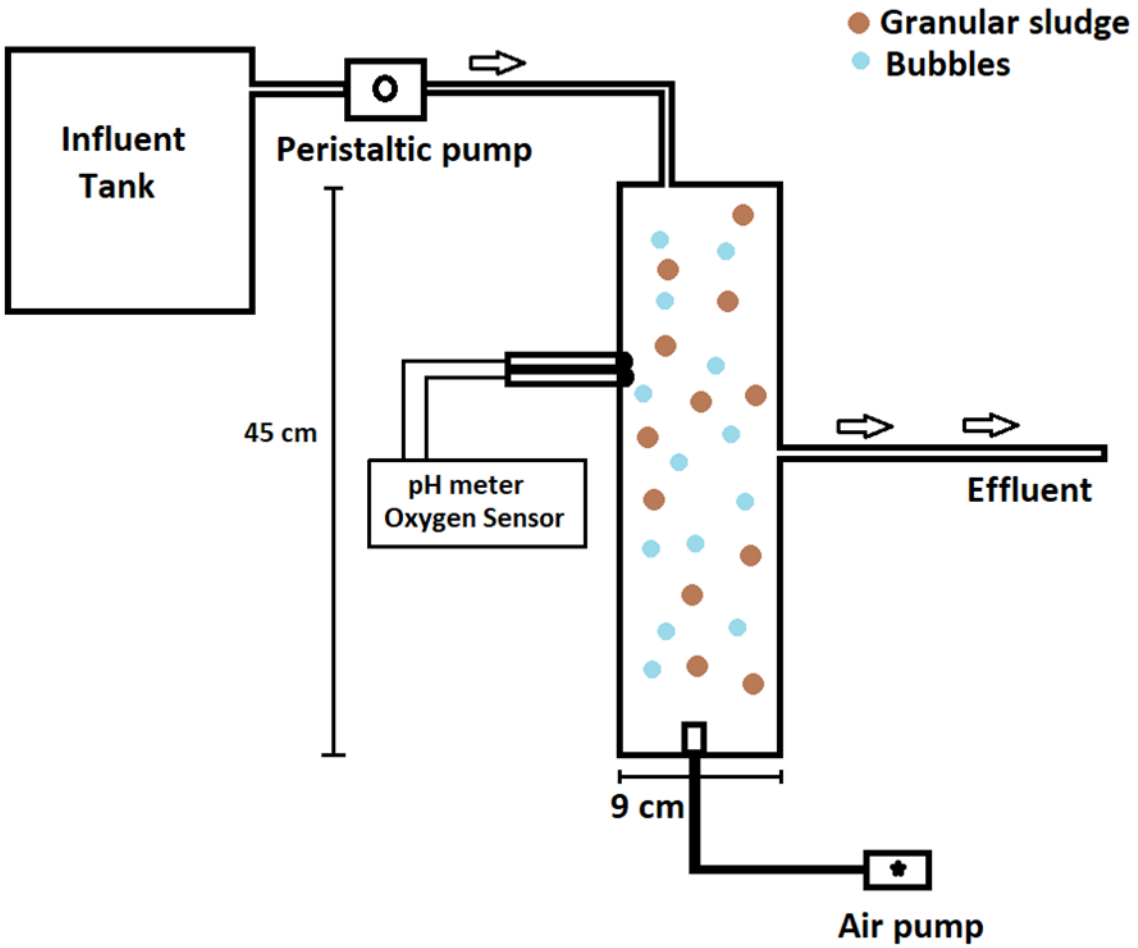

Figure S1. Schematic diagram of sequential batch cylindrical reactors.

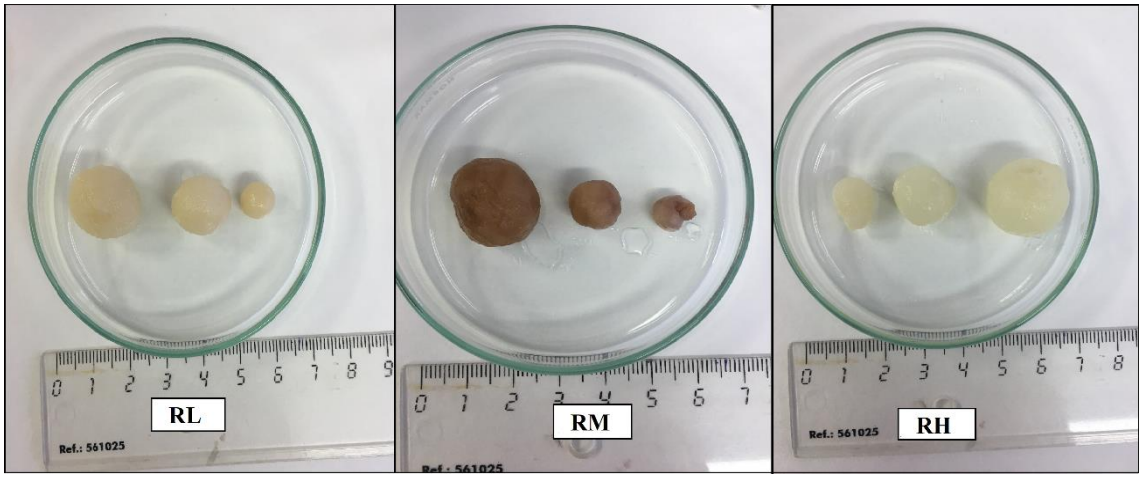

Figure S2. Granular sludge formed at operational day 150, selected by the wide of diameter.

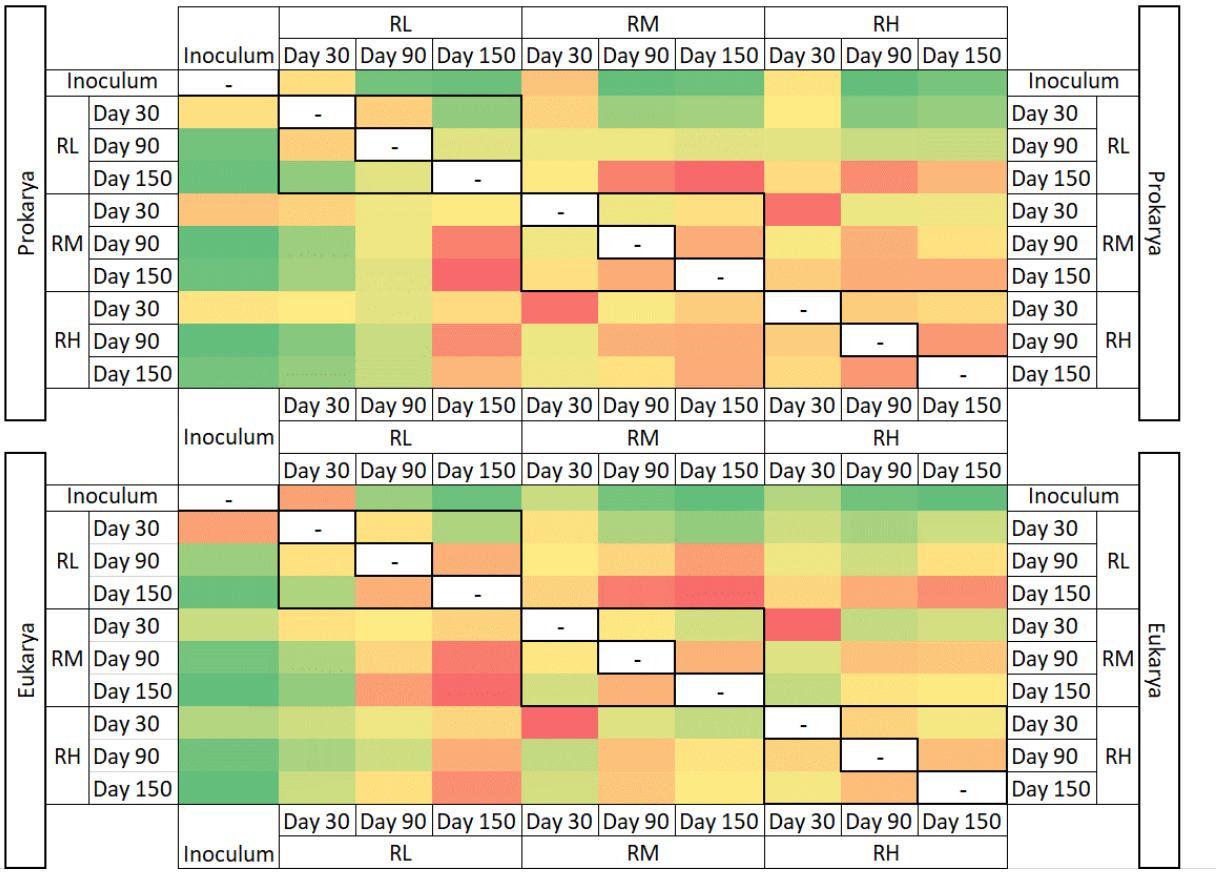

Figure S3. Beta diversity analysis calculated by Whittaker analysis for pair of samples of *Eukarya* and *Prokarya.*

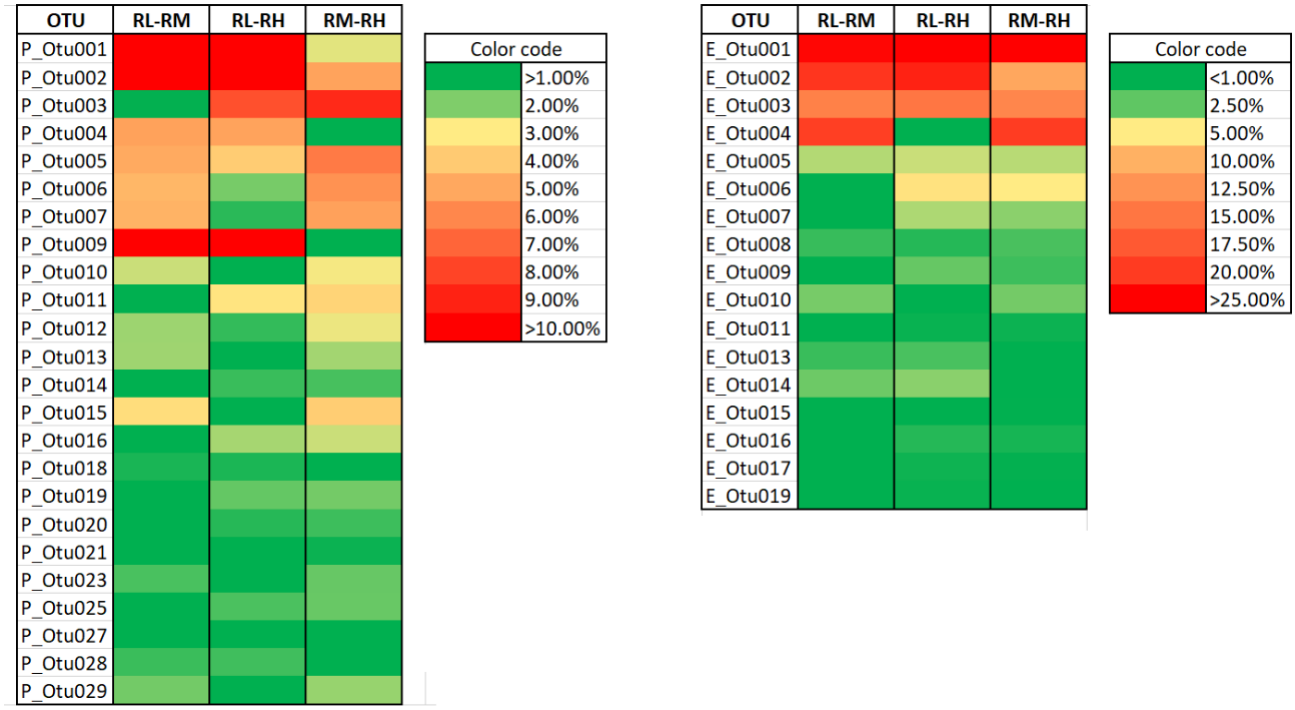

Figure S4. Contribution of sensitive OTUs to dissimilitude (%) between different temperatures of bioreactor for *Prokarya* OTUs (left) and *Eukarya* OTUs (right).

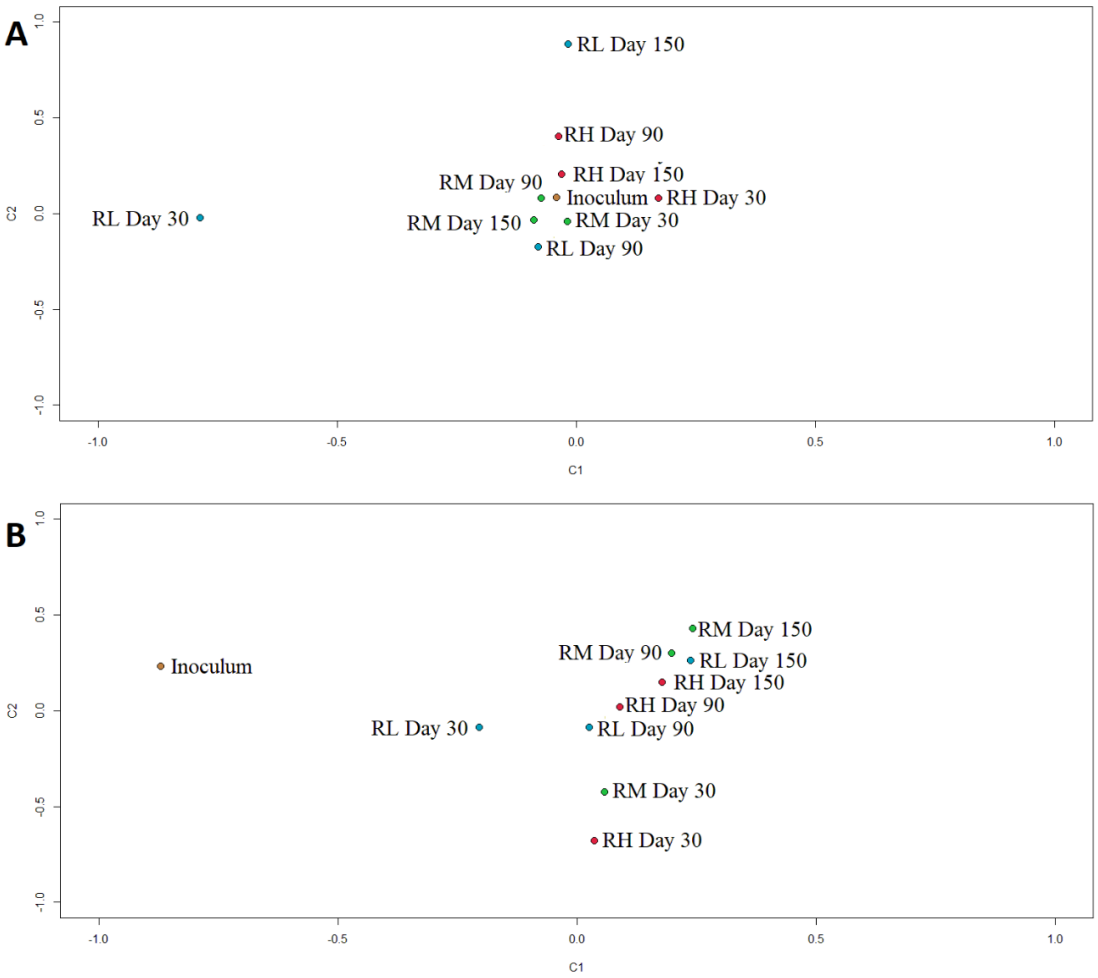

Figure S5. Ordination plots of the biological samples of inoculum and each bioreactor over operational time for prokaryotic samples(A) and eukaryotic samples (B).

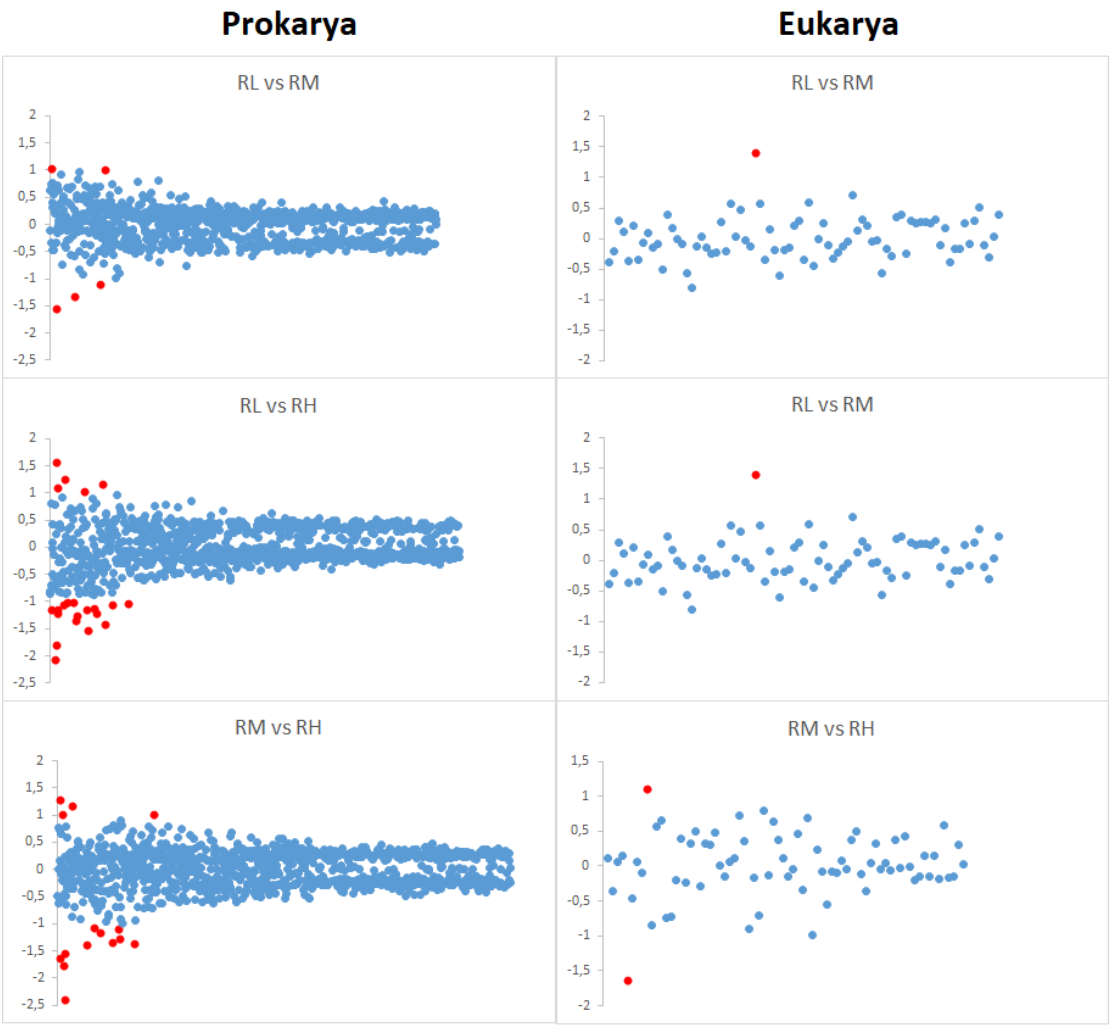

Figure S6. Expected effect size for OTUs statistically different between reactors operated under different temperatures. The graphics on the left showed the number of prokaryotic OTUs with significate differences (red) between RL versus RM, RL versus RH and RM versus RH reactors. The graphics on the right showed the number of eukaryotic OTUs with significate differences (red) between RL versus RM, RL versus RH and RM versus RH reactors.

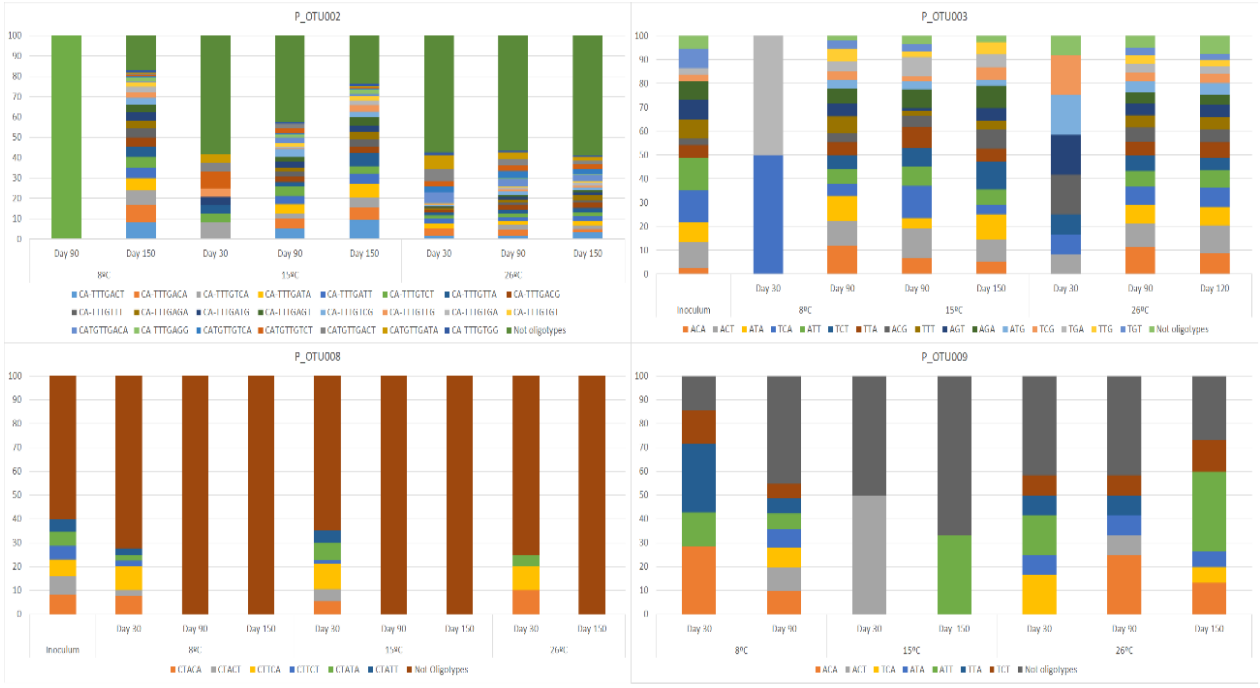

Figure S7A. Prokaryotic oligotypes of sensitive OTUs presented in the biological samples

Figure S7B.Eukaryotic oligotypes of sensitive OTUs presented in the biological samples

Table 1. PERMANOVA analysis of parameters of interest: sensitive prokaryotic and eukaryotic OTUs, copies of target genes, physico-chemical parameters in relation with temperature of bioreactor and operational day

	Prokarya									
	Otu0001		**Otu0002**		**Otu0003**		**Otu0004**		**Otu0005**	
	F	pvalor	F	pvalor	F	pvalor	F	pvalor	F	pvalor
Temperature	3,564	0,004	3,193	0,037	18,580	0,004	5,642	0,004	12,400	0,004
Operational day	1,164	0,329	1,014	0,413	0,378	0,789	0,413	0,917	0,627	0,634
	Otu0006		**Otu0007**		**Otu0008**		**Otu0009**		**Otu0010**	
	F	pvalor	F	pvalor	F	pvalor	F	pvalor	F	pvalor
Temperature	12,870	0,003	16,830	0,003	2636,000	0,004	3,687	0,004	33,030	0,005
Operational day	0,447	0,763	0,237	0,901	0,053	0,889	0,521	0,945	0,067	1,000
	Eukarya									
	Otu0001		**Otu0002**		**Otu0003**		**Otu0004**		**Otu0005**	
	F	pvalor	F	pvalor	F	pvalor	F	pvalor	F	pvalor
Temperature	1,649	0,227	2,017	0,100	1,887	0,121	4,223	0,003	10,150	0,003
Operational day	2,014	0,162	4,516	0,026	0,558	0,699	0,413	0,912	0,156	0,891
	Otu0006		**Otu0007**		**Otu0008**		**Otu0009**		**Otu0010**	
	F	pvalor	F	pvalor	F	pvalor	F	pvalor	F	pvalor
Temperature	33,940	0,003	85,010	0,005	42,860	0,005	42,060	0,005	28,000	0,004
Operational day	0,088	0,959	0,045	0,896	0,490	0,707	0,350	0,773	0,064	0,952
	qPCR											
	16S rRNA Bacteria		**16S rRNA Archaea**		**18S rRNA Fungi**		**AOB (amoA)**		**norB**		**nosZ**	
	F	pvalor	F	pvalor	F	pvalor	F	pvalor	F	pvalor	F	pvalor
Temperature	1,717	0,207	0,545	0,691	0,392	0,862	0,791	0,480	1,860	0,130	10,890	0,008
Operational day	1,397	0,262	3,508	0,046	5,393	0,012	0,261	0,891	0,591	0,731	0,134	0,997
	Physico-Chemical Parameters									
	Nitrogen removal		**Settling**		**Mean size**		**COD removal(%)**		**BOD removal(%)**	
	F	pvalor	F	pvalor	F	pvalor	F	pvalor	F	pvalor
Temperature	3,669	0,003	3,020	0,062	992,800	0,004	3,320	0,003	6,272	0,003
Operational day	2,158	0,151	1,712	0,221	0,025	0,878	0,562	0,917	0,370	0,864

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Chapter III

Polar Arctic Circle biomass enhances performance and stability of aerobic granular sludge systems operated under different temperatures.

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Scientific quality index: **Q1, IF: 6.669**

Abstract

Three bioreactors were inoculated with Polar Arctic Circle-activated sludge, started-up and operated for 150 days at 8, 15 and 26 °C. Removal performances and granular conformation were similar at steady-state, but higher stability from start-up was found when operating at 8 °C. Important changes in the eukaryotic and prokaryotic populations caused by operational temperature were observed, being fungi dominant at 8°C and 15°C, while that ciliated organisms were found at 26°C. The qPCR results showed higher copies of bacteria, and nitrifiers and denitrifying bacteria at cold temperature. The emission of nitrous oxide was linked directly with temperature and the involved microorganisms. This study represents a proof of concept in performance, greenhouse gas emission, granular formation and the role of the Polar Arctic Circle microbial population in AGS technology under different temperatures with the aim to understand the effect of seasonal o daily changes for implementation of AGS at full-scale.

Keywords: aerobic granular sludge; cold-adapted biomass; greenhouse gases emission; massive parallel sequencing; qPCR

1. Introduction

Problems derived from discharge of wastewater treatment plants have increased since the expansion of urban nuclei and the manufacture of several chemical compounds (Loos et al., 2013, Deblonde et al., 2011). Research has put much effort to solve and avoid problems caused to natural environments and water bodies receiving discharges. Some of the problems entailed by urban and industrial wastewater discharges are eutrophication, depletion of oxygen and death of aquatic life (Barrenha et al., 2018). Environmental pollution has triggered devastation, extinction of species and shortage of natural resources in natural places, such as the Polar Arctic Circle.

Usually, urban wastewater treatment is based on conventional activated-sludge technology (Gonzalez-Martinez et al., 2016). However, novel biological technologies are being developed and optimized for the treatment of organic matter and nitrogen, among other pollutants, such as anammox technologies, hybrid floating bed (HFTB), hybrid moving bed biofilm reactor or aerobic granular sludge (AGS) systems (Gonzalez-Martinez et al., 2018a, Rodriguez-Sanchez et al., 2018, Wu, 2016, Liu et al., 2016). In the last decade, the AGS is considered one promising new biotechnology due to high savings in terms of energy (20–50%) and footprint (25–75%) (Sguanci et al., 2019). The AGS system is a novel technology able to degrade organic matter, nitrogen and phosphorous simultaneously in the same chamber (Yan et al., 2016). This advantage is possibly due to granular conformation of the sludge, which allows the presence of nutrient gradients from external layers to internal core, so it is commonly found in the aerobic zone in external layers, anoxic zone in intermedia layers, and anaerobic conditions in the core of granules (Gonzalez-Martinez et al., 2018a). The granular sludge is formed by

aggregation of eukaryotic and prokaryotic microorganisms embedded in a tridimensional matrix attached by extracellular polymeric substances (EPS) (Graaff et al., 2019). AGS systems comprise a dense biomass, which grows in a sequencing batch reactor (SBR), defining a technology with low sludge volume index values, resistance against toxic compounds, higher biomass retention, and operation under high organic load (Tiwari et al., 2019).

Temperature plays an essential role in the selection of microbial communities that form the granules and the overall microbial diversity supported by the AGS bioprocess. It has been found that low temperature dramatically affects the microbial community structure and the microbial activity of natural ecosystems (Wang et al. 2015), thus it also affects these parameters in biological wastewater treatment systems, such as the active sludge process (Zhang et al. 2014). However, biological processes in cold regions such as the Nordic countries, attain very high removal efficiencies in terms of organic matter, N and P at 7 °C (Gonzalez-Martinez et al., 2018a). The impact of temperature variations with respect to inoculum used had consequences in the start-up phase and in the efficiency of depollution in the bioreactor. This parameter directly affects the growth and metabolic mechanisms of microbial communities present in a biological biomass. (Schulte et al., 2015). Thus, the metabolisms involved in the microbial communities of activated sludge had different pathways to degradation of organic matter and nutrients in cold regions, such as the microorganisms acclimatized to Polar region conditions (Gonzalez-Martinez et al., 2018b). Thus, it is relevant to understand the process of

adaptation and change in communities from cold (8 °C) to warm (26 °C) temperatures under different scenarios.

On the other hand, the emission of greenhouse gases (GHG) generates environmental damage mainly by anthropogenic activities. Thus, the quantification of emissions generated by wastewater treatment plants is of essential importance to avoid the production of high volumes of nitrous oxides or nitric oxides, which contribute actively to climate change (Li et al., 2017). It is known that N_2O is the most potent of GHG, with a warming potential >290 times that of carbon dioxide (Castella-Hinojosa et al., 2018). N_2O generated by wastewater treatment plants is caused by the nitrification-denitrification process, produced mainly from aerated and anoxic compartments of biological treatment technologies (Tallec et al., 2008). However, an AGS system is able to perform the complete denitrification process transforming the nitrous oxides into molecular nitrogen gas thanks to the anaerobic conditions which encourage the metabolism of denitrifying microorganisms. The emission of GHG is directly related with the temperature as has been reported by several studies in soil and water environments (Cui et al., 2016, Wang et al., 2018).

To contribute to knowledge of the granulation process under different temperatures from cold-adapted inoculum, and the granulation process in cold environments, long-term experimentation was done to study the dynamics of the microbial communities of activated sludge from Rovaniemi (Lapland, Finland) in the granulation process at native cold temperature (8 °C), mild temperature (15 °C) and warm temperature (26 °C).

Moreover, the efficiency in terms of nitrogen, phosphorous and organic matter removal was monitored, as well as the granules properties during the process and the emission of nitrous oxides. Finally, a correlation study was calculated for qualitative and quantitative data of microbial ecology with operating parameters and physicochemical performance through multivariate analysis.

3. Materials and methods

2.1. Start-up and operation of sequencing batch reactor

The three sequencing batch reactors were of cylindrical shape with a height of 45 cm and a diameter of 9 cm, with an operational volume of 2.5 L, following the design and characteristics of bioreactors described by Muñoz-Palazon et al., 2018b. The hydraulic retention time was 6 hours, with a volume exchange ratio of 60% of total volume in each cycle. The cylindrical reactors were inoculated with 500 mL of activated sludge from Rovaniemi (Lapland, Finland) wastewater treatment plant. The inoculum was cold-adapted (8 °C) microbiota. The air was introduced by fine bubbles at the bottom with an air-flow of 4.5 L min^{-1}. The temperature of the bioreactors was controlled using a room thermostat to the following temperatures: 8 °C (RC), 15 °C (RM) and 26 °C (RW). The oxygen was monitored during the experiment period with values of 7.4±1.4 mg O_2 L^{-1} for the three bioreactors. The pH was controlled in the range of 7.8±0.3 using H_2CO_3. Peristaltic pumps (Watson Marlow, United Kingdom) were used to feed the reactor from

the top. The influent was a synthetic medium simulating high-carbon loading urban wastewater as described by Gonzalez-Martinez et al. (2018a).

The cycle during the first three days consisted of: 20 min of feeding under anaerobic conditions, 180 min of aeration, 10 min of settling time and 5 min of withdrawal. Settling time was then changed from day 4 to day 10 to 5 minutes, and finally reduced to 3 minutes after 10 days of operation and until the end of the operational period.

2.2. Bioreactor performance analysis

Chemical oxygen demand (COD) and biological oxygen demand (BOD_5) were measured three times per week following established protocols (APHA, 2012). The inorganic nitrogen compounds (NO_3^-, NO_2^- and NH_4^+) concentrations were measured in triplicate by ionic chromatography (Metrohm Ion Chromatograph). Total suspended solids (TSS) were determined in duplicate according to standard methods (APHA, 2012). The mean size of granular biomass and settling velocity was measured following the protocol described by Gonzalez-Martinez, et al. (2018a). The dissolved oxygen was controlled by a Crison Oximeter and the pH was measured using an inoLab pH meter.

2.3. Nitrous oxide emissions

Measurements of N_2O concentration in the off-gas were analyzed in triplicate. For this purpose, a cap filter of PVC was installed at the reactor top coated with an air chamber to ensure hermetic conditions. The cap was equipped with a rubber septum to allow air sampling. The reactor was closed with this cap in order to capture off-gas and measure

GHG emissions. Based on previous experiments, off-gas (12 mL) from the reactor headspace was collected with a syringe at 0, 20 and 40 min after reactor closure, within times when gas emissions were linear, and the sample was transferred to 12 mL pre-evacuated vials (Castellano-Hinojosa et al., 2018). N_2O concentrations were analyzed by gas chromatography using a Perkin Elmer Clarus 500 gas chromatograph (Perkin Elmer Instruments, Beaconsfield, UK) equipped with an electron capture detector. Cumulative emissions of N_2O were calculated from the area under the curve after linear interpolation between sampling points (Castellano-Hinojosa, et al., 2019). The N_2O emission were expressed by unit of total suspended solids.

Data from ion chromatography and gas chromatography was using to calculate the daily contaminants by the bioreactors thought mass balance over nitrogen. For the balance the only nitrogen source was ammonium in the influent, also it was assumed that all ammonium influent in the bioreactors was transformed into NH_4^+, NO_2^-, NO_3^-, and NO, N_2O, N_2. To compute the mass balance was used the average values from period steady-stable following the equations:

$$\mathbf{Q_{in}\ TN_{in} = Q_{out}\ TN_{out} + N_{ous\ gas}}$$

$$\mathbf{Q_{in}\ (NH_4^+ - N_{in} + NO_x^- - N_{in}) = Q_{out}\ (NH_4^+ - N_{in} + NO_x^- - N_{in}) + N_2O + (NO + N_2)}$$

2.4. Biomass collection and preparation for molecular biology analysis

Biological samples were taken as 50 mL of activated sludge or granular biomass of operational days 30, 60, 90 and 150. After collection, biomass was submerged in 0.9%

NaCl saline solution and centrifuged at 3500 rpm for 15 minutes. The collected biomass pellets were subjected to DNA extraction in duplicates using FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol. Extracted DNA pools in duplicate were mixed and the nucleic acids were kept at -20 °C and sent to RTL Genomics (Lubbock, TX, USA) for next-generation sequencing process using Illumina MiSeq Equipment and Reagents Kit v3. For next-generation sequencing, the primer pair Bac357-Bac806 was used for the amplification of hypervariable regions of the 16S rRNA gene of Prokarya (Muñoz-Palazon et al., 2019). The primer pair EUK1391-EUKbr was used for the amplification of hypervariable regions of the 18S rRNA gene of Eukarya (Rodriguez-Sanchez et al., 2018).

2.5. Bioinformatics pipeline of next generation-sequencing

The 16S rRNA gene of prokaryotes and 18S rRNA gene of eukaryotes data from next-generating sequencing was treated using mothur v1.39.5 software (Schloss et al., 2016). The paired-end reads were merged into contigs. The contigs underwent a quality-trimming process based on removal of sequences with more than zero ambiguous bases or more than eight bp homopolymers. The remaining sequences were aligned against the SiLVA SEED v128 database, using Needleman conditions and the k-nearest neighbor algorithm with k-mer search using a k-mer size of 8 bp, removing the contigs that failed to start and finish at the position of forwards and reverse primers. The sequences were checked for chimeras using the VSEARCH algorithm implemented in mothur (Rognes et a., 2016). The remaining non-chimeric sequences were classified against the SiLVA nr

v132 database. Then, the remaining sequences were used to construct operational taxonomy units (OTU). The constructions were done using a cutoff of 97% for Prokarya and 95% for Eukarya domains using the abundance-based greedy algorithm implemented in VSEARCH (Westcott and Schloss, 2015, Schloss, 2016). After clustering, singleton OTU were removed from all samples, and the persistent reads within each OTU were used to create a taxonomic consensus.

2.6. Real-time qPCR of target genes involved in depollution process

Real-time qPCR was performed using an Mx3000P qPCR system (Agilent Technologies) and real time PCR data was treated using a MxPro QPCR software version 3.0 (Stratagene, USA), with the primer set of 341F-534R for region V3 of bacterial 16S rRNA gene (Muyzer et al., 1993), ARCH915-(F)- UNI-b-rev (R) for archaeal 16S rRNA (Yu et al., 2008), AmoA1 F-AmoA1 R for determination of ammonia-oxidizing bacteria (Rotthauwe et al., 1997), Crenamo A23 F-Crenamo A616 R for ammonia-oxidizing archaeal (Tourna et al., 2008), FungiQuantF-FungiQuantR for total fungal 18S rRNA gene (Liu et al., 2012), cnorB2F-CnorB6R for denitrifier microorganisms (Braker and Tiedje, 2003), and nosZ1840F-nosZ2090R for complete denitrifier microorganisms with *nosZ* gene Clade I (Henry et al., 2006). The number of copies of each gene were quantified in each DNA pool, using extracted DNA as a template. The qPCR calibration curves were constructed with the plasmid standard using serial ten-fold dilutions (10^2–10^8). The reaction mixture was made in a total volume of 25 mL following Muñoz-Palazon et al. (2018b).

2.7. Statistical Analysis of microbial communities

The study of α-diversity was calculated using the software PAST v3.0 for the determination of diversity, evenness and species richness through Chao-1, Shannon-Wiener, Simpson and Pielou's evenness, calculated for 1000 bootstrap replication.

β-diversity was calculated by Morisita-Horn and Symmetric indices, reported by Barwell et al. 2015, as the most robust indices to capture the difference between pairs of samples for dominant and rare microbial phylotypes. To calculate β-diversity, R software with vegan v2.0 and vegetarian packages was used.

The Similarity Percentages analysis (SIMPER) was calculated to observe the contribution of dominant OTU to dissimilarity between pairs of samples. The OTU tables for prokaryotes and eukaryotes were used for calculation of SIMPER through Bray–Curtis similarity using PAST software v3.4.

The principal component analysis was calculated by the values of OTU zero-correction and centered log-ratio transformation of the OTU tables by generation of 128 Monte-Carlo Dirichlet simulations. Transformed OTU tables were then used for singular value decomposition calculation, which results were represented through principal components analysis plot (PCA) (Muñoz-Palazon et al., 2018b)

2.8. Multivariate redundancy analysis (RDA) and Permutational Multivariate Analysis of Variance (PERMANOVA)

The linkages between Eukarya and Prokarya OTU, number of copies of target genes and physicochemical performance were evaluated by multivariate redundancy analysis. For

this calculation, environmental parameters were transformed to the LOG(X+1). The computation was done by 499 unconstrained Monte-Carlo simulations under a full permutation model in CANOCO 4.5. for Windows.

One-way permutational analysis of variance (PERMANOVA) was calculated by software PAST, with distance of Bray Curtis and under 9999 permutations. The PERMANOVA analysis was calculated to identify significant statistical effects of temperature and operational day over the performance of the systems, the number of copies of target genes and the most abundant Prokarya and Eukarya OTUs.

2.9. Scanning electron microscopy (SEM)

The preparation and visualization of granular samples was done in the Center of Scientific Instrumentation at the University of Granada. First, granules were cut and fixated in a mix solution of 2.5% glutaraldehyde in a pH 7.4 cacodylate buffer 0.1 M at 4 °C for 2 h. Then, they were washed thrice with pH 7.4 cacodylate buffer for 20 minutes. After this, the samples were post-fixated with 1% osmium tetroxide for 2 h, post-fixated with 1% osmium tetroxide for 1 h in darkness and at room temperature, followed by an additional wash with distilled water (three washes of 5 min each). Afterwards, samples were dehydrated by successive baths in ethanol for 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 observation by scanning electron microscope (Carl Zeiss LEO 906E).

3. Results and discussion

3.1. Physicochemical performance and operation of bioreactors

The settling velocity of inoculated activated sludge was around 12 m h^{-1} during the first month of operation, where cores of granulation were not observable. The first small cores of granulation were observable at operational day 35 in RC, linked to an increase in settling velocity (Figure 1A). From operational day 100 until the end of the experiment, the size of granular biomass in the RC reactor was 6.3±0.22 mm diameter. The granular biomass developed in RM achieved the largest mean size (>11 mm) and the fastest settling velocity (>100 m h^{-1}) from operational day 90 until the end of the experiment. The selection of microorganisms and wash-out of filamentous bacteria in RW was slower with respect to RC and RM reactors, suggesting that this deep change in the granulation process could be caused by the strong change of temperature from 8 °C to 26 °C (+19 °C). The core of the RW granules started to be observed at operational day 46, being the slowest granular biomass to form. Possibly, the high temperature caused that microorganisms change the metabolic pathway to adapted to new environmental conditions, managing changes in charges surface of cells, or by change in the production of extracellular polymeric substances, and consequently the settling velocity increasing the floc-forming nuclei, avoiding the strong wash-out of filamentous microorganisms, which is essential for granules formation process. Also, the slowest RW could be due to the floc-forming microbiota were suffered a deep change caused by changes of feeding strategy, hydrodynamic shear force, carbon source and mainly the temperature. The settling velocity for RW had the same trend as RM, with a settling velocity increase from

operational day 100, which was linked with granule size, and with granules reaching mean diameters >9 mm (Figure 1B).

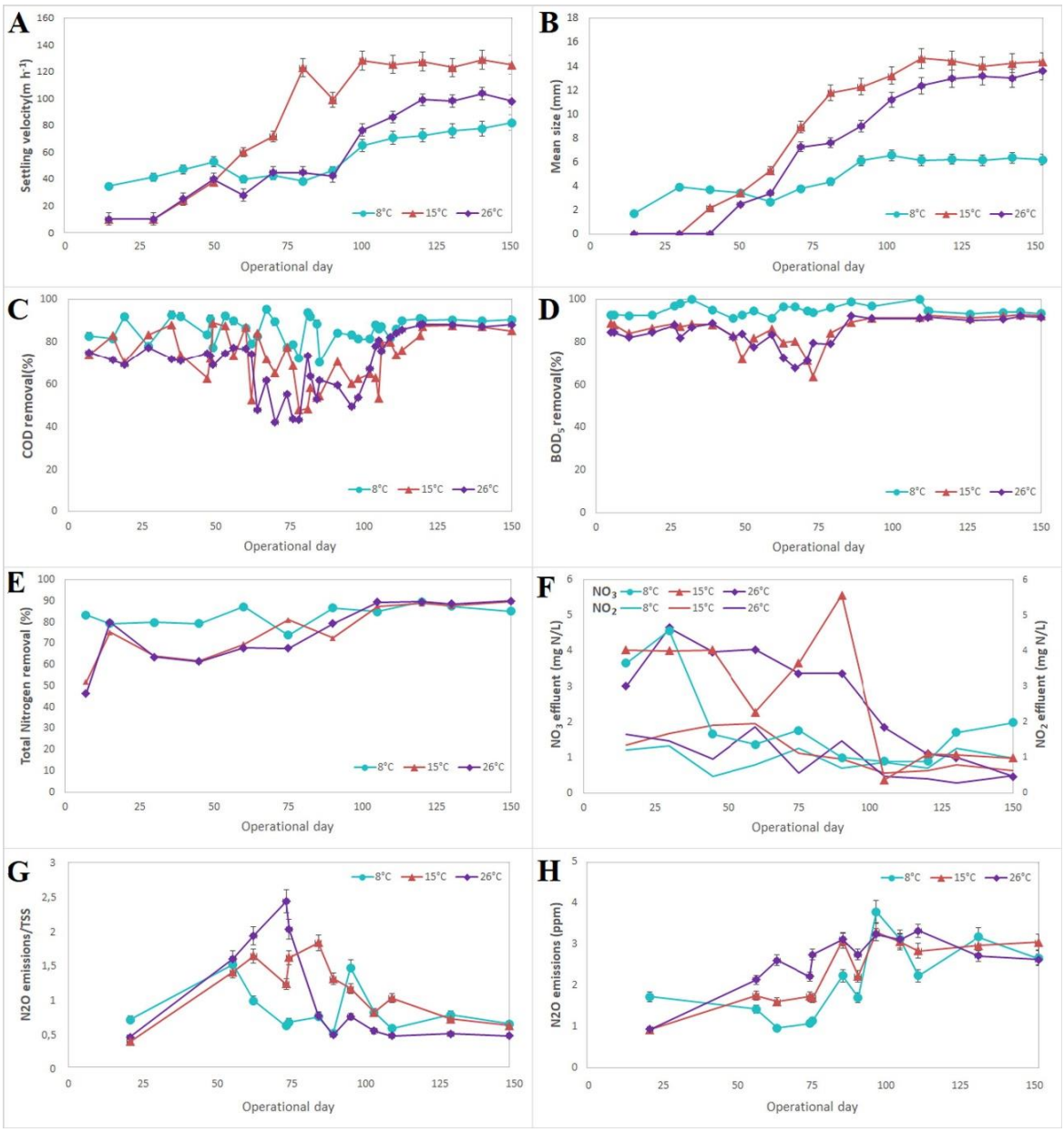

Figure 1. Granules properties: mean size and settling velocity; physic-chemical performance of all bioreactor expressed as chemical oxygen removal; biological oxygen demand removal; total nitrogen removal; and nitrite and nitrate concentration in the effluent expressed in mg-N L^{-1}; emission of greenhouse gases and ratio of N_2O emission and total suspended solids (g L^{-1}) of granular sludge over experimental time of all bioreactor under different temperatures (cold reactor: blue circle; mild reactor: red triangle; warm reactor: purple diamond).

As a global conclusion, stable values in terms of mean size and settling velocity of biomass were reached around day 100 regardless of the operational temperature, however higher operational temperatures seemed to be related to bigger granule size and faster settling velocities. These results suggest that the washout of biomass was fast and strongly correlated with operational temperature.

The BOD_5 removal in the bioreactors during the first 60 days of operation ranged from 60 to 90% of total removal, although after this period the ratio was affected in RM and RW (Figure 1D). During the steady-state phase, BOD_5 removal was higher than 90%, while the highest removal was observed in RC. The removal of organic matter in terms of COD followed a similar trend than BOD_5. Thus, during granulation process the removal performance was higher, but when the granular biomass formed the removal ratio decreased. However, after this it increased again until reaching minimum values of 83% removal (Figure 1C). This trend had been registered in other research about AGS, this results suggest that the high wash-out of microorganisms causes that the biomass concentration in the bioreactor is strongly engaged, so during the first stage when the granules nuclei could be observed the biomass available to remove organic matter or nutrients, such as nitrogen or phosphorous is much lower. After, until the steady-stable the biomass usually is growing until reached a stable MLSS concentration, which suppose the highest organic matter and nitrogen removal ratio. The drop of removal ratio after the first observation of granules is related to biomass concentration in the bioreactor. In terms of total nitrogen removal, RC did not have a wide range of variation in performance regardless of the fact that biomass was suspended or granular (Figure 1E). Despite the low temperature of operation, no accumulation of nitrite and

nitrate in the systems was observed, even though a negative correlation between low temperature and nitrogen removal has been reported by some authors (Gnida et al., 2016), suggesting that the inoculum coming from a cold-adapted environment efficiently removes nitrogen at low operational temperatures. On the other hand, RM and RW had a worse nitrogen removal efficiency than RC until operational day 100 (Figure 1E). Also, no nitrite accumulation was observed in any bioreactor, although higher concentrations of nitrate in the effluent (2–6 mg NO_3^-) was observed during the startup period in all of them (Figure 1F). The total nitrogen and nitrate concentrations (mg-N L^{-1}) in effluents did not reach 3 mg-N L^{-1} during steady-state, being the higher concentration of nitrogen registered in treated water from RC, followed by RM and finally, lower concentration than 1 mg-N L^{-1} in RW. The total nitrogen in the influent was exclusively based on ammonium addition, as the mass balance show a high percent of ammonia oxidation in all bioreactors, although it was not complete. The production of nitrous oxide was proportionally lower in comparison with the production of nitric oxide and nitrogen gas (Table 1).

Table 1. Mass balance on nitrogenous compounds of bioreactors RC, RM and RW in steady-stable.

	TN_{in} (g d^{-1})	TN_{out} (g d^{-1})	NH_4^+-N_{in} (g d^{-1})	NH_4^+-N_{out} (g d^{-1})	NO_x-N_{in} (g d^{-1})	NO_x-N_{out} (g d^{-1})	N_2O-N_{out}(g d^{-1})	gas N_2-NO(g d^{-1})
RC	674,157	106,195	674,157	76,566	0,0	29,629	26,700	541,262
RM	674,157	93,875	674,157	76,610	0,0	17,265	29,800	550,482
RW	674,157	87,609	674,157	0,750	0,0	12,686	27,300	559,249

3.2. Analysis of emission of nitrous oxides

The results linked the emission ratio of N_2O emission with total suspended solids for RC, RM and RW throughout all experimentation periods (Figure 1G, 1H). The results showed that RC during startup had an emission ratio higher in comparison with the other bioreactors, probably due to the higher metabolic activity of cold-adapted microorganisms in shorter operational periods due to the adaptation of the inoculum to low temperature conditions. On the other hand, RM and RW during startup showed lower concentrations of biomass than RC in terms of total suspended solids, which could be linked to lower N_2O emissions. During operation, the emission ratio decreased strongly. In relation to RW, the ratio between emission and TSS was increasing until the end of operation, when the concentration of TSS registered in RW was the highest reached. This fact could be related to the metabolic activity of the microbial community at warmer temperature as was described by Massara et al., 2017, Castellano-Hinojosa et al., 2018, who reported that higher temperature encourages higher metabolic activity in wastewater treatment plants.

RM had a lower ratio between N_2O emission and TSS during the start-up stage. Also, this bioreactor was where lower biomass concentration developed until operational day 75, the trend was decreasing over the experimentation period until it reached a constant steady-stable period. Finally, at operational day 100, the N_2O emission and the ratio of N_2O emission/TSS was stable with values ranging to 2.0–3.5 and 0.5–1.0, respectively.

Finally, as general conclusion related to greenhouse gases in aerobic granular sludge at low temperature it was possible observed that if the biomass was adapted to thermal

conditions of operation, the production of nitrous oxides is similar, as it is shown in Figure 1H, since approximately operational day 100 until the end of operation, regardless temperature. However, from startup period the higher production of nitrous oxide was related to microorganisms not cold-adapted as occurred in reactors RW and RM, suggesting the hypothesize that denitrifying microorganisms with nosZ I gene could not make a complete metabolism to transform nitrous oxide to nitrogen gas.

3.3. Quantitative PCR study of target genes

The absolute quantification of genes that formed the tridimensional matrix of biofilm was made to understand the effect of operational temperature over cold-adapted inoculum during experimentation time. For *Bacteria*, the number of copies of the 16S rRNA gene showed a weak increase in all bioreactors until operational day 60, while the abundance of this gene decreased strongly from operational day 90 until the end of experimentation, with mean values around 10^7 copies g^{-1} in RM and RW (Figure 2). This trend was not as sharp in RC. Granules of RM and RW reached larger mean size, although the granules were fluffier and less dense, on the contrary, RC kept biomass stable in general terms. Interestingly, the number of copies of the archaeal 16S rRNA gene in RC showed a higher order of magnitude than the inoculum and the rest of the samples of RW and RM. This could be produced due to promotion of *Archaea* population in extreme environmental conditions such as cold temperature as occurred in other AGS reactors with cold-adapted inoculum as reported by Muñoz-Palazon et al. (2018). Also some studies had pointed out that the largest proportion and greatest diveristy of archaea

exist in cold environments, as well as molecular analysis had revealed the diverse functional roles and the importance of psychrophilic archaea in a wide cold environment (Cavicchioli et al., 2006). After that, the number of copies of the archaeal 16S rRNA gene showed a decreasing trend from day 30 until the end of operation in all bioreactors, possible due to the presence of other cold-adapted microorganisms more competitive for granule forming under high carbon and nitrogen loading. The activated sludge and the granules at cold temperature during start-up did not show any copies of fungi, although copies were observed in start-up of RM and RW. The presence of fungal populations in biological wastewater treatment has been reported to be strongly correlated with the changes of environmental variables and operation parameters (Maza-Marquez et al., 2016). However, during the operation, the fungal population reached an essential role with 10^5–10^6 copies g^{-1} of granule. *Fungi* had been reported as crucial microorganisms with a structure indispensable for the right development and formation of granules (Gonzalez-Martinez et al., 2018a, Muñoz-Palazon et al., 2018b). Furthermore, fungal populations were reported with high representation in AGS under cold temperature (De Kreuk et al., 2005), corroborating the results obtained through qPCR in this study, in which higher average values of fungal 18S rRNA copies were found.

Figure 2. Quantification of target genes involved in the depollution wastewater.

On the other hand, the ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) were quantified, although copies of AOA did not reach detected levels by MxPro PCR. There was a strong selection of AOB copies from activated sludge to granules in the first stage of operation. The values were sharply affected by the granulation process, being reduced one order of magnitude for RC and RM and three for RW. In RC and RM from operational day 30, the copies of bacterial *amoA* gene were decreasing in value until 10^5 copies g^{-1} of granules remained at the end of experimentation, while bacterial *amoA* copies were more stable for reactor RW remaining stable ranging to 10^5–10^6. Despite the trend of each bioreactor, the final values were similar regardless of temperature. Absolute quantification of NOx-reducer genes showed that the number of *norB* copies, which is in charge of the production of nitrous oxide, were smaller than the number of copies of *nosZ* gene Clade I. Also, the abundance of the *norB* gene was stable during the operation of RC, while the nosZ copies were increasing over time. On the contrary, in RM and RW reactors there was a decrease in the number of copies of *norB* and *nosZ.*

3.4. Dynamics of microbial communities

3.4.1. Prokaryotic community

The diversity of inoculum was high in terms of OTU number. Thus, the phylotypes in the inoculum were of low relative abundance except for *Microthricaceae* and *Methylophilaceae* families, with more than 20% of relative abundance each (Figure 3). Also, *Methylophilaceae* family was represented by OTU with 22% and 6% in the

inoculum. On the other hand, *Arcobacter* and *Acinetobacter* genera assumed the dominance of the community in RC. These phylotypes have been found in AGS at ultra-low temperature (5 °C) (Gonzalez-Martinez et al., 2018). *Arcobacter* has been reported in several wastewater treatment plants. Also, this genus was researched because it is not removed from the bioreactors of activated sludge, thus *Arcobacter* cells pose a potential risk to bio-safety since it does not remain attached to floccules of granules due to pathogen activity (Kristensen et al., 2016). The second most abundant genus *Acinetobacter* was reported to be responsible for disintegration of granules and the deterioration of the granule core (Adav et al., 2010). Also, this phylotype has been described as psychotropic, heterotrophic nitrifier, with tolerance to temperatures below 4°C (Yao et al., 2013). The prokaryotic community changed deeply during the granulation process in RC, showing higher diversity and evenness. Acinetobacter genus and *Comamonadaceae* family were dominant showing a great number of OTU for each one. In the last stage of operation, the community changed with proliferation of the Otu0001 belonging to *Oxalobacteraceae* family. Some members of this family are psychrophilic species (Rosenberg et al., 2014). *Zoogloea*, which belongs to this family, has been reported as a filamentous bacterium characterized as the backbone for floc formation and aerobic granules (Gonzalez-Martinez et al., 2018).

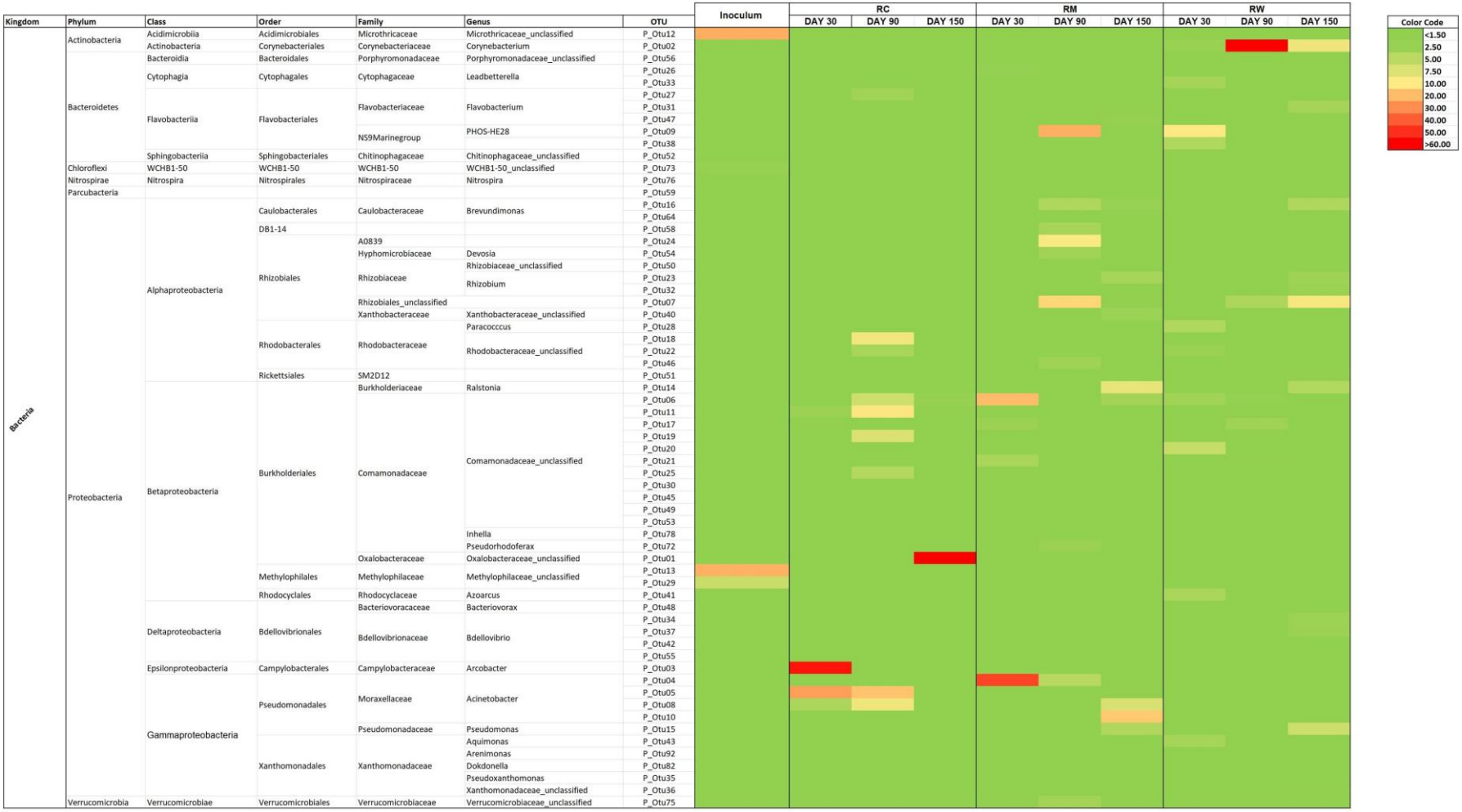

Figure 3. Heat map of dominant OTUs in the system from inoculum until the end of experimentation for each bioreactor for Prokarya with more than 1.50% of total relative abundance (A) and Eukarya with more than 1.00% of total relative abundance (B).

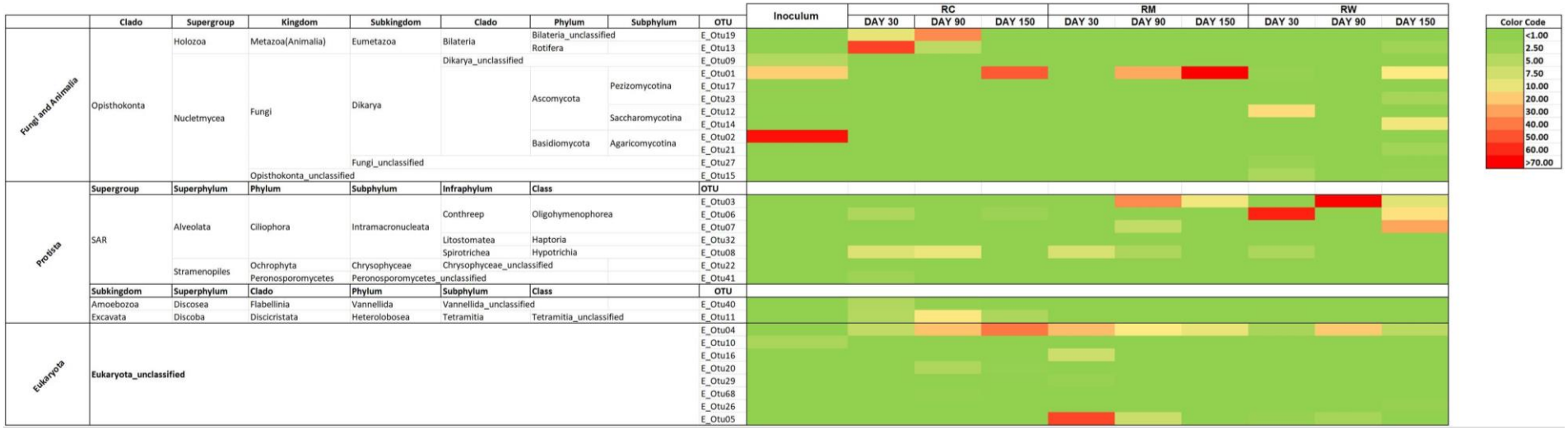

Figure 3. Heat map of dominant OTUs in the system from inoculum until the end of experimentation for each bioreactor for Prokarya with more than 1.50% of total relative abundance (A) and Eukarya with more than 1.00% of total relative abundance (B).

On the other hand, RM suffered changes in the prokaryotic community at operational day 30 with respect to the inoculum, although the trend was similar to reactor RC, proliferating *Acinetobacter* genus and *Comamonadaceae* family. These results suggest that these phylotypes could be involved in granule formation. At operational day 90, the population showed high diversity and evenness. The great diversity was represented by *Acinetobacter* genus, *Rhizobiales* order, PHOS-28 and A0839 family. Extracellular polymeric substances (EPS) could be produced by *Rizhobiales* order in this stage, which has been reported as essential to forming a resistant matrix (Cydzik-Kwiatkowska et al., 2015). Finally, the dynamics showed dominance of different OTUs of Acinetobacter and *Ralstonia* genera. *Ralstonia* have been found in many industrial wastewater treatments such as textile, olive oil mill or acrylic acid (Gan HM et al., 2011; Jalilnejad et al., 2011, Wang et al., 2009).

Compared with the operation at mild and cold temperature, the prokaryotic community in the reactor RW showed an opposite trend against RC and RM due to high representation of several OTU in the system, PHO-28 and *Comamonadaceae* family among them. Dominant OTU were related to *Corynebacterium*, which has been reported in aerobic granules (Ho et al., 2009; Cydzik-Kwiatkowska et al., 2015). Also, aerobic granules based on a culture of *Corynebacterium* have been reported to depollute high-strength organic matter and toxic compounds in high concentrations, suggesting their culture at cold temperature without an acclimatization period (Cydzik-Kwiatkowskaet al., 2015; Muñoz-Palazón et al., 2019). In the last stage at warm temperature, the system

reached high diversity again with different OTU representatives such as *Rhizobiales* order, *Pseudomonas*, *Corynebacterium* and *Ralstonia* genera. Some of these genera, such as *Pseudomonas* and *Ralstonia*, could be correlated with the production of EPS, essential activity to form the tridimensional matrix (Kianpour et al., 2008; Molobela et al., 2010).

The community analysis demonstrated that the biomass granulation selected diverse phylotypes depending on temperature despite the use of the same cold-adapted inoculum. During the stage of operation between day 30 and day 90, a strong selection of phylotypes related to environmental temperature occurred in the AGS system. Some studies reported that the microbial composition in steady-state AGS is unaffected by origin of inoculum (Muñoz-Palazón et al., 2018). These results suggest that the microbial community suffered changes as a function of environmental temperature where the system will be implemented, and not related to inoculum, despite the fact that the depollution performance is faster when the inoculum is temperature-adapted (Muñoz-Palazón et al., 2018b)

3.4.2. Eukaryotic community

In the cold-adapted inoculum, the dominant phylotype was *Agaricomycotina* fungus, which had been found in different technologies for wastewater treatment (Ashhab et al., 2013, Ntougias et al., 2012). However, the dominant fungus during the operation of RC and RW was mainly *Pezizomycotina* a filamentous fungus crucial in the steadiness and steady-state operation in reactors at cold and mild temperature (Gonzalez-Martinez

et al., 2017) formed more than 50% of total relative abundance in the eukaryotic community. Also, the presence of filamentous fungi could be observed in the scanning electron microscopy analysis (Figure 3). However, in RW biomass, the filamentous fungus was exclusively found at operational day 150, with a 12% of total relative abundance, despite the results of the qPCR that showed higher number of copies of 18S rRNA gene of Fungi. These results corroborate the hypothesis of the essential role of fungal populations in this technology especially at mid to low temperature (Gonzalez-Martinez et al., 2018a; Muñoz-Palazon et al., 2018a; De Kreuk et al., 2005). On the contrary, the population in reactor RW at operational day 150 was dominated by three OTU affiliated taxonomically with *Oligohymenophorea*. Also, the presence of *Saccharomycotina* was registered exclusively in this reactor at the end of operation, which has been reported in influent containing high antibiotics concentration under thermal stress (Liu et al., 2013).

3.5. Study of α-diversity, β-diversity and similarity analysis of next-generation sequencing.

The species richness of the prokaryotic community showed the highest values in the inoculum. In RC, the species richness abruptly decreased until the end of operation, registering the lowest values. The trend for RM and RW showed a progressive decline over operational time. The evenness was lower from inoculum sample, in RC a deep decline occurred that recovered on operational day 90, after which the selection of granule-forming microorganisms decreased again. The trend was opposite in RM and

RW since the evenness and diversity was increasing during experimental time, except for the values related to day 90 caused by the overriding proliferation of *Corynebacterium*. The α-diversity study for the eukaryotic community showed stable values over the operational period in terms of diversity and evenness. On the other hand, the species richness showed weak changes from the inoculum, reducing the values in the rest of samples, especially at operational day 30.

For prokaryotic samples, the β-diversity analysis calculated through Morisita-Horn index showed a sharp difference in the dominant phylotypes from inoculum to operational day 30 in all bioreactors. The RC reactor showed dissimilarity in dominant phylotypes during all experimental time, the opposite pattern was found in RM and RW where the dominant phylotypes found were more similar during operational day 30, 90 and 150, although the lowest value was found at operational day 90. This result is corroborated by the study of microbial dynamics. For the eukaryotic community, the β-diversity indices showed greatest similarity in the dominant phylotypes of RC and RM at operational day 150, as verified by the heat map of communities. Greater dissimilarities were found for the reactor RW at the end of operation. In general, the dominant phylotypes of Eukarya for each bioreactor progressively acquired greater similarity during the experiment.

The SIMPER analysis showed the contribution in terms of relative abundance of dissimilarity of eukaryotic and prokaryotic OTU between reactors operated at cold, mild and warm temperature. For Prokarya, the OTU that showed more contribution to

dissimilarity between low temperature versus mid/warm temperature were mainly P_Otu001, P_Otu003 and P_Otu005 with more than >8.00%. These OTU are taxonomically affiliated with *Oxalobacteraceae*, *Arcobacter* and *Acinetobacter*, respectively, and were the phylotypes that marked the greater differences in the prokaryotic community between reactors. Also, the number of OTU that contributed to wide dissimilarities among reactors at low versus high temperature were higher, among them the P_Otu002 affiliated with *Corynebacterium*, contributing to more than 15.00% dissimilarity, which proliferated in the reactor at warm temperature. This OTU marked the difference between reactor at mild and high temperature. The dissimilarities between RM and RW were mainly related to P_Otu002, and P_Otu004. For the eukaryotic community, the SIMPER analysis reflected that the E_Otu001 contributed to dissimilarity in all bioreactors. This OTU was affiliated with *Pezizomycotina* fungus. Also, the E_Otu003 showed high contribution to dissimilarity of high temperature against low/mild temperature, which was affiliated with ciliate microorganisms that was only present in the reactor at warm temperature. The same pattern occurred with E_Otu006, which is *Oligohymenophorea* ciliate. Finally, E_Otu013 and E_Otu019 showed a greater contribution to the reactor at cold temperature as compared to the mid/warm reactors, both OTU belonging to *Bilateria.*

3.6. Ecological analysis of compositional data

The ordination plots for the Prokarya domain shows a similar distance between all samples of bioreactor RM and RW, and the first two biological samples of RL (Figure 4).

The largest distance was found for the last biological sample of RC and inoculum. These results suggest that the community of the inoculum did not affect the development of the prokaryotic community despite temperature, although the lower temperature affected the dynamics of the microbial community. During the first three months, more similarities were found between the samples belonging to RC and RM, however in the last stage, the samples of RM were more similar to RW. The ordination plots of Eukarya samples had a different trend to the Prokarya community. The inoculum sample showed more distance from the rest of the samples. After the granulation process, the biological samples were clustered in three different cores. On one hand, there was a cluster of samples at day 30 and 90 in RC. Also, it was observed the similarity between samples of day 30 belonging RM and RW with day 150 of RC suggesting that at the end of the experiment the communities were similar to those of the granulation process at mild and high temperature. Finally, the samples in steady-state of RM and RW were clustered.

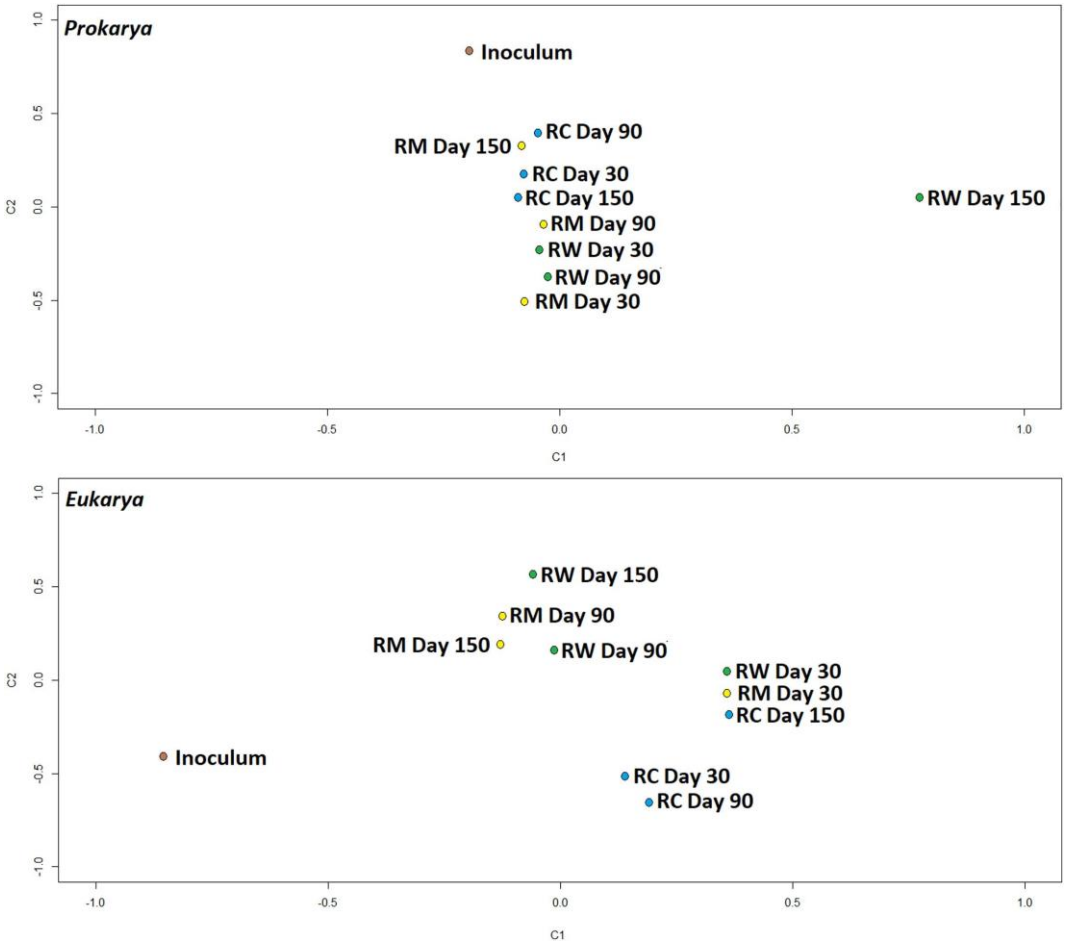

Figure 4. Principal component analysis for the prokaryotic samples (on top) and eukaryotic samples (at bottom)

3.7. PERMANOVA and RDA analyses

The PERMANOVA showed the significant parameters affected by the operational day or temperature. The temperature significantly affected P_Otu002, P_Otu004, E_Otu06, and the number of copies of *norB* genes, which marked nitrous oxide production; while maturation of the systems, in terms of operational days, affects the majority of physicochemical parameters such as COD removal, TSS, accumulation of gases and nitrogen removal, as well as the abundance of bacterial 16S rRNA and AOB genes.

The multivariate RDA was calculated for each bioreactor with the operational parameters, physicochemical performance, eukaryotic and prokaryotic dominant OTUs and biological samples (Figure 5). The RDA of the RC reactor showed strong positive links with the suspended solids, total nitrogen removal and emission of GHG at operational day 150. On the other hand, the E_Otu001 which is taxonomically affiliated with *Pezizomycotina* fungus, revealed a strong relation with total nitrogen removal, as was described by Stein et al. (2016), who reported the role driven by eukaryotic microorganisms which can reduce NO_2^- or NO_3^- to N_2O or N_2. Also, a positive link was shown between a eukaryotic organism related to *Oligohymenophera* ciliate with the concentration of gas accumulated. Similarly, the high ratio of organic matter performance was related to *Pezizomycotina* and *Metakinetoplastina* protist, as well as E_006 and E_Otu011 affiliated with *Artrophoda* phylum and *Pezizomycotina* were related with higher settling velocity and larger size of granules, respectively.

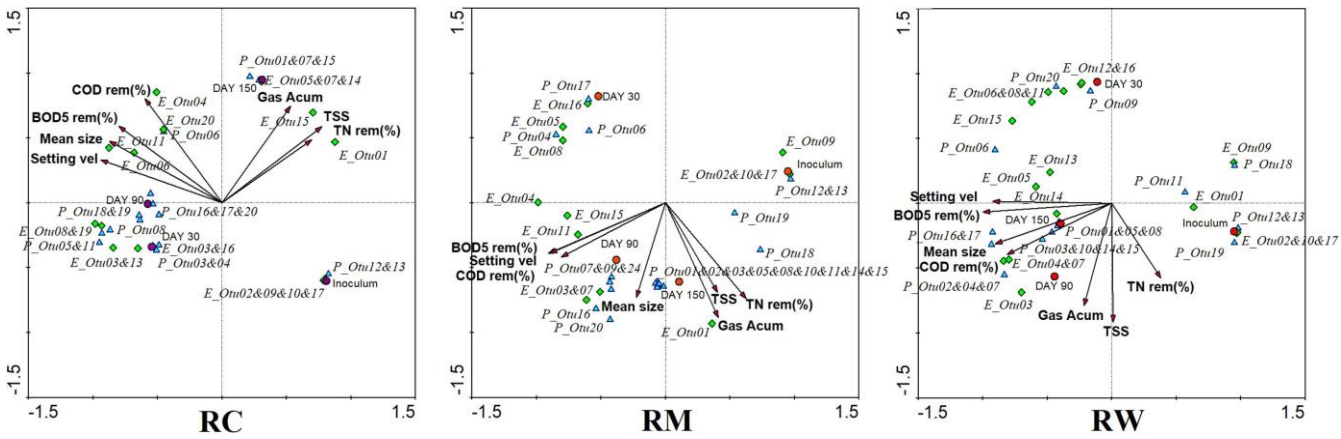

Figure 5. Multivariate redundancy analysis of each bioreactor with dominant OTUS linked with physic-chemical performance for each bioreactor (cold temperature: RC; mild temperature: RM; warm temperature: RW).

For all three bioreactors, removal of organic matter and granular biomass properties were positively correlated, and practically independent of nitrogen removal and GHG emissions. Nitrogen removal and GHG emissions were also positively correlated among them (Figure 5). Several phylotypes were positively correlated with organic matter and nitrogen removal performance under all temperatures, such as P_Otu01, P_Otu07 and P_Otu15, identified as *Oxalobacteraceae* family, *Rhizobiales* order and *Pseudomonas* genus, respectively. P_Otu04 and P_Otu17, related to Acinetobacter genus and *Comamonadaceae* family, respectively, were positively correlated with organic matter removal and negatively with nitrogen removal in RC and RM, however they were positively correlated with both organic matter and nitrogen removal at RW. This result may indicate that similar phylotypes might develop different metabolic roles at different operational temperatures. On the other hand, P_Otu12 and P_Otu13, related to *Microtrichaceae* and *Methylophilaceae* families, respectively, were positively correlated with nitrogen removal and negatively with organic matter removal under all operational temperatures, which relate them with the roles of nitrifiers and/or denitrifiers of the AGS.

3.8. Scanning electron microscopy of mature granules

The biofilm matrix was evaluated with scanning electron microscopy (SEM) analysis. After 150 days of operation of bioreactors under different temperature, six granules were analyzed to observe their spatial conformation and structure of microorganisms. Granules operated at low temperature showed observe the presence of fungi organisms

superficially colonized by bacilli and cocci microorganisms clearly embedded in EPS. The diameter of fungal hyphae was around 2 µm. This spatial situation of microorganisms could generate the diversity of niches presented in the different layers. The full surface of granules formed at cold temperature, showing high density without the presence of filamentous microorganisms. The study of SEM for granules operated at mild temperature were selected as a function of size distribution reached for the medium values, selecting one granule below and above average mean size values, accounting for 8 and 16 mm diameter, respectively. The granules operated at mild temperature shows that the interlayer of granule is formed mainly by filamentous microorganisms which were colonized by a great density of cells in localized areas; the excretion of EPS by bacteria was also perceived. Also, it was possible to observe the complete surface of this kind of granule, which are formed by filamentous microorganism in a compact structure. Finally, the granules operated at warm temperature showed the greatest abundance of bacilli microorganisms and EPS on filamentous fungi hyphae, while the surface was comprised of a dense structure of filamentous microorganisms. Thus, the images reveal that granules at mild and high temperature show high similarities in spatial conformation, EPS production and cellular morphology, while the surface and amount of EPS observed in granules cultivated at cold temperature was lower, evidencing higher differences in sludge depending on the temperature of operation. These images reveal the vital role of filamentous microorganisms in the stability of structure in AGS and the importance of production of extracellular polymeric substance by microorganisms, regardless of origin of activated sludge and operational temperature.

4. Conclusions

Three AGS reactors were stated up with cold-adapted activated sludge from Rovaniemi to observe the performance under different environmental temperatures. The faster granulation process and better removal performance was achieved by RC, following by RM, however RW had a much larger start-up period. The eukaryotic community was similar between RC and RM, while RW was dominated by ciliate microorganisms. Prokaryotic community was completely affected by the temperature at operation. Therefore, the startup of an AGS full-scale reactor should be seed with activated sludge thermally adapted to the area where it is to be implemented.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary data

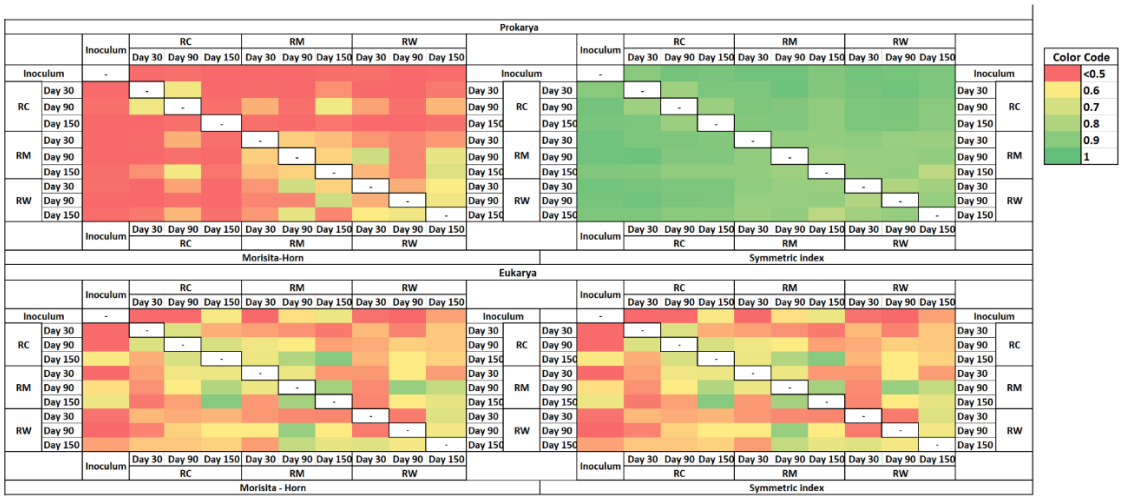

Figure S1- β-diversity calculated thought Morisita Horn index and Symmetric index for dominant and rare phylotypes respectively. The results of Prokarya pairs of samples is shown on top, and the results of Eukarya pairs of samples is shown at bottom.

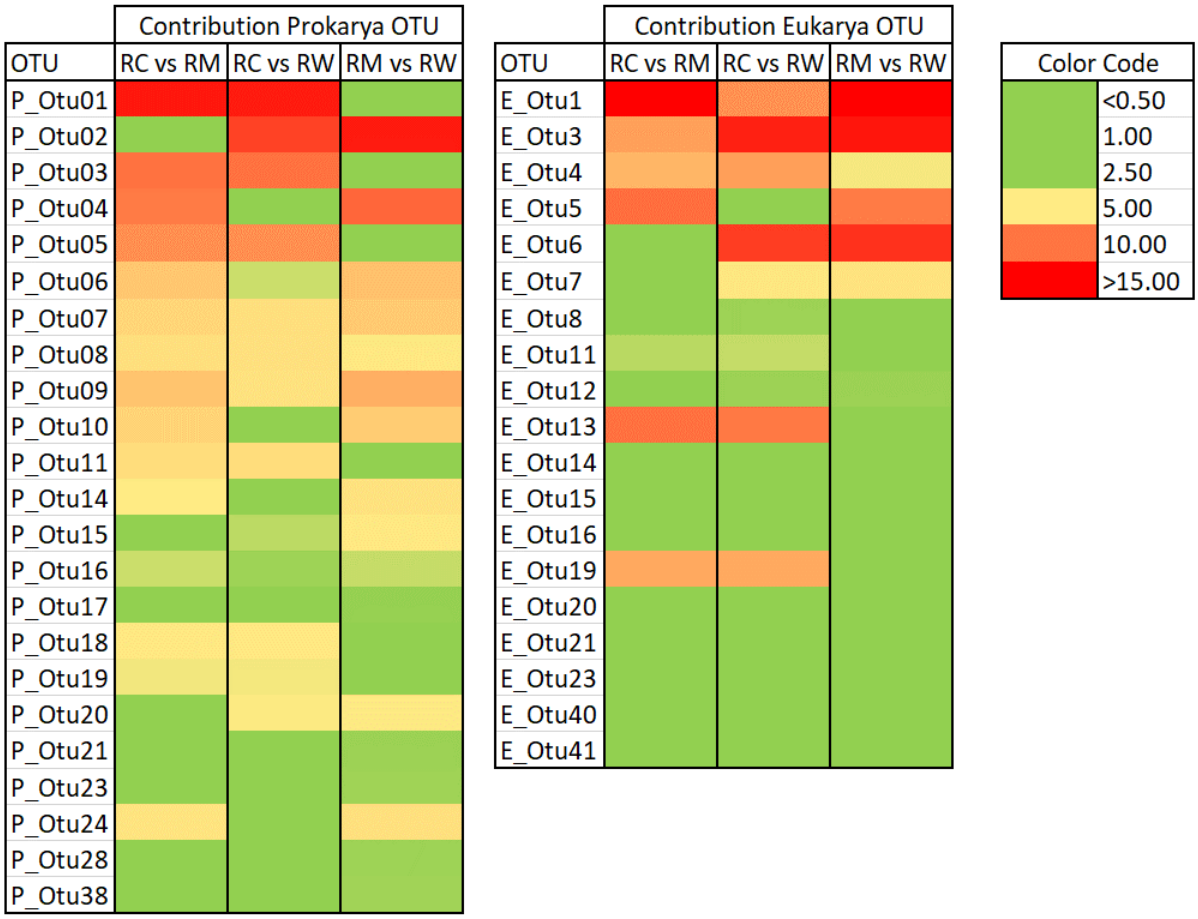

Figure S2- SIMPER analyses for pairs of bioreactors to show the contribution of dominant OTUs to dissimilarities, on the left the Prokarya SIMPER analysis, on the right the contribution of eukaryotic OTU for pair of bioreactors.

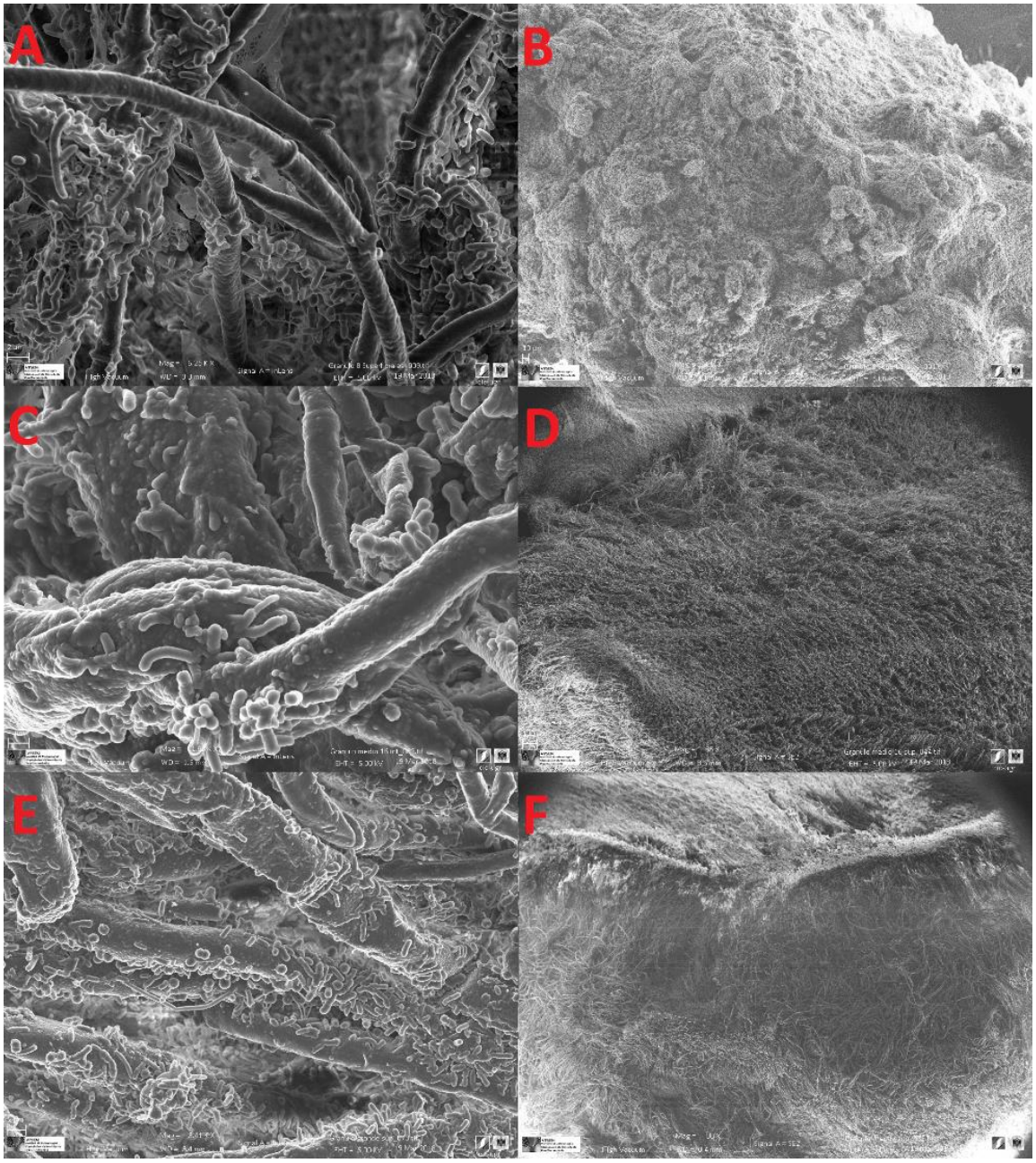

Figure S3.- Scanning electron microscopy of surface granules at operational day 150 of bioreactors operated at cold temperature (A, B); mild temperature (C, D) and at warm temperature (E, F).

Table S1.- α-diversity analysis calculated thought Chao-1, Shannon Wienner, Pielou's evenness and Simpson indices for inoculum and the operational day under different temperatures

	Inoculum	RC			RM			RW		
		Day 30	Day 90	Day 150	Day 30	Day 90	Day 150	Day 30	Day 90	Day 150
Prokarya										
Chao 1	768,2	276,8	502,7	136,8	698,4	352,2	386,6	746,2	448,5	326,4
Shannon Wiener	3,987	1,619	3,367	0,4943	2,692	3,433	4,01	4,578	2,043	4,053
Pielous Evenness	0,07054	0,0202	0,0598	0,01261	0,02279	0,09139	0,1523	0,1376	0,01952	0,1882
Simpson	0,891	0,6201	0,9214	0,1413	0,7509	0,9084	0,9487	0,9719	0,5481	0,9661
Eukarya										
Chao 1	68,4	35	26	48,25	37,33	33	24	36,2	24,2	51,14
Shannon Wiener	1,313	1,577	1,657	1,29	1,419	1,587	1,171	1,423	1,151	1,837
Pielous Evenness	0,05551	0,1862	0,2496	0,09821	0,1477	0,1527	0,1535	0,1297	0,1374	0,1365
Simpson	0,6318	0,6714	0,6978	0,6505	0,6629	0,6915	0,6084	0,6472	0,6111	0,713

Table S2- For each operational temperature, the one-way Permutational Analysis of Variance (PERMANOVA) with 9999 permutations calculated by Bray-Curtis similarity index were calculated to link significant effect of number of copies of targeted genes, physic-chemical performance, prokaryotic and eukaryotic communities in aerobic granular sludge sequential batch reactor. (Temp: Temperature, O.P.: operational day)

	P_Otu01		P_Otu02		P_Otu03		P_Otu04		P_Otu05		P_Otu06		P_Otu07		P_Otu08		P_Otu09		P_Otu10	
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Temp	0,43	0,89	4,6	0,0072	1,18	0,29	2,8	0,002	0,70	0,71	0,75	0,46	0,83	0,53	1,49	2,00	3,07	0,028	1,11	0,67
O.P	2,57	0,06	0,43	0,91	0,74	0,72	0,84	0,53	1,61	0,17	0,67	0,36	1,14	0,36	0,34	0,96	0,39	0,91	1,14	0,46
	E_Otu01		E_Otu02		E_Otu03		E_Otu04		E_Otu05		E_Otu06		E_Otu07		E_Otu08		E_Otu09		E_Otu10	
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Temp	0,86	0,44	0,34	0,89	1,04	0,40	0,84	0,43	1,70	0,11	2,86	0,013	0,950	0,466	0,873	0,482	0,531	0,787	1	1
O.P	1,76	0,18	5,74	0,10	1,63	0,31	0,76	0,47	1,44	0,21	0,60	0,79	0,988	0,456	2,284	0,112	1,834	0,216	1	1
	Mean size		Settling vel		COD rem(%)		BOD rem(%)		TSS		Accumulation gases		TN rem(%)							
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*						
Temp	0,36	0,94	0,80	0,56	2,04	0,20	2,95	0,13	0,41	0,89	0,076	0,98	0,36	0,71						
O.P	2,72	0,11	0,29	0,97	2,93	0,074	1,94	0,22	6,75	0,003	29,33	0,003	13,35	0,007						
	16S rRNA Bacteria		16S rRNA Archaea		18S rRNA Fungi		AOB (*amoA)*		*nor B*		*nos Z* Clado I									
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*								
Temp	0,59	0,69	0,63	0,67	0,73	0,50	0,49	0,81	2,61	0,045	1,20	0,32								
O.P	2,92	0,019	2,26	0,10	0,74	0,96	2,49	0,037	1,06	0,39	1,12	0,36								

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5. General discussion

The goal of this research was to study the effects of different temperatures and thermal adaptations of inoculums during the start-up and operation of aerobic granular sludge (AGS) technology for treating high-organic load urban wastewater.

Thanks to its special properties, the novel AGS system has become a promising technology in the field of biological wastewater treatment. A few years ago interest concerning AGS technology began to grow, bringing with it new research into the design of novel bioreactors, optimization of operating variables and alterations of the microbial community involved in the granular sludge.

Many authors have described the instability of the technology encountered during long-term operation, thought to be due to several factors such as the growth of filamentous microorganisms, low shear forces, and high organic-loads or temperatures (Luo et al., 2014; Adav et al., 2010; De Kreuk et al., 2005). However, the influence that microbial communities exert on the granular stability of the sludge is practically unknown. Additionally, many questions have been raised regarding the effect temperature has on the microbial community that composed the granular sludge, as well as the physical-chemical performance of the aerobic granular sludge.

In order to answer these questions, AGS bioreactors were start-up in a column sequencing batch reactors inoculated with different thermally-adapted activated sludges and operated them at different temperatures. Specifically, the effect of temperature on the granulation process, microbial community and physical-chemical performance of the technology was studied using: 1) mild-adapted, cold-adapted and a mixture of both activated sludges operated at 15°C (Chapter I), 2) mild-adapted activated sludge operated at low (8°C), mild (15°C) and warm (26°C) temperatures, and

3) cold-adapted activated sludge operated at cold (8°C), mild (15°C) and warm (26°C) temperatures.

De Kreuk et al. (2005) previously reported that thermal changes in an AGS system can lead to losses and wash-out of the biomass along with decreased organic matter and nitrogen removal capacities. However, results in our study demonstrate a progressive adaptation by the inoculums, and consequently by the bioreactors, to different operational temperatures.

The experiment performed using inoculums adapted to different latitudes (Finland and Spain) showed a process of biomass selection leading to high settleability, removing slow settling microorganisms and decreasing the mixed liquor suspended solids (MLSS). These results were confirmed in the experiments described in Chapters II and III. Moreover, our results show that selection of floc-forming microorganisms usually occurred faster in reactors operating at colder temperatures when compared to warm temperatures (26°C), possibly as a result of certain metabolic and physiologic changes occurring in the microorganisms at high temperatures determining faster cell aggregation. For instance, changes to the cell surface or production of EPS may lead to faster settling velocities and also an optimal wash-out of biomass. In the same way, our results suggest that the thermal adaptation of inoculums affected the formation of granular nuclei. Thus, it was observed that the first cores of granulation were produced faster when the bioreactors were operated at the same temperature they were previously adapted to. Thus, the thermal profile of areas where AGS systems plan to be implemented should be well-studied in order to optimize the start-up process (Wang et al., 2010).

Our results also indicate that the size of the granules formed varies as a result of the temperature. In all of our experiments, the smallest granules were formed in bioreactors operating at a low temperature (8°C) regardless of the thermal-adaptation of the inoculum, and the second smallest were formed in reactors operating at a mild temperature (15°C). In contrast, settling velocity is correlated with the density and compactness of the granules and thus did not follow a similar trend. Usually, faster settling velocities were achieved by granules in reactors operating at mild and higher temperatures, a fact that could be explained through higher secretion of EPS under these conditions (Liao et al., 2011) leading to increased weight of the granules.

Organic matter removal was strongly affected by the operating temperature of the bioreactor. The organic matter removal ratio, measured as COD and BOD_5, showed sturdy positive correlations with the thermal-adaptation of inoculum and the operational temperature. Specifically, our results showed higher organic matter removal rates in the bioreactors operating at the same temperature as the inoculum's origin. These results suggest that both the origin of the inoculum and the operating temperature of the bioreactor can affect organic matter removal during the set-up phase. However, under stable conditions similar removal rates were achieved in all bioreactors with the exception of bioreactors inoculated with mild-adapted activated sludge and operated at low temperature. Our results clearly agree with previous observations by Costa et al. (2014) suggesting that inoculum selection is an essential part of implementing AGS systems at full scale.

Some of the most relevant features of AGS systems are their high nitrogen removal capacity through nitrification and denitrification in a single chamber under the same

operational conditions, as well as their ability to remove phosphates using microbial biomass. Gnida et al. (2016) have reported that nitrogen removal at low temperatures is challenging for bioprocesses, demonstrating the negative relationship between nitrogen removal and temperatures below 10°C. However, our results show that low or high temperatures did not drastically affect total nitrogen removal during the start-up and stable stages in the AGS bioreactors, as previously suggested by Gonzalez-Martinez et al. (2018a). Our results show that reactors containing inoculums thermally-adapted to their operational temperature had higher removal ratios than reactors operating at differing temperatures, although total nitrogen removal in AGS systems is also affected by other operational parameters, including the dissolved oxygen, hydrodynamic shear forces and circular movement, all of which are characteristics linked to the density and compaction of the granules (Kim et al., 2011).

Nitrate production in the AGS systems was related to the characteristics of the inoculum used during the start-up stage of each bioreactor. As such, higher nitrate accumulation was seen in reactors inoculated with mild-adapted activated sludge while lower nitrate accumulation occurred in reactors inoculated with cold-adapted activated sludge, regardless of the operating temperature. These results may help explain why nitrogen removal performance is very high in WWTP located in Nordic countries such as Finland, regardless of the low ambient temperatures (Gonzalez-Martinez et al., 2018b). Nitrite accumulation values ranging from 5 to 0.5 mg L-1 were not observed in any of the bioreactors.

We studied greenhouse gas (N_2O) production by the bioreactors and found that, during the start-up period, production of N_2O (mg L^{-1}) was higher in bioreactors running at

warm temperatures. This finding may be related to the higher metabolic activities seen in microbial communities at warmer temperatures, as described by Massara et al. (2017). In a similar sense, Castellano-Hinojosa et al. (2018) have reported that higher temperatures increase the metabolic activity within wastewater treatment plants, thus denitrifying microorganisms possessing the *nosZ* gene had insufficient time to completely metabolize nitrous oxide in to nitrogen gas.

Absolute quantification, used to study genes at the end of the experiments, revealed that the number of bacterial 16S rRNA gene copies was higher in the AGS reactors operated at low temperature or seeded with a cold-adapted inoculum. Regardless of bioreactor temperature, copy number of the archaeal 16S rRNA gene usually decreased during the granulation process, suggesting that *Archaea* populations are promoted under extreme conditions (Cavicchioli et al., 2006) and compete with other bacteria in high organic loading environments in AGS systems.

Previous studies have considered fungi responsible for the granular breakage and biomass instability seen in AGS systems (Sharaf et al., 2019; De Kreuk et al., 2005). For the first time, our study analyzed the fungal community in AGS systems through detection, identification and quantification during both the initial granulation process and in mature granules. Our quantification studies show increased fungal copies during initial granulation, increasing from one to five orders of magnitude during the first 30 to 60 days of operation, after which trends diverged for all inoculums and operational temperatures. The greatest number of fungal copies were registered in mild-adapted inoculums. This could possibly be due to the high level of fungal copies in these inoculums. The number of fungal copies in biological wastewater treatment is certainly

highly variable relating to the environmental conditions. The ecological role of fungal communities in biological wastewater treatment has often been neglected, despite the fact that fungal populations are usually metabolically versatile and ubiquitous (Maza-Marquez et al., 2016). Fungal filaments are involved in cell aggregation (Weber et al., 2009) and thus play a key role in the formation of granular sludge under aerobic conditions. Using scanning electron microscopy, we saw that the core of the granules was composed of filamentous fungi with hyphae evidently colonized by bacteria. In this sense, we can suggest that fungal population is essential for the stable development of granules due to exert the structure of core and it is employed as carrier.

In the current study, quantification analyses were also performed on microorganisms involved in the nitrification-denitrification process. The results of our qPCR experiments on total number of *amoA* gene copies show that the ammonia-oxidizing bacteria in mature granules were strongly linked to the original inoculums (see Chapter I and II), although there was no effect on total nitrogen removal ratio. Interestingly we did not detect any ammonia-oxidizing archaea, possibly indicating how little importance these microbes play in nitrification processes under the operating conditions applied in our study.

The number of denitrifying microorganisms correlated with the operational temperature, as copy numbers of the *norB* gene were higher in reactors operating at cold temperatures, followed by granular sludge cultivated at mild temperatures and warm temperatures, although this trend was notably weaker. In all bioreactors, *norB* copies increased in the initial stages, after which the copy number declined to maintain values similar to the inoculum. The copy number of organisms with the *nosZ clade I* gene

was generally higher in mild-adapted inoculums than in cold-adapted inoculums. Moreover, reactors operating at mild and warm temperatures tended to contain a similar or decreased number of copies compared to their inoculums at the end of the experiment, while in reactors operating at low temperatures copy number increased regardless of the inoculum.

The diversity and evenness indices of α-diversity for the eukaryotic communities remained stable across the operational period, independent of both temperature and inoculum, although it could be verified (see Chapter I) as the fungal population suffered a strong depletion in both diversity and evenness. In contrast, the diversity and evenness indices for the prokaryotic communities clearly correlated with both the operating temperature and the inoculum. As such, we observed prokaryotic communities with decreased indices of diversity and evenness in bioreactors inoculated with cold-adapted biomass, while trends depended on the operating temperature in bioreactors inoculated with mild-adapted activated sludge, as low indices of diversity were found at low temperatures and indices similar to that of the inoculum were found at mild and high temperatures. According to Gonzalez-Martinez et al. (2018a; b), these results might be explained through adaptation and survival processes of microbial communities living in extreme environments, such as low temperatures, that can decrease microbial diversity and possibly change the metabolic activity of these cold-adapted populations.

We found that both the origin of the inoculum and the operational temperatures determined the phylotypes shaping the microbial dynamics within the granules using studies with next-generation sequencing. In general, bacterial communities were dominated by *Corynebacterium, Acinetobacter, Arcobacter, Pseudomonas, Meganema,*

Thiothrix, Thauera, Phraetobacter and Arenimonas genera. Some genera, such as *Corynebacterium*, *Acinetobacter* and *Meganema,* were present in communities across different temperatures and origins of inoculum. *Corynebacterium* is a phylotype relevant in wastewater treatment plants as it possesses nitrate reductase and organic nitrogen hydrolase, which have been demonstrated to improve nitrogen removal (Liu et al., 2018). Additionally, *Corynebacterium* had been linked with phosphate metabolism. The *Acinetobacter* genus seems to play an important role in both auto-aggregation activity and protein secretion (Weber et al., 2017), although it remains necessary to control the proliferation of this phylotype as a result of its contribution to granule disintegration, mainly at high-organic loads (Adav et al., 2010). In the same way, *Meganema* was present in the colonies of almost all experiments, a fact that reiterates the importance of this genus in granular sludge (Figueroa et al., 2015). *Meganema* has previously been found to be linked to important roles in AGs systems such as increasing the mean granule size, storing organic carbon, denitrifying organisms, and breaking granules from nuclei (Figueroa et al., 2015; Szabo et al., 2017). *Arcobacter* and *Thiothrix* have been previously reported in systems of granular sludge inoculated with cold-adapted inoculums and operated under low temperatures (Gonzalez-Martinez et al., 2018a).

The archaeal community changed sharply from floccular biomass to granular sludge, disappearing methanogenic archaea in granular sludge (Liu et al., 2017), being *Thermoproteaceae* family the dominant archaea phylotype in mature granules.

Finally, identification of the eukaryotic communities demonstrated both the relevance of the fungal community in granule formation, as well as the importance of the

protozoans in controlling suspended bacteria and peripheral cells, thereby supporting a vigorous trophic chain (Papadimitriou et al., 2007). The fungal community was generally dominated by the *Pezizomycotina* subphylum and *Trichosporonaceae* family, regardless of the inoculum. The remaining sequences showed fungi of uncertain position, which are unexplored fungi. Genera of the *Trichosporonaceae* family have been described in wastewater treatment plants (Maza-Marquez et al., 2016), as well as specifically in granular sludge at cold temperatures (Gonzalez-Martinez et al., 2018a). Furthermore, our complete study of the eukaryotic community showed that protists played an essential role in controlling the population and consuming particulate matter, through processes previously described demonstrating granule disintegration by high-organic loads (Muñoz-Palazon et al., 2019).

In summary, we suggest that the previous thermal-adaptation of the microbial biomass used in the start-up of an AGS system is closely related to the properties of the granular sludge and its microbial diversity in a stable bioreactor. Also, operational temperature is a key parameter to consider during the design, implementation and exploitation of full-scale AGS bioreactors in order to obtain a more efficient start-up period and achieve better nutrient removal with lower greenhouse gas emissions. In this context, it is of great importance to evaluate the temperatures at which bioreactors will be operated in order to correlate this parameter with the microbiological characteristics of the inoculum. However, the considerations made here should be confirmed on a full-scale WWTP level under real conditions due to the inherent temperature fluctuations.

6. General Conclusions

The results reported in this Ph.D. thesis provide the following conclusions:

- The microbial characteristics of the biomass and their previous thermal-adaptation are decisive parameters in terms of nutrient performance and stable microbial conditions for the start-up of AGS reactor. However, the operational conditions, and particularly the temperature, determine the microbial diversity and granular conformation at long-term operation period.

- The granulation process using an inoculum adapted to specify range of temperature will be affected with change of temperature during the operation. If the inoculum is not adapted to operational temperature, the start-up period will be longer; although once the system will be stable the performance in terms of stability and organic matter and nutrients removal is similar.

- The removal of nitrogen by the AGS is high, reaching more than 80% in all studied cases, even at low temperature.

- The smaller granule size was registered at low temperature independently of inoculum. The low temperature did not affect the regular shape and density of granules, while that settling velocity wass faster in granules cultivated at mild and high temperature

- The presence of *Acinetobacter*, *Corynebacterium* and *Pseudomonas* genera with a high relative abundance was observed in bioreactors operated at cold, mild and warm temperature, suggesting the crucial role of these genera in the

granules. However, during the granulation process, the microbial diversity decreased due to the strong selection of microorganisms involved in granular formation.

- The number of archaeal 16S rRNA copies, in general trends, decreased during the granulation process, showing a smaller number of copies than in activated sludge. However, when the bioreactors were inoculated with mild-adapted sludge the number of gene copies was kept stable during operation. Finally, ammonia-oxidizing archaea were not detected in any bioreactor.

- The absolute bacterial copies of 16S rRNA in the granular bioreactors inoculated with mild-adapted sludge was almost stable during all operation. However, if the inoculum was cold-adapted the number of copies was higher at low temperature, followed by mild temperature and lastly warm temperature.

- Bacterial gene involved in the ammonia oxidation decreased in number of copies at the end of operation in mature granules formed with cold-adapted inoculum, while increasing in the bioreactors inoculated with mild-adapted activated sludge.

- *nor B* and *nos Z* genes increased weakly during the granular formation at low temperature, while at mild and warm temperature the number of their copies decreased.

- Fungal population had an essential role in the initial granular formation and in the main structure of granulation core, exercising the surface and bridge for the

bacterial colonization, as it was demonstrated through quantitative analysis and scanning electron microscopy.

- Ciliated microorganisms had a strong dominance in the eukaryotic community, corroborating their role for the population control that conform the granules.

- The inoculation of AGS bioreactors using temperature-adapted inoculums produces a reduction in the start-up period, as well as better performance and stability of the systems.

7. Conclusiones generales

Los resultados obtenidos durante la tesis doctoral proporcionan las siguientes conclusiones:

• Las características microbianas de la biomasa y su previa adaptación térmica son parámetros decisivos en términos de rendimiento de eliminación de nutrientes y condiciones microbianas estables para la puesta en marcha de reactores de fango granular aeróbico. Sin embargo, las condiciones operacionales y particularmente la temperatura determinan la diversidad microbiana y la conformación granular en el período operacional a largo plazo.

• El proceso de granulación utilizando un inóculo adaptado a un perfil térmico se verá afectado con el cambio de la temperatura operacional. Si el inóculo no se adapta a la temperatura operacional, el período de puesta en marcha será más largo; aunque una vez que el sistema sea estable, el rendimiento en términos de estabilidad y eliminación de materia orgánica y nutrientes será similar.

• La eliminación de nitrógeno por sistemas de fango granular aeróbico es alta, llegando a más del 80% en todos los casos estudiados, incluso a baja temperatura.

• El tamaño medio de gránulo más pequeño se registró en reactores operados a baja temperatura, independientemente de la procedencia del inóculo. La baja temperatura no afectó a la densidad, ni a la forma regular de los gránulos. La velocidad de decantación es más rápida en gránulos cultivados a temperatura media y alta.

• Se observó la presencia de los géneros *Acinetobacter*, *Corynebacterium* y *Pseudomonas* en alta abundancia relativa en biorreactores operados a temperatura fría, media y cálida, lo que sugiere el papel esencial que ejercen estos géneros en la biomasa

granular. Durante el proceso de granulación, la diversidad microbiana disminuyó debido a la fuerte selección de microorganismos involucrados en la formación granular.

• El número de copias del gen 16S rRNA de arqueas, en general disminuyeron durante el proceso de granulación, mostrando un número menor de copias en gránulos que en el fango activo. Sin embargo, cuando los biorreactores se inocularon con fango activo adaptado a temperatura media, el número de copias de genes se mantuvo estable durante la operación. Finalmente, no se detectaron arqueas oxidadoras de amonio en ningún biorreactor.

• Las copias del gen 16S RRNA de bacterianas en los sistemas granulares inoculados con fango activo adaptado a clima medio fueron prácticamente estables durante toda la operación. Sin embargo, el inóculo adaptado a bajas temperaturas incrementó el número de copias durante la operación, seguido del biorreactor a temperatura media y, por último, temperatura cálida.

• El gen bacteriano involucrado en la oxidación de amonio disminuyó en número de copias al final de la operación en gránulos maduros formados con inóculo procedente de clima frío, mientras que aumentó en biorreactores inoculados con fango activo adaptado a clima medio.

• los genes *nor B* y *nos Z* aumentaron débilmente durante la formación de biomasa granular a baja temperatura, mientras que a temperatura media y cálida disminuyó el número de copias.

• La población de hongos tuvo un papel esencial en la formación inicial del gránulo, así como en la estructura principal del núcleo de granulación, ejerciendo el filamento tanto de superficie como de puente para la colonización bacteriana, como se demostró mediante análisis cuantitativo y la microscopía electrónica de barrido.

• Los microorganismos ciliados tuvieron un fuerte dominio en la comunidad eucariota, lo que corrobora su papel para el control de la población que conforma los gránulos.

• La inoculación de biorreactores de tecnología de fango granular aeróbica utilizando inóculos adaptados a la temperatura operacional produce una reducción en el período de puesta en marcha, así como un mejor rendimiento y estabilidad de los sistemas.

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