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Efficient carbon, nitrogen and phosphorus removal from low C/N real domestic wastewater with aerobic granular sludge

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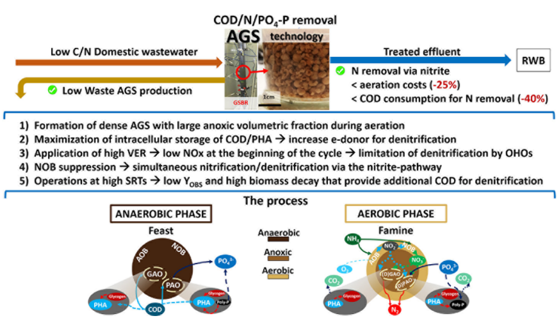
GRAPHICAL ABSTRACT



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ABSTRACT

This work reports on simultaneous nitrification, denitrification and phosphorus removal treating real domestic wastewater with low carbon/nitrogen (C/N) ratio by aerobic granular sludge (AGS). Operations at high sludge retention time (SRT = 61 ± 24 days) resulted in low biomass yield per chemical oxygen demand removed (COD_{rem}) (0.21 ± 0.01 $gCOD_x/gCOD_{rem}$), lower COD demand for denitrification as well as high effluent quality in terms of total suspended solids (TSS) (22 ± 7 mgTSS/L). The average ratio between the biodegradable soluble COD stored anaerobically as polyhydroxyalkanoates (PHAs) and the N removed was 3.1 ± 0.6 $gCOD_{sto}/gN_{rem}$, suggesting that nitrification/denitrification occurred partly via the nitrite pathway. Results revealed that stable AGS process with high C/N/P removal efficiency of 84/71/96% can be obtained besides a low organic loading rate (0.43 ± 0.11 g COD/L/d) and influent C/N ratio (3.8 ± 1.6 g/g), resulting in a high effluent quality characterized by 25 ± 6 mg sCOD/L, 0.09 ± 0.07 $mgPO_4$-P/L, 9 ± 2 mgTIN/L (10 ± 2 mgTN/L) and 22 ± 7 mgTSS/L.

1. Introduction

Aerobic granular sludge (AGS) technology is a promising and viable alternative to conventional activated sludge (CAS) systems for biological wastewater treatment (Pronk et al., 2015a). AGS is a pseudo-spherical biofilm made of self-aggregated microorganisms, having high density, high settling capacities and the possibility to have a multi-layered structure with different redox potential conditions due to e-donors and e-acceptors radial concentration gradients. Therefore, this technology is ideal for simultaneous removal of carbon (C), nitrogen

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(N) and phosphorus (P) due to the presence of aerobic, anoxic and anaerobic micro-environments (Winkler et al., 2018). The conventional cultivation strategy based on hydraulic selection pressure without prior acclimation of the inoculum biomass (Heijnen and van Loosdrecht, 1998) leads to a rapid fast-settling granules formation, but having a low stability in the long-term operation (Bassin et al., 2019). Stability of granular sludge represents the real bottleneck of this technology and, to overcome this issue, a metabolic selection strategy has been proposed from various research groups (Bassin et al., 2019; de Kreuk and van Loosdrecht, 2004; Sguanci et al., 2019; Winkler et al., 2018). According to this strategy, the goal is to enrich the bacterial consortium of inoculum biomass, with slow-growing microorganisms having higher granulation properties, such as polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), which are recognized as key players in granulation process (Layer et al., 2019). PAOs and GAOs have a similar metabolic pathway, and they work according to the succession of anaerobic and aerobic phases (Lopez-Vazquez et al., 2009). During the anaerobic phase, PAOs and GAOs perform the storage of volatile fatty acids (VFAs) produced from fermentation of organic matter, as polyhydroxyalkanoates (PHAs). At the meantime, PAOs release PO_4-P in the bulk derived from the hydrolysis of intracellular poly-P that provide the energy for VFAs storage as PHAs, while GAOs gain the energy for storage mainly from the oxidation of internally stored glycogen (Lopez-Vazquez et al., 2009). Then, during the subsequent aerobic (with dissolved oxygen) or anoxic phase (with nitrite or nitrate), PAOs uptake the dissolved PO_4-P with a positive net mass balance and grow, while GAOs replenish their glycogen pool and grow, both groups gaining the energy from the oxidation of the PHA stored during the anaerobic phase (Smolders et al., 1995). Once stable granules are formed according to the metabolic selection strategy (Bassin et al., 2019; Sguanci et al., 2019), another crucial aspect in a C,N,P removal process is represented by N-removal from low carbon/nitrogen (C/N) wastewater, as in the case of domestic wastewater from combined sewer systems (Sguanci et al., 2019). In this case, the principal issue is the process limitation by low electron-donor availability during the denitrification step, and this represent a major challenge for wastewater treatment plants (WWTPs). Denitrification via nitrite (denitritation) would result in higher N-removal efficiency since it requires 40% less chemical oxygen demand (COD) compared to the conventional nitrate pathway (Lochmatter et al., 2014). The key is that the theoretical catabolic COD demand per g NO_2-N in denitritation and per g NO_3-N in denitrification is 1.71 g and 2.86 g COD, respectively (Metcalf and Eddy, 2003). Moreover, partial nitrification (PN) process reduces the aeration consumption by 25% (Bartrolí et al., 2010). The strategy to obtain the nitrite pathway is to suppress the nitrite oxidizing bacteria (NOB) activity, while retaining and promoting the ammonia oxidizing bacteria (AOB) activity (Bartrolí et al., 2010). At this purpose, various methods can be applied, such as by controlling the concentration of NOB inhibitors (e.g. free ammonia (FA) and free nitrous acid (FNA)) (Ribera-Guardia et al., 2016), by working at low DO/NH_4-N ratio (Bartrolí et al., 2010) given the higher oxygen affinity of AOBs compared with NOBs, by controlling the aeration duration in sequencing batch reactors (SBRs) (Blackburne et al., 2008; Lochmatter et al., 2014). Recently, great attention is paid to denitrification (both denitratation or denitritation) by denitrifying PAOs (DPAOs) and denitrifying GAOs (DGAOs) clades, by utilizing endogenous e-donor source (PHA) (Rubio-Rincón et al., 2017a). Operational strategies aimed at maximizing anaerobic intracellular COD storage (COD_{sto}) (i.e. prolonged/mixed anaerobic conditions) and minimizing growth yield (i.e. working with long sludge retention time (SRT)) would also favour N-removal due to the increase of COD availability as PHAs for denitrification by DPAOs and DGAOs (Rubio-Rincón et al., 2017a; Sguanci et al., 2019). To date, few studies address the nutrients removal from low C/N domestic wastewater with AGS (Coma et al., 2012; Derlon et al., 2016). Moreover, researches aiming at simultaneous N and P removal with AGS at long SRTs are lacking. The main objective of this work is to assess the feasibility to achieve high C,N and P removal efficiencies treating low C/N real domestic wastewater with AGS technology. Attention was paid to waste aerobic granular sludge production, as well as performances and microbial composition of mature granules.

2. Materials and methods

2.1. Lab-scale granular sequencing batch reactor (GSBR)

A granular sequencing batch reactor (GSBR) of initial working volume of 4 L and height/diameter (H/D) ratio of 18.75, was operated for a long-term period of 522 days at the wastewater treatment plant (WWTP) of San Colombano (Florence, Italy). The GSBR cycle structure is shown in on-line e-supplementary data. The criterion of metabolic selection was followed to cultivate stable and well-selected aerobic granules, as demonstrated by Sguanci et al. (2019). A common GSBR cycle was structured in two macro-phases: i) an up-flow anaerobic feeding and an anaerobic mixing phase with recirculation of oxygen-free process-gas to favour the metabolic selection of slow-growing microorganisms such as PAOs and GAOs; ii) an aerobic phase with external air supply for dissolved oxygen (DO) control, aimed at promoting nitritation by AOBs despite of nitratation by NOBs and aerobic/anoxic phosphates uptake by (D)PAOs. In this phase a simultaneous denitrification by DPAOs/DGAOs can occur. The aeration phase is composed of two sub-phases: a DO-controlled sub-phase (Aeration 1) and a final "tail" without DO control (Aeration 2), where oxygen is progressively depleted to a minimum thus maximizing the anoxic volume inside the biofilm of aerobic granules and enhancing the denitrification of residuals NO_x; iii) each cycle ends with settling, withdrawal of the effluent and an idle phase before the next start.

2.2. Inoculum, influent wastewater composition and operational conditions

The inoculum was a mixture of 1L of settled activated sludge collected from San Colombano (Firenze) WWTP, and 150 mL of AGS cultivated in a previous study (Sguanci et al., 2019). The average composition of feeding low C/N domestic wastewater is reported in Table 1a), where the letter C stands for biodegradable COD (bCOD). Globally, the experimental period was divided into two macro-stages (Stage I and Stage II), operated at different SRT. From day 0 to day 221 (Stage I) the average SRT was 22 ± 17 days, whereas from day 222 to day 522 (Stage II) the average SRT was increased to 61 ± 24 days to evaluate the effect of high SRT on the Y_{obs} in aerobic granular sludge process. The operational cycle length and the volumetric exchange ratio (VER) ranged from 3 h to 6 h and from 64% to 79%, respectively, in order to guarantee a higher organic loading rate and to promote the aerobic granulation. During the first 42 days of operation (start-up), the cycle duration was fixed to 3 h, with a VER of 64% and a hydraulic retention time (HRT) of 4.7 h. Then, the cycle duration and HRT were increased to 4 h and 6.2 h, respectively, to favour the hydrolysis of organic particulate matter of the influent. From day 177 onwards, the working volume and VER were increased up to 4.5 L and 79%, respectively. From day 311 until the end of the experimental period the cycle duration and the HRT were increased to 6 h and 7.6 h, respectively. Regarding the applied hydraulic selection pressure (HSP), expressed as the minimum settling velocity inside the reactor, it was maintained on values between 1.15 and 2.74 m/h during Stage I, and between 2.74 and 3.08 m/h during Stage II. These HSP values are among the lowest compared to literature references (Ni et al., 2009; Pronk et al., 2015a; Derlon et al., 2016; Bengtsson et al., 2018), and the metabolic selection was thus the main criterion for aerobic granular sludge cultivation. In a previous research (Sguanci et al., 2019) the authors have demonstrated that high granular sludge stability can be

Table 1
a) Average composition of domestic wastewater fed to GSBR reactor. b) Operational conditions and removal efficiencies of GSBR reactor.

Parameter		Stage I Days: 0 - 221	Stage II Days: 222 - 522
a)			
tCOD	[mg/L]	133 ± 12	178 ± 27
sCOD	[mg/L]	111 ± 20	128 ± 29
pCOD	[mg/L]	22 ± 7	50 ± 9
bCOD	[mg/L]	87 ± 8	129 ± 8
bsCOD	[mg/L]	77 ± 21	108 ± 27
TN	[mg/L]	31 ± 6	34 ± 9
NH_4-N	[mg/L]	27 ± 5	30 ± 9
bCOD/TN	[-]	2.8 ± 1.3	3.8 ± 1.6
PO_4-P	[mg/L]	7.2 ± 0.8	1.8 ± 1.2
TSS	[mg/L]	18 ± 5	48 ± 31
VSS	[mg/L]	15 ± 5	36 ± 26
b)			
SRT	[d]	22 ± 17	61 ± 24
HRT	[h]	4.7 – 5.1	5.1 – 7.6
VER	[%]	64 – 79	79
HSP	[m/h]	1.15 – 2.74	2.74 – 3.08
Cycle length	[h]	3 – 4	4 – 6
Feeding	[min]	15 – 90	15 – 15
Anaerobic mixing	[min]	20 – 60	30 – 60
Aeration 1	[min]	30 – 160	85 – 250
Aeration 2	[min]	30 – 130	40 – 120
Settling	[min]	15 – 25	13.3 – 20
Discharge	[min]	3.3 – 6.7	3.3
Idle	[min]	1 – 11.7	1.7 – 3.3
DO set-points*	[mg/L]	1.4 – 1.5	1.0 – 1.2
		2.4 – 2.5	2.3 – 2.5
COD removal	[%]	69 ± 9	84 ± 5**
N removal	[%]	31 ± 12	71 ± 6**
P removal	[%]	59 ± 28	96 ± 3**
TSSeff	[mg/L]	29 ± 14	22 ± 7**

* Minimum (above) and maximum (below) DO ranges during the Aeration 1.
** Average values calculated at pseudo-stationary conditions (days 487–522).

obtained by operating at low HSP and by applying the metabolic selection as described in the previous section.

2.3. Analytical procedures and measurements

COD, total nitrogen (TN), ammonia–nitrogen (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N), phosphate (PO_4-P), total suspended solids (TSS), volatile suspended solids (VSS), sludge volume index after 30 (SVI_{30}) and 5 (SVI_5) minutes, were measured according to the Standard Methods (APHA/AWWA/WEF, 2017). Total COD (tCOD) was determined by analysing the raw sample; soluble COD (sCOD) was determined after filtration at 0.45 µm; particulate COD (pCOD) was determined by difference between tCOD and sCOD; biodegradable soluble COD (bsCOD) was calculated by difference between sCOD and the inert soluble COD, assumed equal to the effluent sCOD. Total inorganic nitrogen (TIN) was calculated by summing NH_4-N, NO_2-N and NO_3-N concentrations. The size distribution of granules was measured using an image analysis procedure (Tijhuis et al., 1994). pH and dissolved oxygen (DO) were constantly measured trough Hamilton® probes and registered inside the memory of a panel logic controller (PLC). Calculations of SRT, Y_{obs}, anaerobic COD consumption (Smolders et al., 1994; Metcalf and Eddy, 2003; Henze et al., 2015), and the protocol steps for microbial community analysis (Wang et al., 2007; Martin, 2011; Takahashi et al., 2014; Albanese et al., 2015; Rognes et al., 2016), are reported in on-line e-supplementary data.

3. Results and discussion

3.1. Aerobic granular sludge features

3.1.1. Evolution of TSS, VSS, settleability and particle size distribution

The GSBR was inoculated with a mixture of 1L of activated sludge and 150 mL of AGS cultivated in a previous study (Sguanci et al., 2019), in order to speed-up the granulation process. Image analysis revealed that granules mean dimensions were included within the dimensional class of equivalent diameter (d_{eq}) 0.2–0.5 mm after 10 days of operation, while on day 500 the mean d_{eq} was 1.5 mm, typical of mature aerobic granules (on line e-supplementary data). The flocculent fraction of mixed liquor (d_{eq} < 0.2 mm) decreased from 16% (day 10) to 9% (day 500). The reason of this evidence was twofold: on the one hand, almost all the bsCOD was stored anaerobically by PAOs/GAOs, as discussed in the following sections, and no soluble organic matter was available during the aerobic phase for flocculent OHOs growth; on the other hand, the growth of denitrifying fast-growing OHOs is also limited by the minimization of NO_x in the effluent and by diluting the residual NO_x in the reactor through the application of a high VER at the beginning of GSBR cycle. Although flocs are known to more readily adsorb particles for hydrolysis (de Kreuk and van Loosdrecht, 2004), the low TSS_{eff} concentration arises mainly from: 1) low influent TSS concentration (18 ± 5 mg/L during Stage I and 48 ± 31 mg/L during Stage II) (Table 1a) compared to other studies (Pronk et al., 2015a,b, 236 mg/L; Derlon et al., 2016, 140 mg/L). 2) limitation of fast-growing flocculent/filamentous OHOs in favour of slow-growing organisms (i.e. PAOs, GAOs) that are known to be key for stable and dense aerobic granules formation (de Kreuk and van Loosdrecht, 2004). Furthermore, the high SRTs and mixed anaerobic contact time applied allowed the hydrolysis of solids as pCOD adsorbed on the surfaces of aerobic granules. On the other hand, the lower is the flocculent/fluffy fraction with poor settling properties, the lower will be its washout from the GBSR at a proper HSP, thus resulting in a lower TSS_{eff} concentration. Nevertheless, considering the lower specific surface of fully granular sludge compared to systems with the presence of a flocculent fraction, future studies should explore the effluent TSS achievable in the case of higher solids load.

During the first 10 days of operation, after the start-up, an initial washout of flocculent biomass was observed. Then, the VSS concentrations progressively increased to 4.4 gVSS/L (day 310) (Fig. 1a). Excess sludge withdrawals were performed in order to control the SRT roughly every two weeks. The most intense sludge withdrawal was performed on day 402 for the conduction of ex-situ batch tests and provoked a sensible decrease of VSS inside the reactor, down to 2.2 gVSS/L. At the end of the experimental period the aerobic granular sludge concentration reached 3.5 gVSS/L and during the last month of operation the process was considered in pseudo-stationary conditions.

Despite of the long SRT applied during the Stage II (61 ± 24 days), an increase of VSS/TSS ratio from 79% ± 4 up to 90% ± 3 was observed (Fig. 1a) and aerobic granules appeared compact and stable (online e-supplementary data). This confirmed that operations at high SRT, in the case of the treatment of wastewater from combined sewer systems, would result in the maximization of hydrolysis and storage of particulate organic matter thus favoring the growth of PAOs and GAOs (Sguanci et al., 2019).

By observing the time courses of SVI_5 and SVI_{30} (Fig. 1b) the initial worsening of the settleability of mixed liquor observed during the first two weeks of operations was likely due to the lack of poly-aluminium chloride (PAC) dosage, differently from the conditions under which the activated sludge inoculum was previously cultivated, as observed in a previous study (Sguanci et al., 2019). In fact, the flocculent biomass used as inoculum in this study originated from a WWTP where PAC dosage is applied to remove phosphorus and to improve the sludge settling properties. During the following period SVI_5 and SVI_{30} decreased sharply, reaching values on day 50 of 30 and 24 mL/g TSS,

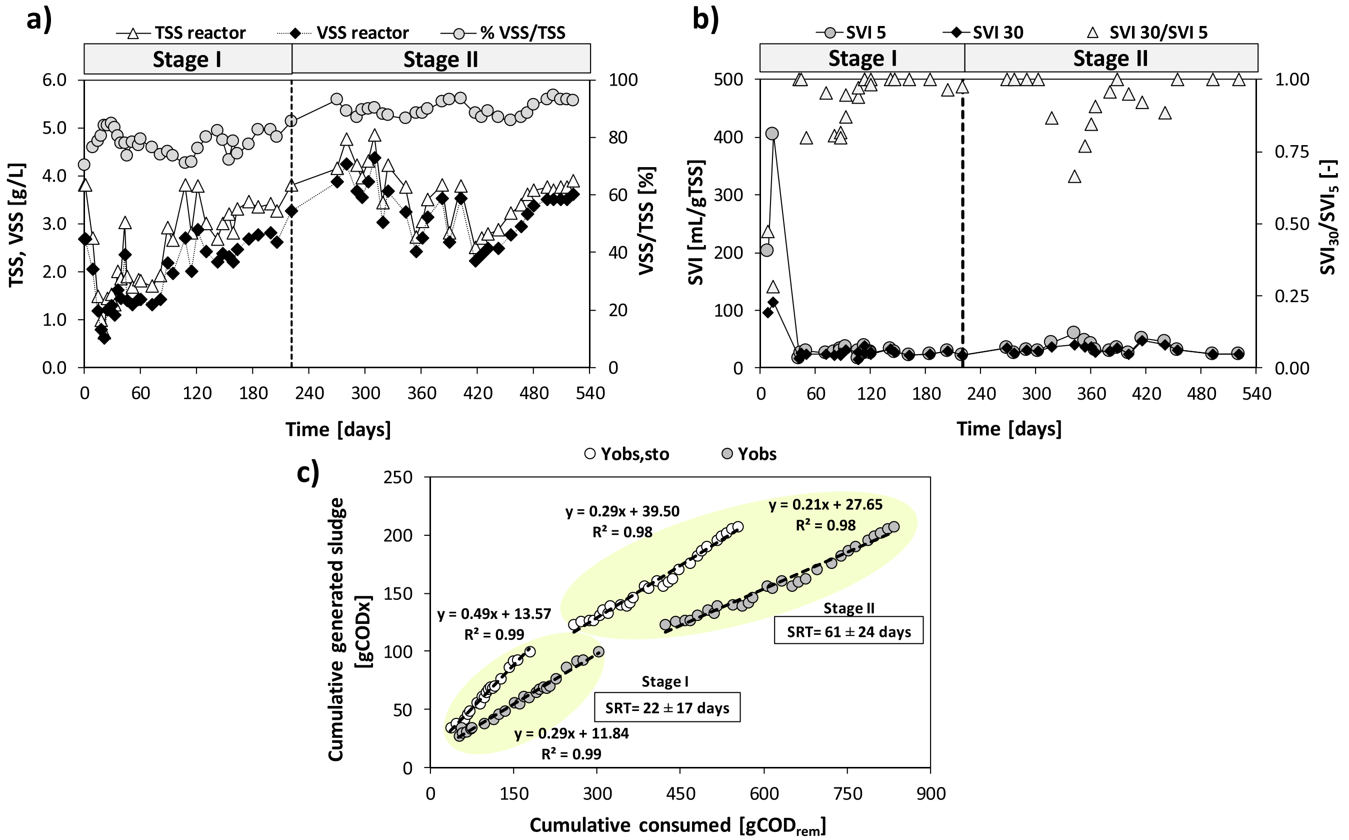

Fig. 1. a) Evolution of TSS and VSS; b) SVI_5 and $SVI_{30;}$ c) Total observed sludge yield (Y_{obs}) and observed sludge yield taking into account the COD stored by slow-growing microorganisms ($Y_{obs,sto}$) calculated as the slope of the curves of cumulative generated sludge versus cumulative consumed COD.

respectively. Contextually, the SVI_{30}/SVI_5 ratio progressively rose up to one (day 114), denoting a system granules-dominated. However, on day 343, a worsening of the SVI_{30}/SVI_5 ratio was registered, due to the occurrence of technical problems. In fact, in the period between days 318 and 343, a biofilm layer grown on the surface of the DO probe, resulted in an underestimation of the DO levels present in the bulk liquid of the reactor. This issue lead to an increase of NO_x concentrations in the effluent, that were denitrified by OHOs during the initial anaerobic phase of the following cycle. The occurred conventional denitrification favoured the growth of OHOs. While, especially during the granulation phase, some OHOs are considered positive for granules formation and stability due to the production of extracellular polymeric substances (EPS), other OHOs are fast-growing filamentous bacteria that could threaten the stability of granules (Winkler et al., 2018), and preferentially grow in the form of poorly settling flocs and/or fluffy external layer of granules (de Kreuk and van Loosdrecht, 2004). The growth of OHOs is favoured by the simultaneous presence in the bulk of COD and e-acceptors (oxygen, nitrite, nitrate), as occurred during days 318-343, thus resulting in the observed deviance of the SVI_{30}/SVI_5 ratio from one (Fig. 1b). After solving the issue, the system recovered its settling properties, reaching a SVI_5 = SVI_{30} = 36 mL/gTSS the day 389. Another technical problem occurred on day 398, causing a partial breakage of granules without a loss of biomass with the effluent. This issue resulted in another deviation of SVI_{30}/SVI_5 ratio at the end of the experimental period (Fig. 1b), thus confirming the resilience of AGS systems to operating irregularities.

3.1.2. Granular sludge production and observed yield evaluation

Once pseudo-stationary conditions were obtained (Stage II), long SRT of 61 ± 24 days was maintained. Throughout all the experimental period, the "feast"/"famine" regime of bulk organic matter (COD) was controlled guaranteeing a complete anaerobic feast phase internal storage of biodegradable bsCOD (>90%), followed by an aerobic famine (Fig. 2). Focusing on the GSBR excess sludge production, Fig. 1c) shows the total observed sludge yield (Y_{obs}) and the observed sludge yield taking into account the COD stored during the anaerobic phase ($Y_{obs,sto}$) calculated as the slope of the curve of cumulative generated sludge versus cumulative consumed COD, throughout all the experimental period.

Except for the start-up phase (days 0–40) where Y_{obs} was between 0.46 and 0.62 $gCOD_x/gCOD_{rem}$ due to uncomplete anaerobic internal storage of bsCOD and thus the abundance of fast-growing microorganisms (i.e. OHOs), Y_{obs} gradually decreased. It is worth noting that two slopes for Y_{obs} were registered during the experimental campaign, depending on the applied SRT. The first one was 0.29 ± 0.02 $gCOD_x/gCOD_{rem}$, during the first 221 days operating at an average SRT of 22 ± 17 days. The second one was 0.21 ± 0.01 $gCOD_x/gCOD_{rem}$ for the remaining days of operation, corresponding to an average SRT of 61 ± 24 days in stationary conditions. These values of Y_{obs} were at least 60% less than those usually obtained for CAS systems (Metcalf and Eddy, 2003), and about 40–45% less than reported full-scale AGS applications (Pronk et al., 2015a). The increase of SRT was applied with multiple purposes: enhance the hydrolysis of pCOD, lower the excess sludge production and, therefore, reduce the metabolic demand for denitrification, as discussed in section 3.2.3. When operating at high SRT microorganisms need more energy for their maintenance requirements and biomass decay processes become more important resulting in the decrease of net biomass synthesis. This was conceived as viable technique for minimisation of sludge production in biological wastewater treatment (Henze et al., 2015). Observing the obtained results for $Y_{obs,sto}$, also in this case two slopes were observed corresponding to values of 0.49 $gCOD_x/gCOD_{sto}$ (SRT = 22 ± 17 days) and 0.29 $gCOD_x/gCOD_{sto}$ (SRT = 61 ± 24 days). Although $Y_{obs,sto}$ does not represent the real sludge production, the comparison with the total Y_{obs} could provide useful information on the growth of storing organisms

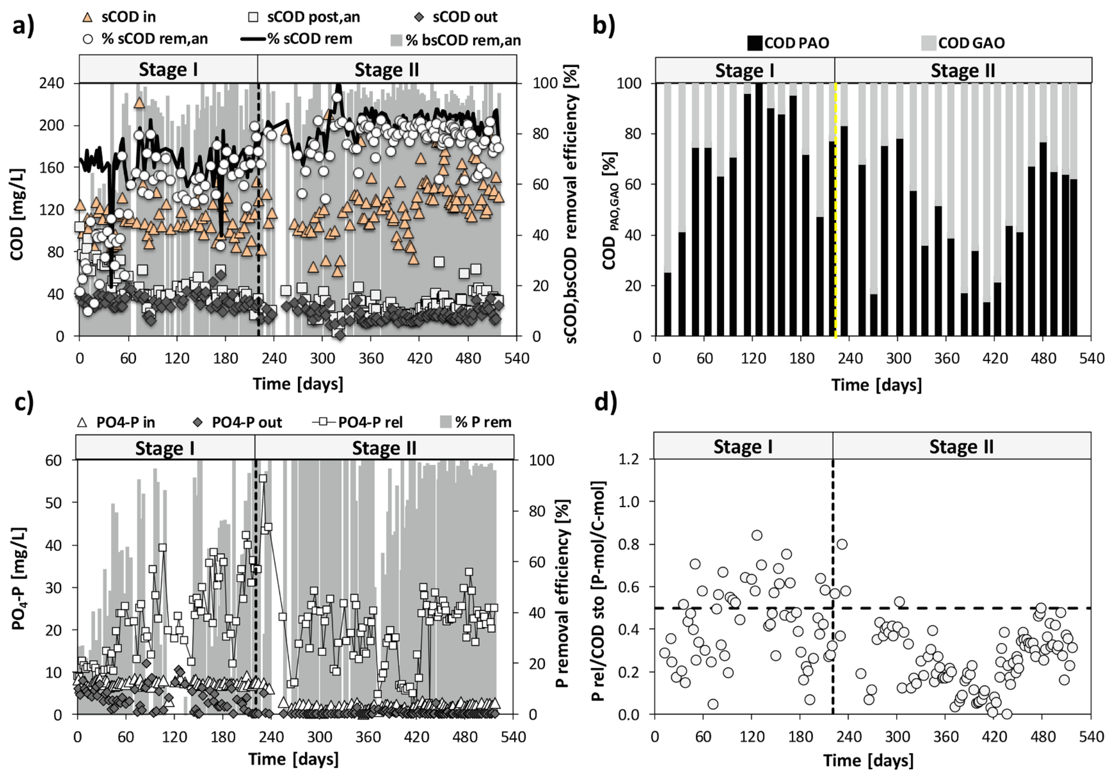

Fig. 2. (a) Evolution of sCOD in the influent (sCOD in), after anaerobic mixing (sCOD post,an), in the effluent (sCOD out), sCOD and bsCOD removal efficiencies; (b) repartition of COD_{sto} between PAOs (black) and GAOs (grey) as averages calculated over a two-weeks period; (c) Time course of phosphates in the influent (PO_4-P in), effluent (PO_4-P out), released after the anaerobic phase (PO_4-P rel), and P removal efficiency; (d) evolution in time of P-release/C-uptake ratio.

such as PAOs and GAOs. By comparing both the observed yields obtained at the two SRTs, the ratio between total Y_{obs} and $Y_{obs,sto}$ increased from 0.59 to 0.72 by increasing the SRT. The closer this ratio is to one the more the sludge production is attributable to the growth of PHA-storing microorganisms. Therefore, the observed increase of this ratio denoted a greater enrichment of PAOs and GAOs in the biomass. This result confirms the fundamental importance of SRT in selection of microorganisms, as also reported by Pronk et al. (2015b). Furthermore, the use of real domestic wastewater as feed, could result in the enrichment of different PAOs and GAOs microorganisms than those commonly enriched in systems fed with synthetic medium (e.g. *Ca.Accumulibacter* (PAO) and *Ca.-Competibacter* (GAO)), such as fermentative PAOs and GAOs whose metabolisms are quite different (Nielsen et al., 2019).

3.2. Long-term GSBR performances

The GSBR was operated for 522 days. During Stage I (0–221 d) the GSBR worked at a SRT of 22 ± 17 days; during Stage II (222–522 d) the SRT was increased up to 61 ± 24 days. In the following sections, organic matter, phosphorus and nitrogen removals are discussed. Table 1b) resumes the operational conditions and the performances of the GSBR throughout the whole experimental period. A detailed cycle analysis performed on day 500 (Stage II) is reported in online e-supplementary data.

3.2.1. Organic matter removal

The influent, the effluent and the soluble COD concentrations after the anaerobic mixing phase, as well as the overall and the anaerobic removal efficiencies of sCOD and soluble biodegradable COD (bsCOD) are reported in Fig. 2a). During Stage I, the increase of VER from 64% to 79% operated on day 177 resulted in an increase of the bsCOD anaerobic removal efficiency from 74 ± 24% up to 95 ± 7%. This was due to the higher dilution of NO_x accumulated during the previous cycle, thus limiting the availability of e-donors for denitrifying OHOs and creating the proper anaerobic redox conditions to promote the anaerobic uptake of biodegradable COD by PAOs/GAOs, as also observed by Sguanci et al. (2019). During Stage II, the increase of SRT did not involve a sensible variation of COD_{sto} efficiency (92 ± 7%). This particular observation leads to suppose that VER increases have a more direct effect on the biodegradable soluble COD storage efficiency than SRT increase. Regarding the anaerobic removal efficiency of sCOD, lower values were registered both in Stage I with the VER of 64% (49 ± 18%) and the VER of 79% (68 ± 8%), and in Stage II (77 ± 7%), mainly due the presence in the influent of inert soluble COD that was not stored anaerobically, nor biodegraded aerobically, thus accumulating in the effluent (25.4 ± 9.8 mg/L). However, the sCOD removal efficiency throughout the whole GSBR cycle (black solid-line in Fig. 2a) was very close to the sCOD removed anaerobically (69 ± 9%, in Stage I, and 84 ± 5%, in Stage II). This confirmed that almost all the biodegradable sCOD was removed anaerobically by OHOs, PAOs and GAOs. In order to discern the anaerobic COD removal exerted by PAOs and GAOs, thus excluding the COD consumption by OHOs to denitrify the NO_x present in the bulk at the beginning of GSBR cycle, mass balance was performed according to the equations (4,5,6,7) (on line e-supplementary data). The analysis of these calculations revealed that the organic matter storage, expressed as stored biodegradable soluble COD (COD_{sto}) during the anaerobic mixing phase, was performed by both PAOs and GAOs microbial populations, as showed in Fig. 2b). By comparing the results for both microorganisms, it was observed that during Stage I (SRT = 22 ± 17 days), the average anaerobic COD_{sto} was mainly performed by PAOs (71 ± 30%) and by GAOs (29 ± 23%). During Stage II (SRT = 61 ± 24 days) the average anaerobic COD_{sto} was well balanced between PAOs and GAOs (51 ± 30% and 49 ± 27%, respectively). Although PAOs growth and activity is well known to be enhanced by operating at high SRTs (Smolders et al., 1995), in this case the decrease of PAOs anaerobic storage activity for the benefit of GAOs is likely due to the influent COD/P ratio. Indeed, it is reported that an influent COD/P ratio in the range 10–20 gCOD/gP is favourable to the growth of PAOs, whereas ratios higher than 50 gCOD/gP promotes the growth of GAOs (Lopez-Vazquez et al., 2009). In this study, the average influent COD/P ratio during Stage I and Stage II was 16 ± 3 mgCOD/mgP and 88 ± 43 mgCOD/mgP, respectively. This was probably the main reason for the different PAOs/GAOs anaerobic activity in the two experimental stages.

3.2.2. Phosphorus removal

In order to better understand the dynamic activity evolution of PAOs, the PO_4-P released during the anaerobic phase and subsequently removed during the aerobic phase is shown in Fig. 2c). According to the data of the anaerobic activity of PAOs discussed in the previous section and reported in Fig. 2, during Stage I the influent COD/P ratio was low (16 ± 3 mgCOD/mgP) and an increasing PO_4-P release was registered. Observing Fig. 2d), as in Stage I the P-mol_{rel}/C-mol_{sto} ratio was on average equal to 0.60 P-mol_{rel}/C-mol_{sto}, higher than the stoichiometric 0.50 P-mol_{rel}/C-mol_{sto} founded for PAOs by Smolders et al. (1995), it could be asserted that PAOs dominated the storing organisms consortium with an average PO_4-P removal efficiency of 59 ± 28%. In Stage II, where the influent COD/P increased up to 88 ± 43 mgCOD/mgP, the average P-mol_{rel}/C-mol_{sto} was 0.28 P-mol_{rel}/C-mol_{sto}, lower than 0.50 P-mol_{rel}/C-mol_{sto}, thus confirming the increase in GAOs activity. Despite the increase of GAOs activity, the average PO_4-P removal efficiency increased up to 96 ± 3% (effluent phosphorus concentrations: 0.09 ± 0.07 mg PO_4-P/L). This result could appear in disagreement with the scientific literature, as GAOs have historically been seen as competitors of PAOs during the anaerobic organic matter storage and, therefore, as the main cause for EBPR failure (Lopez-Vazquez et al., 2009). However, as discussed by Nielsen et al. (2019), a moderate amount of GAOs may be a good sign for efficient EBPR treating real wastewater because their presence indicate the excess of carbonaceous substrate with respect to what is required for P removal. With the increasing of influent COD/P ratio in Stage II (Table 1a), the low PO_4-P availability limits COD consumption by PAOs, thus favouring the growth of GAOs on the remaining COD. In this perspective, since influent COD is not limiting for efficient P-removal, GAOs are not real competitor against PAOs and their enrichment in the sludge is functional to the efficient anaerobic COD uptake and subsequent denitrification and thus positive to the process performance (Nielsen et al., 2019). As a matter of facts, despite the variability of influent wastewater composition between Stage I and Stage II (i.e. lower phosphate and higher COD levels in Stage II) and the observed increase in GAOs activity thus granules stability and biological phosphorus removal efficiencies were not affected, indicating GAOs as potentially beneficial for successful EBPR process with granular sludge in such circumstances, in line with recent reports (Nielsen et al., 2019).

3.2.3. Nitrogen removal

Fig. 3a) reports the evolution in time of nitrogen forms and nitrogen removal efficiencies throughout all the experimental period. Regarding the nitrification process, during Stage I it was registered an average ammonium removal efficiency and ammonium removal rate of 44 ± 20% and 0.04 ± 0.03 $mgNH_4$-N/L/d, respectively. These values increased up to 67 ± 25% and 0.06 ± 0.03 $mgNH_4$-N/L/d in Stage II. The increase of SRT, from Stage I to Stage II, acted positively on the selection of nitrifying microorganisms and, once pseudo-stationary conditions were obtained, an ammonium removal efficiency of 84 ± 9% was registered. Average nitrogen removal efficiencies of 31 ± 12% (Stage I) and 50 ± 20% (Stage II) were achieved. During the last month of operations (days 487–522), the reactor worked under pseudo-stationary conditions and the nitrogen removal efficiency was 71 ± 6% (effluent nitrogen concentrations: 8.6 ± 2.0 mgTIN/L and 9.8 ± 2.2 mgTN/L). The nitrogen removal registered in the present study is comparable with other literature results for higher C/N wastewater treatment with AGS (68 – 71% obtained by Chen et al. (2011) with C/N = 10; 64 – 86% obtained by He et al. (2018) with C/N = 9.7). Thus, considering the very low C/N of the treated wastewater (2.8 ± 1.3 g bCOD/gTN during Stage I, and 3.8 ± 1.6 g bCOD/gTN during Stage II), the obtained results are noteworthy, compared with the reference literature (Derlon et al., 2016; Lochmatter et al., 2014; Pronk et al., 2015a). This result was achieved by reaching a balance between nitrification and simultaneous denitrification operated by denitrifying DPAOs and DGAOs organisms. At this purpose, Fig. 3b) shows the ratios between the simultaneous denitrification and the total denitrification, and the ratio between the total denitrification and the nitrification. Focusing on the pseudo-stationary phase in Stage II (days 487–522), it was observed a sensible increase of the simultaneous denitrification/total denitrification up to 81 ± 16%. Since the bsCOD was almost completely stored during the anaerobic mixed phase (Fig. 2), the simultaneous denitrification was operated mainly by the storing microorganisms such as DPAOs and DGAOs that used the COD_{sto} in the form of PHA as e-donor for NO_x reduction during the aerobic phase of the cycle, as indicated by the positive correlation between the simultaneous denitrification and the COD_{sto} (Fig. 3c). As previously discussed, the pCOD fraction in the influent that is not converted during the anaerobic phase and thus leaks to the aerobic phase could represent an additional source of e-donor for simultaneous denitrification. The exact quantification of pCOD contribution to denitrification under aerobic conditions is difficult, but it should represent a minor fraction since most of pCOD is likely oxidized via aerobic pathway in the aerobic bulk or in the aerobic external layer of the biofilm where it may be adsorbed. The applied operational strategy, aimed at maximizing the anaerobic storage of COD and dynamic control of the DO concentration (between 1.4 and 1.6 mg/L) during the aerobic phase, promoted high nitrogen removal efficiencies for the low C/N treated wastewater. In similar conditions, the maximization of intracellular COD_{sto} and nitrification/denitrification via nitrite was key in achieving the high reported nitrogen removal efficiency for the treatment of domestic wastewater with such low C/N ratio, compared to previous studies (Coma et al., 2012; Lochmatter et al., 2014; Wang et al., 2018). Based on the obtained $Y_{obs,sto}$ (0.29 ± 0.01 $gCOD_x/gCOD_{sto}$), the estimated COD demands for denitrification via-nitrite and via-nitrate (on line e-supplementary data) were 2.42 and 4.05 gCOD/gN, respectively. From mass balance calculations and taking into account a fractionation of aerobic/anoxic COD_{sto} consumption of 35/65% obtained by estimating DO penetration depth of 100 μm according to Derlon et al., 2016; Winkler et al., 2012 for the mean granules diameter of 1.5 mm, the average COD_{sto} used for denitrification during the aerobic phase was calculated as 3.1 ± 0.6 $gCOD_{sto}/gN_{rem}$. By comparing this result with the above calculated COD demands for N removal via the nitrite and nitrate pathway, it was estimated that about 56% of nitrogen was removed via-nitrite and 44% via-nitrate. This fraction of nitrification/denitrification via nitrite correspond to 14% and 22% reduction in aeration costs and COD consumption, respectively, for nitrogen removal. The occurrence of nitrite shunt is supported by the accumulation of nitrite at the end of the phase Aeration 1 during the GSBR cycle (e-supplementary data). This analysis would explain the achievement of the high nitrogen removal efficiency for such low C/N wastewater. Higher nitrogen removal efficiency over nitrite (95%) was obtained by Lochmatter et al. (2014). However it should be noted that in their reported study the wastewater had a much higher C/N = 8, thus facilitating high N removal efficiencies.

In order to better evaluate the AOBs and NOBs activities, the NOBs suppression and the denitrification rates via-nitrite and via-nitrate of the GSBR, specific batch tests were conducted at the end of the experimental period. Regarding the nitrifying microorganisms, a specific ammonium oxidation rate of 4.36 ± 0.26 $mgNH_4$-N/gVSS/h and a specific nitrate production rate of 3.56 ± 0.09 $mgNO_3$-N/gVSS/h, were measured. These results highlight that NOBs were not absent in the microbial consortium of AGS, but their activity was partly suppressed likely due to a synergistic effect of various strategies, such as: i) maintaining low DO concentration (1.4–1.6 mg/L) during the aeration

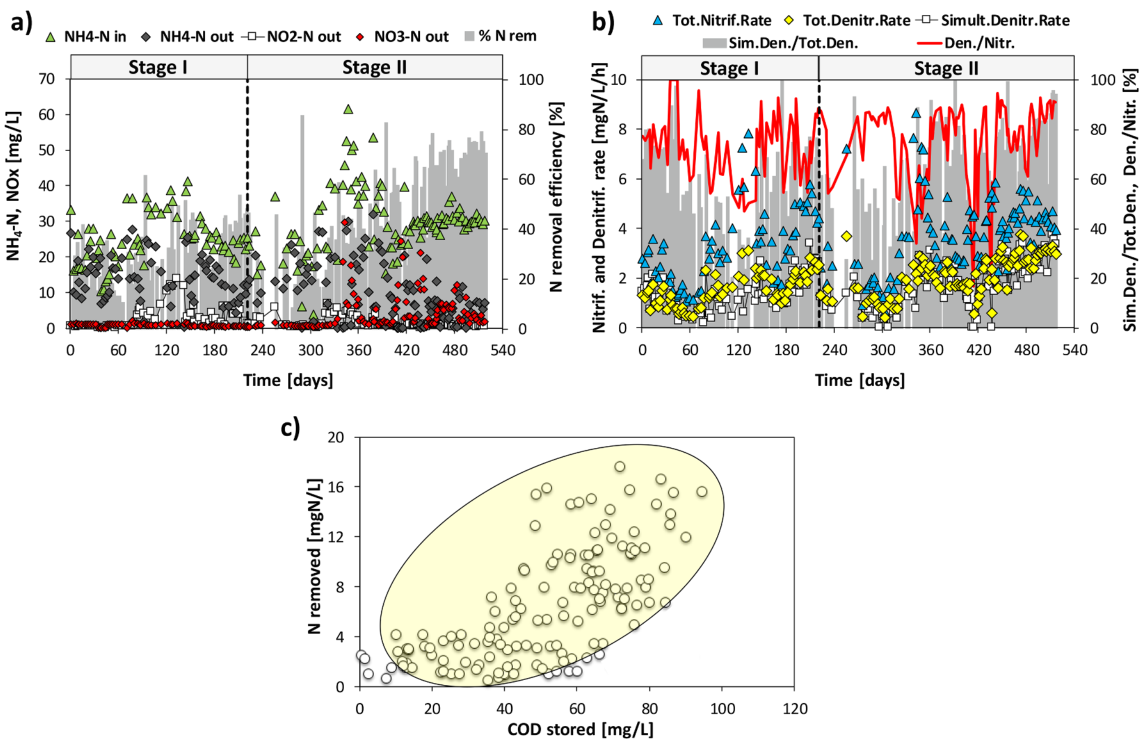

Fig. 3. Time courses of: (a) nitrogen forms (NH_4-N in,out, NO_2-N out,NO_3-N out) and N removal efficiency; (b) nitrification and denitrification rate; (c) correlation between nitrogen removed and COD stored.

phase where NOBs may be outcompeted by AOBs, PAOs/GAOs and OHOs, the latters hydrolizing aerobically the pCOD adsorbed in the outer surface of granules; ii) maintaining low DO/NH_4-N ratio to suppress NOBs activity (0.09–0.21 gO_2/gNH_4-N) as reported in on-line e-supplementary data and as suggested by Bartrolí et al. (2010); iii) the competition with DPAOs and DGAOs populations that reduce nitrites to nitrogen gas. Furthermore, another reason for NOBs suppression can be found in the low SRT of flocculent biomass (8–10 days) in the granular reactor, that determine a selective NOBs washout, as observed by Laureni et al. (2019) working with a moving bed biofilm reactor – integrated fixed film activated sludge (MBBR-IFAS) with a coexistence of biofilms and flocs operated for a mainstream partial nitritation/anammox (PN/A).

Denitrifying batch tests revealed a specific maximum nitrate removal rate of 2.26 $mgNO_3$-N/gVSS/h and a specific maximum nitrite removal rate of 2.25 $mgNO_2$-N/gVSS/h. Reasoning in terms of volumetric rates, a nitrate removal rate of 15.80 $mgNO_3$-N/L/h and a nitrite removal rate of 15.72 $mgNO_2$-N/L/h were obtained. It is worth to mention that nitrite did not accumulate during the denitrification tests with nitrate as e-acceptor (data not shown). Several studies analyzed the issues of denitritation and/or denitratation by DPAOs and DGAOs (Ribera-Guardia et al., 2016; Rubio-Rincón et al., 2017a). It is reported that the capability and relative kinetics in performing denitrification via-nitrite or via-nitrate is clade-specific mainly due to differences in enzymatic properties. DPAOs clade I are able to remove nitrogen via-nitrite and via-nitrate while DPAOs clade II are able to perform denitritation only, for instance. Regarding DGAOs, kinetics of nitrate reduction are generally higher than those of nitrite reduction (Ribera-Guardia et al., 2016), therefore they can perform partial denitratation (from NO_3-N to NO_2-N) thus providing nitrites for DPAOs clades. In the present study, no accumulation of nitrite in the denitrification test with nitrate as the sole e-acceptor dosed and nitrate reduction kinetics not higher than nitrite ones, suggested the denitrifying community as not dominated by GAOs. From these results and the above reported literature findings, it is possible to assert that nitrogen removal via-nitrite calculated during the pseudo-stationary conditions was likely due to DPAOs. This statement is corroborated by the dominance of PAOs activity observed during the last three months of operation in Stage II, as showed in Fig. 2b) and in Fig. 2d).

3.3. Bacterial community after long-term operation

In Fig. 4 the relative abundances (RA) at phylum (Fig. 4a) and genus (Fig. 4b) taxonomic levels are reported. By analysing the microbial consortium of aerobic granular sludge at the end of experimental period (day 522), it was observed that the predominant phyla were *Bacteroidetes* (43.24%) and *Proteobacteria* (40.17%). Other significant phyla were represented by *Planctomycetes* (2.67%), *Parcubacteria* (2.04%), *Ignavibacteriae* (1.76%), *Chloroflexi* (1.25%), *Verrumicrobia* (1.02%), *Spirochaetes* (0.93%) and *Firmicutes* (0.82%). A more detailed comparison down to the genus level was conducted to reveal more information about the microbial community structure and potential metabolic functionalities. Fermentative bacteria from the family *Saprospiraceae* (2.9%), and likely fermentative bacteria from the unclassified OTUs at genus level belonging to the *Bacteroidetes* phylum (5.7%) and *Parcubacteria* (2.0%), were probably involved in the anaerobic hydrolysis of slowly biodegradable particulate matter. These microorganisms were recently selected by operating at high SRT and treating real low C/N domestic wastewater with a high slowly biodegradable particulate COD fraction (Layer et al., 2019), thus in similar operating conditions to those of the present study. A relevant RA of bacteria from genus *Thiothrix* (8.6%) was obtained. Rubio-Rincón et al. (2017b) found that some species of this genus, such as *Thiothrix caldifontis*, could behave like PAOs with a mixotrophic metabolism fixing atmospheric carbon dioxide for phosphorus removal, and using an intracellular sulphur pool as energy source. Furthermore, these species are likely present in low C/N wastewater containing reduced S-compounds (Rubio-Rincón et al., 2017b), like the wastewater originating from septic tanks such as those used in the sewer system of Florence city (Italy) and thus being part of the real municipal wastewater used as influent in our GSBR. In the present study, both the influent wastewater charachteristics and the operating conditions, might likely suggest that similar putative PAOs

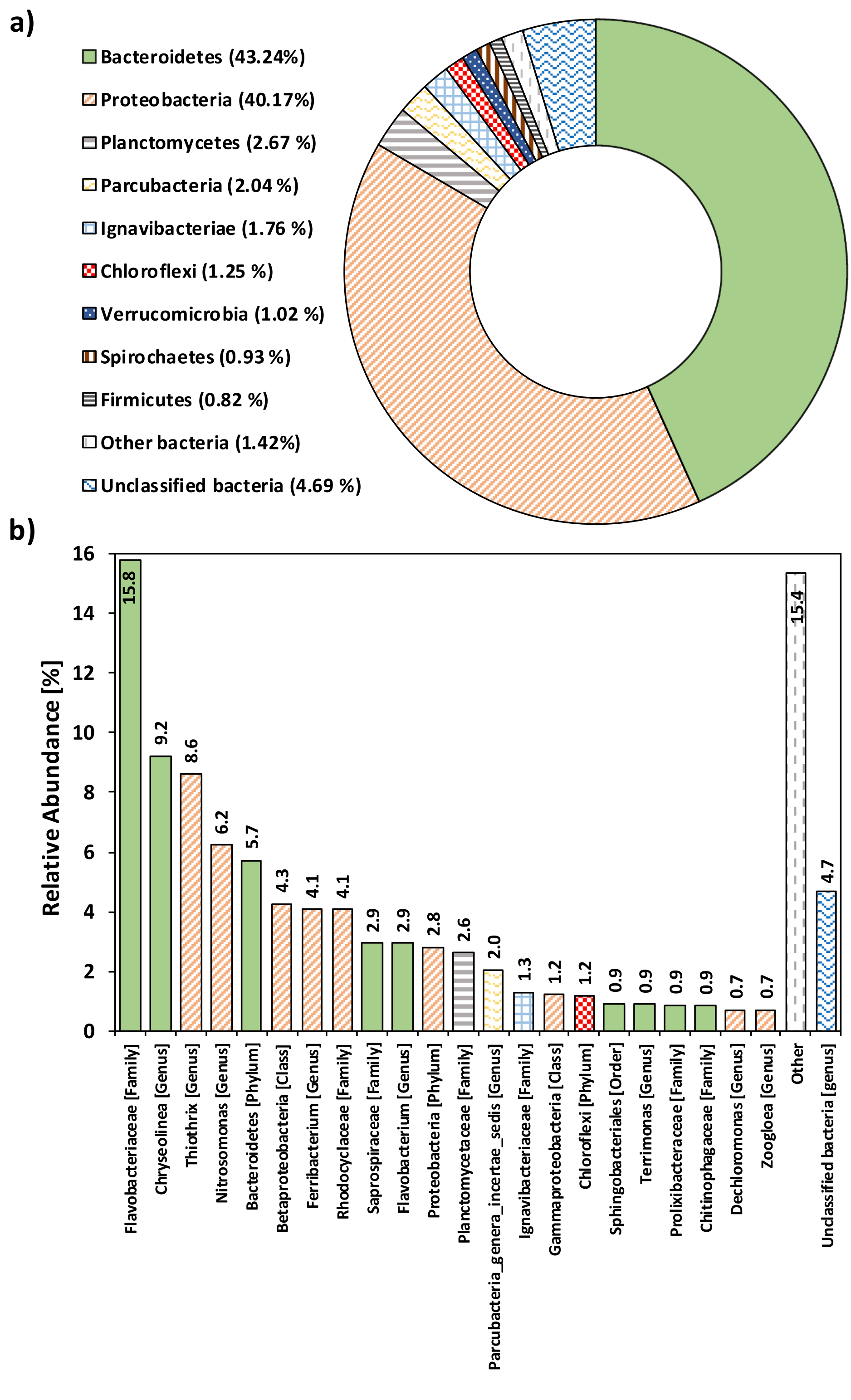

Fig. 4. Composition of bacterial community at phylum (a) and genus (b) taxonomic levels. Relative abundance (%) reported in (b) is referred at the genus taxonomic level for all microorganisms. The taxonomic classification reports the more stringent level between Phylum, Class, Order and Family, resulting from database search for each of the not classified OTU at genus level. The "Unclassified bacteria" at Genus level comprise those OTUs being classified only at the Kingdom taxonomic level. The "Unclassified bacteria" at both Phylum and Genus level comprise those OTUs being classified only at the Kingdom taxonomic level. The group "Other" comprises, at any taxonomic level investigated, all the OTUs with a relative abundance below 0.8 (a) and 0.7 % (b).

clades were selected. Other putative PAOs and DPAOs genus is *Dechloromonas* (0.72%), as stated by Nielsen et al. (2019), whereas some PAOs and GAOs having a fermentative metabolism, could likely be present in the unclassified genera belonging to the family *Rhodocyclaceae* (4.1%) and to the classes *Beta-proteobacteria* (4.3%) and *Gamma-proteobacteria* (1.20%) (Nielsen et al., 2019). The selection of fermentative PAOs and GAOs, favoured by the influent composition (e.g. the presence of slowly biodegradable particulate matter as fermentable substrate) and by the unusually long SRT applied, might have outcompeted the classical PAOs (*Ca. Accumulibacter*) and GAOs (*Ca. Competibacter*) (Layer et al., 2019). Regarding the nitrifying organisms, a high RA of AOBs genus *Nitrosomonas* (6.2%) was detected, whereas classical NOBs were not observed. More specifically, no *Nitrospira* phylum was detected and very low relative abundances of *Bradyrhizobiaceae* (0.01%), as family group of *Nitrobacter*, and *Gallionellaceae* (0.04%), as family group of *Ca. Nitrotoga,* were noticed. These results confirm that the operating conditions, as those applied in the present study, led to AOBs dominance and NOBs suppression. However, from nitrogen removal results discussed in the previous section, it was reported that only 56% of nitrogen was removed via-nitrite and the remaining 44% via-nitrate. The nitratation step was then likely performed by bacteria belonging to unclassified genus from phylum *Chloroflexi* (1.2%). Some bacteria of this phylum, such as *Nitrolancea Hollandica,* are able to perform mixotrophic nitratation and, therefore, are associated to NOBs (Daims et al., 2016). Furthermore, anaerobic environments and low influent C/N, such as those applied in the present study, might be advantageous to *Chloroflexi* enrichment (He et al., 2019). An open point for further discussions is related to the not negligible presence of unclassified genus from family *Planctomycetaceae* (2.6%). These bacteria belongs to phylum *Planctomycetes* (2.7%) that is the same phylum of anammox bacteria (Hu et al., 2013). However, the common anammox microorganisms belong to family of *Brocadiaceae* (e.g. genera *Ca.Brocadia, Ca.Jettenia, Ca.Kuenenia*), but recent studies revealed that at least an uncultered bacterium from *Planctomycetaceae* has anammox activity (Khramenkov et al., 2013). Therefore, if this hypothesis is true, in the present study a probable occurrence of an anaerobic ammonium oxidation with nitrite cannot be excluded. In this case, without taking into account the nitrate production and the ammonium/nitrite autotrophic removal by anammox metabolism, the nitrate production by NOB may have been overestimated as well as the calculations presented for the estimation of the denitrification pathway via-nitrite and via-nitrate could be intrinsically incorrect. The presence of unclassified genus belonging to the family *Flavobacteriaceae* (15.8%) and of genus *Flavobacterium* (2.9%), as well as unclassified genus belonging to the order of *Sphingobacteriales* (0.93%) and to the family *Chitinophagaceae* (0.85%), is likely due to the coexistence of algae into the GSBR (Huang et al., 2015) as observed during the last period of operation.

3.4. Operational strategies for the treatment of low C/N domestic wastewater

Three most remarkable results were obtained treating low C/N real domestic wastewater with AGS:

- High nitrogen removal efficiency: 71 ± 6%
- Low excess sludge production: 0.21 ± 0.01 $gCOD_x/gCOD_{rem}$
- Low solids concentration in the effluent: 22 ± 7 mgTSS/L

The efficient nitrogen removal obtained arises from the followings: 1) formation of dense and thick biofilm in the form of granular sludge resulting in large volumetric fraction under anoxic conditions during the aerobic phase of the cycle; 2) maximization of the intracellular storage of influent biodegradable COD in order to increase e-donor availability for denitrification during the aerobic phase of the cycle when production of nitrite and nitrate take place; 3) the anoxic period operated at the end of the cycle without DO control and the high VER applied resulted in negligible NO_x concentrations at the beginning of the next cycle to limit the consumption of influent COD by OHOs; 4) NOBs suppression to achieve simultaneous nitritation/denitritation, thus decreasing the COD requirements for N removal; 5) operations at high SRT in order to stimulate biomass decay thus obtaining additional COD from hydrolysis. The carbonaceous substrates produced from biomass decay during the anaerobic phase can be stored intracellularly by PAOs/GAOs increasing their denitrification capacity, while the COD produced during the aerobic phase can stimulate conventional heterotrophic denitrification in the anoxic portion of the granule that represent the largest fraction of the biomass volume where most of the decaying biomass reside.

The low observed yield reported arises mainly from the long SRT applied. It is worth to note that in this respect the long SRT has two effects. The first is the renowned observation that the observed growth yield is inversely proportional to sludge as mechanistically modelled by many authors (Metcalf and Eddy, 2003; Henze et al., 2015). The second effect of long SRT applied is the possibility to enrich storing microorganisms with fermentative capabilities which intrinsic maximum growth yield are thought to be lower than conventional storing microorganisms with fully respiratory metabolism (Nielsen et al., 2019). Generally, operations at high SRTs imply a lower sludge production but a higher e-acceptor demand. However, in the case of low C/N wastewater treatment for carbon, nitrogen and phosphorus removal this aspect could be mitigated because the e-acceptor is mainly represented by NO_x, rather than dissolved oxygen, also to perform anoxic P-uptake. Therefore, by operating at high SRTs it could be possible to achieve high nitrogen and phosphorus removal efficiencies with low dissolved oxygen supply.

The high solids removal efficiency reported in this study arises from the prolonged anaerobic phase and the long SRT applied and the implementation of a fully granular system with only a minor fraction of flocculent sludge (< 10%). In fact, the long contact time under anaerobic conditions stimulate the adsorption of solids on the biomass and being the available biomass surface mostly residing on the granular fraction, it can be slowly hydrolysed and degraded thanks to the long SRT applied. Furthermore, since the prolonged anaerobic phase allows for the almost fully uptake of bCOD by storing microorganisms residing in the granules, the filamentous growth of OHOs respiring bCOD under aerobic conditions is prevented, thus avoiding the conversion of influent COD into poorly settling solids.

4. Conclusions

This study reports on the long-term treatment of low C/N real domestic wastewater by means of AGS technology. Simultaneous removal of COD, PO_4-P, N with efficiencies of 84%, 96%, 71%, respectively, resulted in high effluent quality characterized by 25.1 ± 6.1 mg sCOD/L, 0.09 ± 0.07 mgPO_4-P/L, 8.6 ± 2.0 mgTIN/L (9.8 ± 2.2 mgTN/L) and 22.1 ± 6.9 mgTSS/L. The long SRT applied (61 ± 24 days) resulted in an observed yield as low as 0.21 ± 0.01 $gCOD_x/gCOD_{rem}$ thus increasing the COD availability for catabolic processes such as denitrification. The N removal was performed using the COD stored during the anaerobic phase, partly via the nitrite pathway.

CRediT authorship contribution statement

Riccardo Campo: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Sara Sguanci:** Data curation, Formal analysis, Investigation, Methodology. **Simone Caffaz:** Supervision, Writing - review & editing. **Lorenzo Mazzoli:** Writing - review & editing, Microbiological & bioinformatics analysis. **Matteo Ramazzotti:** Writing - review & editing, Supervision, Microbiological & bioinformatics analysis. **Claudio Lubello:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **Tommaso Lotti:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing - review & editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.122961.

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