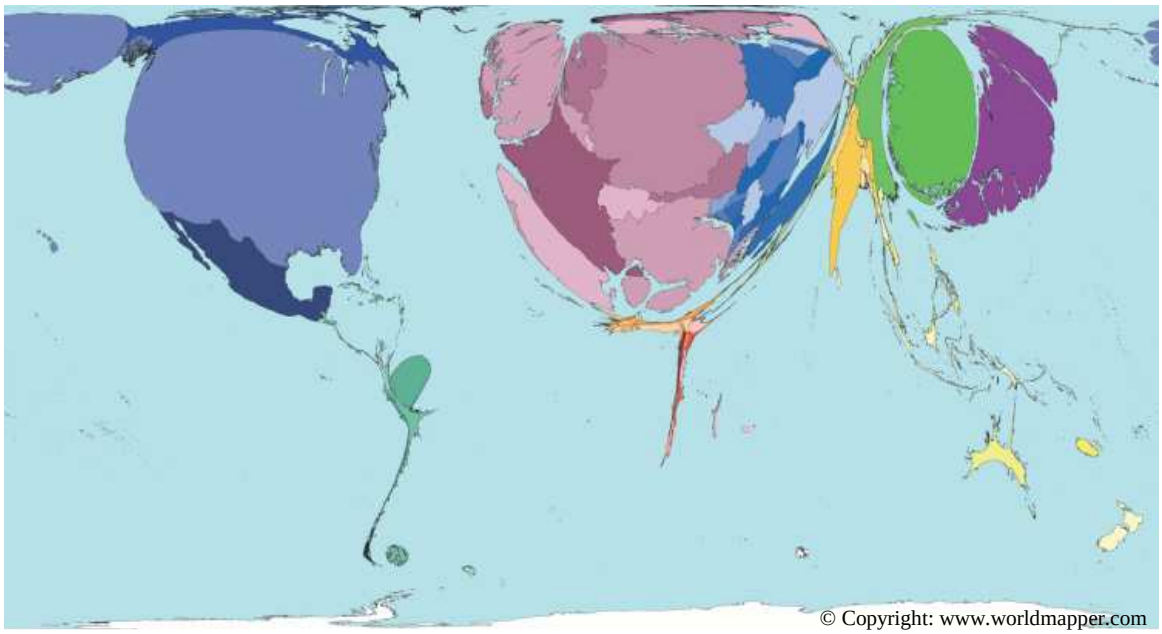
Doctoral School in Environmental Engineering

# Anaerobic side-stream reactor: a sustainable solution for sewage sludge reduction

**Roberta Ferrentino**




UNIVERSITY OF TRENTO - Italy
Department of Civil, Environmental and Mechanical Engineering

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*A mia madre e mio padre*

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# About the Author

**Roberta Ferrentino** was born on the 26th of February 1985 in Avellino, Italy. In 2010 she received her master's degree in Environmental Engineering from the University of Salerno, Italy. During her master thesis, she worked with the group of the Sanitary Environmental Engineering Division. Here, she studied an innovative wastewater treatment process based on the simultaneous application of membrane, ultrasound and adsorption, under the supervision of dr. eng. Milena Landi, prof. ing. Vincenzo Naddeo and prof. ing. Vincenzo Belgiorno. She spends the following years working in the public administration, dealing with private construction and land management. In 2013, Roberta Ferrentino started her PhD research at the University of Trento under the supervisor of prof. dr. eng. Gianni Andreottola, at the Department of Civil, Environmental and Mechanical Engineering. Her research focused mainly on the application of an anaerobic side-stream reactor in the water line of a conventional activated sludge process in order to reduce the sewage sludge production.

# Publications & relevant work

**Patent**

Andreottola G, **Ferrentino R.**, Langone M., (2016) *Plant and method for sludge reduction in wastewater treatment – UTN (University of Trento) System*

**Journal Articles**

**Ferrentino R.**, Langone M., Villa R., Andreottola G. (2016) *Simultaneous biological mechanisms for sludge reduction in anaerobic side-stream reactor.* Submitted.

**Ferrentino R**., Langone M., Gandolfi I., Bertoldini V., Franzetti A., Andreottola G. (2016) *Shift in microbial community structure of anaerobic side-stream reactor in response to changes to solid retention time and interchange ratio*. Submitted

**Ferrentino R.**, Langone M., Andreottola G. (2016) *Temperature effects on PAO and DPAO activity in Anaerobic Side-Stream Reactor.* Submitted

**Ferrentino R.**, Langone M., Merzari F., Tramonte L., Andreottola G. (2016) *A review of Anaerobic Side-Stream Reactor for excess sludge reduction: configurations, mechanisms and efficiency*. Critical Reviews in Environmental Science and Technology Vol. 46 (4)

Ragazzi M., Rada E.C., **Ferrentino R.** (2015) *Analysis of real scale experiences of novel sewage sludge treatments in an Italian pilot Region*. Desalination and Water Treatment, Vol. 55 (3), pp. 783 - 790 DOI:10.1080/19443994.2014.932717

Langone M. , **Ferrentino R.**, Trombino G., Waubert De Puiseau D., Andreottola G., Rada E. C., Ragazzi M. (2015) *Application of a novel hydrodynamic cavitation system in wastewater treatment plants.* U.P.B. Scientific Bullettin, Series D, Vol. 77 (1), pp. 225 - 234

**Proceeding**

**Ferrentino R.**, Langone M., Andreottola G., Rada E. C. *Anaerobic side-stream reactor in wastewater treatment: review*. Proceeding in 9th International Conference on Urban Regeneration and Sustainability, Siena (Italy), 23 – 25 September 2014.

Langone M. , **Ferrentino R.**, Trombino G., Waubert De Puiseau D., Andreottola G., Rada E. C., Ragazzi M. *Application of a novel hydrodynamic cavitation system in wastewater treatment plants.* Proceeding in 6th International Conference on Energy and Environment 2013, University Politehnica of Bucharest, Bucharest (Romania), 7 – 8 November 2013.

Sbrissa E., Langone M., Cosoli P., Andreottola G., **Ferrentino R.**, Rada E.C., Ragazzi M., *Critical analysis of anaerobic digester supernatant treatment*. Proceeding in the 6th International Conference on Energy and Environment 2013, University Politehnica of Bucharest, Bucharest (Romania), 7 – 8 November 2013.

Ragazzi M., Rada E.C., **Ferrentino R.** *Analysis of real scale experiences of novel sewage sludge treatments in an Italian pilot Region.* Proceeding of the 13th International Conference on Environmental Science and Technologies, Athens (Greece), 5 – 7 September 2013.

**Posters**

Langone M., **Ferrentino R.**, Mancuso G., Trombino G., Andreottola G. *Disintegration of waste-activated sludge using a novel hydrodynamic cavitation system*. Poster in 2th IWA Specialized International Conference- Ecotechnologies for Wastewater Treatment, Verona (Italy), 23-27 June 2014.

**Ferrentino R.**, Langone M., Andreottola G. *Implementation of an anaerobic side-stream reactor in CAS system.* Poster in 2th IWA Specialized International Conference- Ecotechnologies for Wastewater Treatment, Verona (Italy), 23-27 June 2014.

**Master Thesis Co-Supervisor**

*Completed*

Stalliviere, S. a.a. 2015/2016. (Environmental Engineering). *Indagine sperimentale sulle modalità di selezione dei batteri DPAO negli impianti di depurazione.* Supervisor: Andreottola G.

Rossetto, S. a.a. 2014/2015. (Environmental Engineering). *Sperimentazione a scala pilota del processo biologico ASSR (Anaerobic Side-Stream Reactor) per la minimizzazione dei fanghi di supero.* Supervisor: Andreottola G.

Gelmini, E. a.a. 2013/2014. (Environmental Engineering). *Studio sperimentale di un processo biologico di trattamento delle acque reflue basato sull'attività di batteri solfato riduttori*. Supervisor: Andreottola G.

# Abstract

# Abstract

Over the last two decades, the production of excess sludge has increased rapidly due to a more stringent legislation on effluent quality and a growing number of new plants, becoming an economic and an environmental critical issue. Processing excess sludge could account for half up to 65% of the total operation costs of a wastewater treatment plant. Technologies to reduce the excess sludge had been widely studied. Several studies reported that the technologies integrated in the wastewater handling units should be cost effective and preferable rather than the techniques integrated in the sludge handling units, as they allow to reduce the sludge production rather than treat it. Thus, the development and the optimization of a technology able to reduce the sludge production in the water line is now challenging. A lot of technique have been developed such as biological, thermal, high temperature oxidation, mechanical treatments, ultrasonication, ozonation or by using chemical compounds. Some of these have been proven not energy saving, while others can negatively affect the effluent quality of the process due to the formation of by-products. Among others, biological treatments are a challenging strategy for sludge reduction. In recent years, several studies showed that including an anaerobic bioreactor in the returned activated sludge line of a conventional activated process could significantly enhance the sludge reduction without causing negative effects on operational performances. Today, this configuration is known as anaerobic side-stream reactor (ASSR) process. Several laboratory applications highlighted that the sludge yield of the ASSR process could be reduced up to 60% compared to a conventional activated process. Despite the highest percentage of sludge reduction achieved, the process is still little applied to real scale because its main operating parameters and sludge reduction mechanisms are still unclear.

This study focused on the verification of ASSR process, the mechanisms of sludge reduction and the microbial structure of the process. During the first part of the research, a laboratory experimental system was designed and implemented. A sequencing batch reactor (SBR), to simulate the water line of a real wastewater treatment plant, and an ASSR as a sludge treatment unit composed the system. Unlike most of the previous studies, the system was fed with real urban wastewater in order to obtain results that reflect as much as possible what can really happen to a municipal WWTP. Through a critical analysis of the literature, the influence of two important operating parameters, such as the solid retention time (SRT) of the ASSR and the interchange rate (IR), which means the percentage of biomass cycled into the ASSR, had been uncovered

Given this, the experimental system was started up and reached a stable condition after 60 days.

The research was developed in three different phases that lasted for about 90 days each. The experimental lab system was tested under three configurations: i) 10% sludge interchange rate and

SRT in the ASSR of 10 days; ii) 20% sludge interchange rate and SRT in the ASSR of 5 days and iii) 40% sludge interchange rate and SRT in the ASSR of 2.5 days. The observed sludge yield (Yobs) of each phase was evaluated and was equal 0.21 g TSS/g COD, 0.14 g TSS/g COD and 0.12 g TSS/g COD in Phase I, II and III, respectively. These results confirmed that the process could significantly decrease the sludge production and a reduction up to 62% could be achieved. To explain the results obtained in terms of sludge reduction, different tests and analysis were performed. The release of soluble COD and ammonia in the ASSR have highlighted that the endogenous decay and cell lysis mechanism occur in the ASSR. Extraction of EPS, with CER and BASE methods, showed a release of protein and polysaccharides in the bulk solution that increased passing between Phase I and III. At the end of each experimental phase, batch tests were carried out to evaluate the activity of phosphorus accumulating organisms (PAO) and denitrifying phosphorus accumulating organisms (DPAO).

Recirculation in SBR-ASSR selects DPAO microorganisms. This was a result of great interest because DPAO could enhance the biological nutrient removal since nitrogen and phosphorus can be simultaneously removed. Furthermore, DPAO has lower cell yield than PAO resulting in lower sludge production. Results showed an activity of PAO, DPAO and other slow growers such as sulfate reducing bacteria. All these results suggested that the high percentage of sludge reduction could be explained as a combination of aspects, such as the cell lysis, the cryptic growth, the selection of slowing microorganisms and EPS destructuration. The SRT and the IR could be considered as main parameters and their variation could significantly affect the performance of the process. Microbial analyses were carried out to investigate the bacterial and archaeal structure of the ASSR sludge during each phase.The results confirmed the presence of several bacteria that are typically heterotrophic responsible of hydrolysis and fermentative process of organic matter. Several slow growers bacteria were also detected. Moreover, according to the batch tests on PAO and DPAO activity, a relevant increase in Phase III of some genera able to enhance the biological phosphorous removal has been observed.

In summary, the research found that the ASSR process is a sustainable solution for the sewage sludge reduction due to an efficient and a low sludge production, able to ensure both carbon, nutrients and phosphorous removal applying an extremely simple technology, easy to realize both in new and in existing wastewater treatment plants.

# I. Chapter

# Executive summary

# Executive summary

Until today, the conventional activated sludge (CAS) process has been widely applied to wastewater treatments although the large amount of sewage sludge produced by it. The handling, treatment and disposal of sewage sludge are challenging waste management problems common to many countries. The implementation of Urban Waste Water Treatment (UWWT) Directive 91/271/EC (CEC, 1991) forced all the Member States to improve their wastewater collecting and treatment systems. As a result, the annual sewage sludge production increased. It was estimated that the worldwide sewage sludge production could exceed 13 million tons dry solids up to 2020 (Milieu Ltd., WRc and RPA, 2010; Leonard, 2011). In view of this, the Council of the European Union defined the reduction of sewage sludge production one of the first priorities in waste management hierarchy.

Up to day, many technologies have been developed to reduce and/or treat sewage sludge. One promising option to reduce the sludge production in wastewater treatment plant (WWTP) is the application of an anaerobic side-stream reactor (ASSR) in the returned activated sludge line of a CAS system. Several schemes different from each other for the reactor type used to simulate the water line has been proposed. Chudoba et al. (1992) were the first to implement the ASSR in a CAS system, naming the process as OSA (oxic-settling- anaerobic). This configuration was proposed also by others as An and Chen (2008) or Ye et al. (2008). Further other studies were performed using others reactors to simulate the water line such as membrane bioreactor (MBR) (Chen et al., 2003 and Saby et al., 2003), in a moving bed biofilm reactor (MBBR) (Li et al., 2014) and in a sequencing batch reactor (SBR) (Novak et al., 2007; Goel and Noguera, 2006). All these studies reported that the ASSR introduction could increase significantly the biological sludge reduction up to 60% without causing negative effects on sludge settling and effluent properties (Novak et al., 2007). The CAS-ASSR configuration allows a percentage of sludge reduction ranging between 14- 50 % to be obtained (Chudoba et al. 1992; Wang et al. 2008; Ye et al. 2008; Torregrossa et al. 2012).

According to Semblante et al. (2014), the observed sludge yield ($Y_{obs}$) of a lab application of the CAS-OSA configurations ranged from 0.21-0.25 gTSS/gCOD (Chudoba et al. 1992) and 0.53 gMLSS/gCOD (Wang et al. 2008). Torregrossa et al. (2012) implemented the same configuration as a pilot plant continuously fed with a real municipal wastewater achieving 35% of sludge reduction ($Y_{obs}$ 0.36 gTSS/gCOD), compared to the control system. Ye et al. (2008) had a cumulative sludge production of 1.842, 1.597 and 2.062 g SS/d, depending on the SRT applied, which were 23%, 33% and 14%, respectively, lower than the control system (2.392 g SS/d).

The MBR-OSA configuration performed by Saby et al. (2003) achieved a cumulative sludge reduction ranging between 28 – 58% with a $Y_{obs}$ 0.17-0.29 gTSS/gCOD, depending on the oxidation potential reduction (ORP) value. Among the different configurations implemented in laboratory studies, the SBR-OSA configurations reported up to 60% sludge reduction, making the best result. Novak et al. (2007) reported $Y_{obs}$ of 0.11 gVSS/gCOD and a reduction of excess sludge production of 20 – 45%. Similar results were obtained in the studies performed by Chon and Park (2012) and Chon et al. (2011a, 2011b). Recently, Zhou et al. (2015b) observed that applying a modified OSA system (A + OSA) with an anoxic tank prior to oxic tank, a Yobs of 0.21 gSS/gCOD, 32% lower than a conventional process with anoxic and oxic phases in the water line (A/O) (0.32 gSS/gCOD) was obtained.

Up to now there are two patent applications that are based on lab-scale implementation of the process. The Cannibal® process, patent by Curtis et al. (2007; 2011), is a combination of biological and physical treatment, and is the first implementation of an ASSR to full scale. The Cannibal® process is composed of a CAS system, a solid separation module, an ASSR with 10 d SRT and a remote control system that monitor the ORP at different levels/compartments of the side reactor. Recently Chon and Park, (2012) proposed a new patent, called UMass process, characterized by an ASSR with a short SRT (2.5 days). Their results clearly indicated that the UMass sludge reduction system, which employs a short SRT (2.5 days), is very effective in reducing sludge generation, being even more effective than the sludge reduction obtained in the Cannibal® process. Thus, the lower SRT of the UMass process needs a smaller ASSR than the Cannibal® process and consequently lower capital and operating costs.

However, despite the good percentage of sludge reduction obtained, the process is not yet applied at full scale as the mechanisms behind the biological reduction are still unclear. In fact several and contrasting explanations have been provided about the sludge reduction such as uncoupling metabolism, cell lysis and cryptic growth, domination of slow-growing microorganisms and destruction of EPS, but a complete mechanism is not clearly reported and related to an operative control strategy.

Chudoba et al. (1992) based their explanation on the uncoupling metabolism mechanism. The Authors reported that in the anaerobic reactor, where food and oxygen are insufficient, microorganisms are subjected to a physiological shock. In these conditions, they spent energy to satisfy their maintenance functions and not for the synthesis of new cells. When they are returned in the aerobic reactor, they have enough substrate and rebuilt their energy reserves. However this mechanism is strictly connected to the selection of particular microorganisms.

Therefore the sludge cycling in anaerobic and aerobic phases results in a metabolic selection of those microorganisms able to favour catabolic pathways. On the contrary, Wang et al. (2008) reported that the sludge decay is the main cause of sludge reduction in OSA system, accounting for 2/3 reduction. Subjecting the sludge to alternate aerobic and anaerobic conditions, the concentrations of soluble COD, $NH_4^+$-N, TP, protein and carbohydrate in the supernatant gradually increased proving that the sludge lysis and acidogenesis occur and lead to minimization of excess sludge. Furthermore, Chon et al. (2011) showed that degradation of EPS, iron and/or aluminium-associated materials occurs under aerobic and anaerobic cycling conditions, while it does not occur in a CAS system even with long SRT. Thus, the Authors reported that the EPS destructuration could be responsible of the lower sludge yield, too. Furthermore, several operative parameters have been defined as the main such as the ORP, the SRT, the $HRT_{ASSR}$, and the IR. However, only for the ORP it could be possible to define an optimal value in the ASSR for sludge reduction of -250 mV. On the contrary, a wide range of SRT of the whole system has been reported, does not allowing defining a unique value. On the other hand, previous results reported that low IR and high HRT in the ASSR allow high sludge reduction efficiency to achieve.

Given this great confusion about the ASSR process, the aim of the present thesis was to test a SBR-ASSR laboratory scale system under different configurations that were made varying the solid retention time (SRT) of the ASSR and the interchange rate (IR). Thus, three different phases were considered: 10% sludge interchange rate and SRT in the ASSR of 10 days; ii) 20% sludge interchange rate and SRT in the ASSR of 5 days and iii) 40% sludge interchange rate and SRT in the ASSR of 2.5 days. Each Phase lasted for about 90 days. The main mechanisms involved in the sludge reduction were studied, focussing the attention on endogenous decay, destruction of extracellular polymeric substance (EPS) and the domination of slow-growing microorganisms. The performances of the process in terms of carbon and nutrient removal efficiencies were also evaluated in order to verify that the process could meet the discharge regulatory limits. Furthermore, an analysis and a comparison of the microbial community structure in each experimental phase were performed. Finally, the effects of the temperature on the kinetic and stoichiometry of two main groups of bacteria were carried out.

**A review of Anaerobic Side-Stream Reactor for excess sludge reduction: configurations, mechanisms and efficiency - Chapter 2 – Published in 2015, *Critical Review in Environmental Science and Technologies***

In the frame of a modern waste management, an important sector concerns the sewage sludge minimisation. In recent years a lot of techniques have been developed to reduce the sludge production such as biological, thermal, thermochemical, high temperature oxidation and mechanical treatments, ultrasonication and ozonation or using chemical compounds. Among those, the use of an Anaerobic Side-Stream Reactor (ASSR) in the conventional activated sludge line is a challenging biological technology able to minimize sludge production in wastewater treatment plants. The ASSR can be easily realized in both new and existing plants as it consists of an anaerobic side-stream reactor for sludge treatment and reduction where a portion or, in some cases, all the return sludge of the activated sludge process is subjected to alternating aerobic, (anoxic) and anaerobic conditions. Studies show that, combining a conventional activated process with an ASSR, sludge yield could be reduced by up to 40 - 60% without any negative effects, neither on the effluent quality nor on the settling characteristics of the activated sludge. The process has been applied using various configurations. Further, different explanations about the reduction mechanisms behind the process have been provided. This paper is a review of the existing applications of the ASSR in laboratory scale and patents in order to describe the configurations implemented, the performance of the process in terms of sludge reduction and carbon and nutrient removal, the main operating parameters and the mechanisms of sludge reduction observed.

**Simultaneous biological processes for sludge reduction in anaerobic side-stream reactor - Chapter 3 – Submitted**

An anaerobic side-stream reactor (ASSR) was operated for 300 days coupled to a conventional activated sludge (CAS) to investigate the impact of SRT in the ASSR and the related sludge interchange ratio (IR) on the sludge reduction process. Different phases varying the SRT and the IR were carried out, revealing that an SRT in the ASSR of 2.5 d and an IR equal to 40% was the most suitable case in terms of sludge reduction, achieving a 66% of sludge reduction as compared to a CAS system. An increasing release of ammonia, soluble COD and soluble EPS was detected in the ASSR increasing the amount of biomass cycled to the ASSR, showing the importance of the cell lysis process. The release of orthophosphate in the ASSR was also detectable, which was explained by the activity of the total phosphorous accumulating organism (TPAO). Specific batch

tests demonstrated that increasing the biomass cycling in the ASSR, the percentage of denitrifying phosphorous accumulating organism (DPAO) over the aerobic phosphorous accumulating organism (PAO) increased. The activity of sulphate reducing bacteria (SRB) was also investigated. All the results observed led to a new explanation of the sludge reduction process achieved by inserting an ASSR in the returned sludge line of a CAS system. This study tried to connect the sludge decay, the cell lysis, the EPS destruction and the presence of slow growing microorganism in order to define a mechanism based on synergy and coexistence of all of them.

**Shift in microbial community structure of anaerobic side-stream reactor in response to changes in solid retention time and interchange ratio – Chapter 4 -Submitted**

A laboratory scale sequencing batch reactor (SBR) - anaerobic side-stream reactor (ASSR) system was operated for 300 days under three different values of anaerobic solid retention time ($SRT_{ASSR}$) and interchange ratio (IR) to investigate carbon and nutrient removal efficiencies and sludge reduction as compared with the microbial community structure of the ASSR. Under each experimental phase, the SBR-ASSR system was effective in the removal of COD, ammonia nitrogen and phosphorous. The best carbon and nutrient removal efficiencies were obtained under the last experimental phase when the $SRT_{ASSR}$ was 2.5 days and IR equal to 40% (Phase III). In this phase, the highest reduction of sludge production was also observed (66%). Quantitative PCR analyses encoding 16 rRNA gene revealed a wide diversity of phylogenetic groups in each phase. However, an increasing selection of fermenting bacteria able to release EPS, denitrifying phosphate accumulating bacteria (DPAOs) and heterotrophic denitrifying bacteria was observed from Phase I to Phase III. Further, specific qPCR analyses targeted *apsA* gene showed an increase of sulphate reducing bacteria (SRBs) in Phase III. The total number of Archaea was almost the same for each experimental Phase. However, a shift from hydrogenotrophic methanogens to methylotrophic and acetoclastic methanogens was detected.

**Temperature effects on PAO and DPAO activity in Anaerobic Side-Stream Reactor – Chapter 5 - Submitted**

In this study, the effect of the temperature on total phosphorous accumulating organisms (TPAOs), both aerobic phosphorous accumulating organisms (PAOs) and anoxic denitrifying phosphorous accumulating organisms (DPAOs) were investigated. Four different temperatures, 5, 10, 15 and 20°C were tested in batch assays using a selected biomass from an ASSR process performed at lab-scale at room temperature. Batch tests were carried out in anaerobic, aerobic and

anoxic conditions to evaluate the phosphorous release of TPAOs, the uptake of PAOs and the uptake of DPAOs, respectively. Results showed that the phosphorous release and uptake kinetics were influenced from the variation of the temperature, while temperature did not influenced significantly the anaerobic and the anoxic processes stoichiometry. In general, decreasing the temperature, a decreasing in the P-uptake and release rates was observed.

In anaerobic conditions, the P-release rate was 0.06, 0.08, 0.20 and 0.30 mg PO4 3- -P/ (g TSS h) at 5, 10, 15 and 20 °C, respectively. Under aerobic conditions the P -uptake was 0.95, 1.47, 2.41 and 4.53 mg $PO_4^{3-}$ -P/ (g TSS h) at 5, 10, 15 and 20 °C, respectively. In anoxic conditions the P-uptake was 0.24, 0.53, 1.55 and 3.01 mg $PO_4^{3-}$ -P/ (g TSS h) at 5, 10, 15 and 20 °C, respectively. Arrhenius temperature coefficients θ for anaerobic, aerobic and anoxic metabolism were found to be 1.114, 1.121 and 1.165, respectively. Results revealed that DPAO activity was more affected by a lower temperature than PAO activity as a higher Arrhenius coefficient was estimated.

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# II. Chapter
# A review of Anaerobic Side-Stream Reactor for excess sludge reduction: configurations, mechanisms and efficiency

This chapter was based on:

Ferrentino R., Langone M., Merzari F., Tramonte L., Andreottola G. (2016) *A review of Anaerobic Side-Stream Reactor for excess sludge reduction: configurations, mechanisms and efficiency.* Critical Reviews in Environmental Science and Technology Vol. 46 (4)

# A review of Anaerobic Side-Stream Reactor for excess sludge reduction: configurations, mechanisms and efficiency.

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ABSTRACT

In the frame of a modern waste management, an important sector concerns the sewage sludge minimisation. In recent years a lot of techniques have been developed to reduce the sludge production such as biological, thermal, thermochemical, high temperature oxidation and mechanical treatments, ultrasonication and ozonation or using chemical compounds. Among those, the use of an Anaerobic Side-Stream Reactor (ASSR) in the conventional activated sludge line is a challenging biological technology able to minimize sludge production in wastewater treatment plants. The ASSR can be easily realized in both new and existing plants as it consists of an anaerobic side-stream reactor for sludge treatment and reduction where a portion or, in some cases, all the return sludge of the activated sludge process is subjected to alternating aerobic, (anoxic) and anaerobic conditions. Studies show that, combining a conventional activated process with an ASSR, sludge yield could be reduced by up to 40 - 60% without any negative effects, neither on the effluent quality nor on the settling characteristics of the activated sludge. The process has been applied using various configurations. Further, different explanations about the reduction mechanisms behind the process have been provided. This paper is a review of the existing applications of the ASSR in laboratory scale and patents in order to describe the configurations implemented, the performance of the process in terms of sludge reduction and carbon and nutrient removal, the main operating parameters and the mechanisms of sludge reduction observed.

*Keywords:* aerobic and anaerobic sludge cycling, activated sludge, Anaerobic Side-Stream Reactor (ASSR), Oxic Settling Anaerobic process, sludge minimisation, sludge reduction mechanisms.

## 1. Introduction

Nowadays, the most used process for treating civil and industrial wastewaters is the conventional activated sludge (CAS) that allows a high organic carbon removal efficiency to be obtained producing, on the other hand, a large amount of excess sludge to be disposed. The production of excess sludge in municipal wastewater treatment plants (WWTPs) has increased rapidly in recent years, due to a more stringent legislation on effluent quality and a growing number of new plants, becoming a critical issue. Processing excess sludge could account for 25 - 65% of the total operation costs of a WWTPs (Chen et al., 2001; Saby et al., 2003; Yang et al., 2011) and its disposal costs has become more and more expensive due to restriction in reuse and disposal.

In recent years a lot of techniques have been developed to reduce sludge production in WWTPs (Carrère et al. 2010; Foladori et al. 2010), such as biological (Wei et al. 2003; Semblante et al. 2014), thermal (Neyens and Baeyens 2003), thermochemical (Rocher et al. 1999), high temperature oxidation (Hii et al. 2013) and mechanical treatments (Weemaes and Verstraete 1998), ultrasonication (Pilli et al. 2011), ozonation (Chu et al. 2009b) or using chemical compounds (Liu 2003). In order to reach a significant sludge reduction, some of these have been proven to be not energy saving technologies, while others can negatively affect the effluent quality of the process due to the formation of by-products. Among others, biological treatments are a challenging strategy for sludge reduction in WWTPs.

A promising biological technology based on sludge cycling between aerobic, anoxic and anaerobic conditions has been developed to minimize activated sludge production (Chon et al., 2011). It fundamentally consists of an aeration basin as activated sludge reactor, a settling tank and an anaerobic side-stream reactor (ASSR) for sludge treatment and reduction where a portion or, in some cases, all the raw activated sludge (RAS) is recycled.

Several laboratory applications demonstrated that using an ASSR, the sludge yield (Yobs) could be reduced by up to 40% (Chudoba et al., 1992), 55% (Chen et al., 2003; Saby et al., 2003) and 60% (Novak et al. 2007) compared to a CAS process. The process showed to be useful in terms of sludge reduction, simple to realize in existing WWTPs, energy saving and without negative effects on effluents quality. Different explanations about the reduction mechanisms have been proposed, such as the enhancement of endogenous decay, the metabolic uncoupling, the feasting/fasting mechanism, the destruction of extracellular polymeric substances (EPS), the domination of slow-growing microorganisms and the predation of bacteria (Van Loosdrecht and Henze 1999).

However, the mechanism underlying the biological sludge reduction is not clearly identified yet. Thus, the aim of this paper is to present an overview of the existing application of ASSR in order to describe the implemented configurations, the performance of the process, the observed mechanisms of sludge reduction and the main involved parameters.

## 2. Anaerobic side-stream treatment

### *2.1. Lab scale configurations*

The integration of an ASSR in the water line of a WWTP allows the alternate cycle of the sludge between aerobic, anaerobic and anoxic conditions. Westgarth et al. (1964) possibly for the first time reported that sludge cycling from aerobic to an anaerobic environment could reduce the rate of sludge production by half as compared with a CAS process.

After some years, Chudoba et al. (1992) realized a laboratory unit, called oxic settling anaerobic process (OSA). It consists of an activated sludge system with the anaerobic sludge holding tank within the sludge return line named CAS – OSA. Whole (Fig. 1a) or most (Fig. 1b) of the settle sludge, extracted from the settling reactor, is pumped into the anaerobic tank and then, it is recycled in the CAS and thus resubjected to aerobic conditions (Wang et al. 2008; Ye et al. 2008; Torregrossa et al. 2012).

An alternative configuration to the CAS-OSA system is the MBR– OSA configuration that employed a submerged membrane module in the aerobic tank, a settling tank and a sludge holding tank (Fig 1c). The settling tank is used to thicken the sludge and to maintain a high sludge content in the sludge holding tank which consists of an anaerobic zone in the sludge return line. The excess sludge is directly discharged from the aerobic tank. The settled sludge in the clarifier is pumped to the ASSR while the supernatant is recirculated to the aeration tank. This configuration allows any solids loss through the effluent by the gravitational clarifier, presents the advantage of separating the hydraulic retention time (HRT) from the sludge retention time (SRT), allowing the excess sludge production to be accurately determinate (Chen et al. 2003; Saby et al. 2003; An and Chen 2008).

The most used configuration in literature and in laboratory scale applications is the so called SBR – OSA configuration (Fig 1d). It consists of a sequencing batch reactor (SBR), that treats the incoming wastewater operating with several cycles per day, mainly four, with four phase (fill, react, settle and decant), and an ASSR fed by a portion of the settled sludge. An equal volume from the ASSR is returned to the SBR. The main difference between this configuration and the CAS-OSA or the MBR-OSA configurations is the lower space requirement in the water line due to the absence of the secondary settling and the alternate loading mode instead of continuous. The reaction phase of the SBR cycle could be only aerobic (Novak et al. 2007), to perform carbon removal and nitrification, or aerobic and anoxic. In the latter case both carbon and total nitrogen removal could be performed (Sun et al. 2010; Chon et al. 2011b; Kim et al. 2012) or simultaneous carbon, nitrogen and phosphorous removal (Goel and Noguera 2006; Datta et al. 2009) .

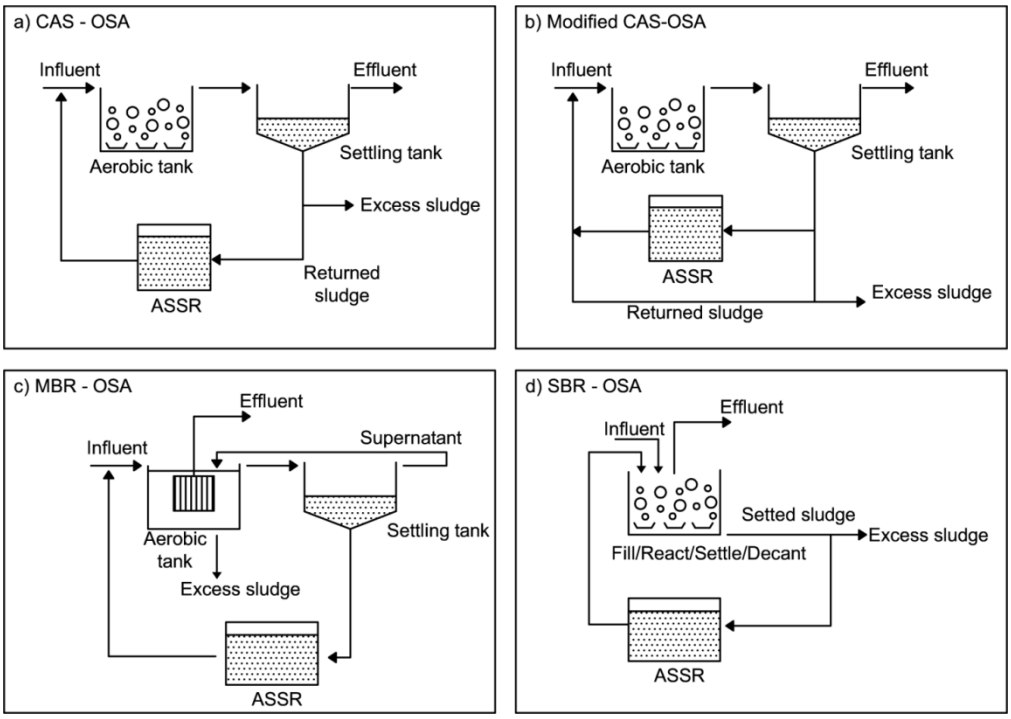


Fig. II.1 Schematic diagrams of (a) CAS - OSA system; (b) Modified CAS - OSA system; (c) MBR-OSA system; (d) SBR-OSA system

Coma et al. (2013) developed a new process called Biminex™ (Fig 2a) for simultaneous nutrient removal and biological minimization of sludge production. The process is a modified configuration of the University of Cape Town (UCT) process, that provided the nutrient removal by combining anaerobic, anoxic and aerobic processes in the water line in three separate reactors (Tchobanoglus et al. 2003). The Biminex™ process simulates the same conditions of the UTC process in the water line but, to enhance the sludge reduction, a portion of the sludge, extracted from the settling reactor, is discharged as excess sludge and a portion (RAS) is recycled to the system. The recycled RAS is divided into two lines: the first one allows the recirculation of the sludge into the anoxic tank of the water line while the second one leads to the ASSR before being returned to the anaerobic reactor of the water line.

This configuration had been improperly compared to the SBR-OSA developed by Datta et al. (2009), where the SBR cycle consists of an anaerobic, anoxic and aerobic phase. Even if the water line has the same operative conditions, there are two main differences between Biminex™ and the SBR-OSA system: the presence/absence of three separate volumes to perform the anaerobic, anoxic and aerobic reactions in the water line and, moreover, the continuous/batch loading rate of the settled sludge in the ASSR.

Recently, another configuration has been developed by Zhou et al. (2015). It consists an anoxic tank prior to the oxic tank in the water line and an ASSR where a portion of the RAS is cycled.

This configuration is called A+OSA system and allows both the nitrogen removal and the biological sludge reduction. (Fig 2b).

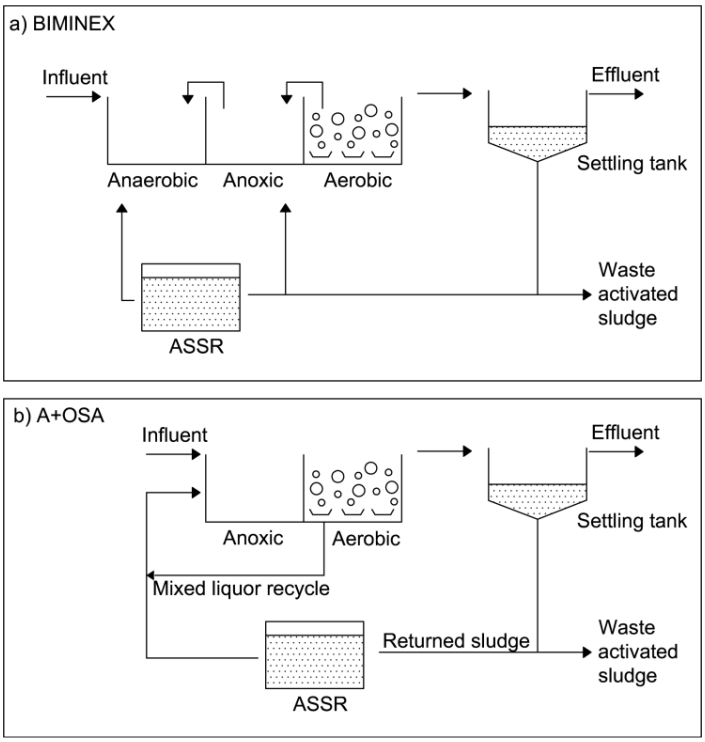


Fig. II.2 Schematic diagrams of (a) BIMINEX system; (b) A+OSA system

*2.2. Engineering applications*

Some laboratory applications are also the bases of two different patents. The Cannibal® process patent by Curtis et al. (2007; 2011), which is a combination of biological and physical treatment, is the first implementation of an ASSR to full scale. The Cannibal® process is composed of the following parts: (1) a CAS system, (2) a solid separation module; (3) an ASSR with 10 d SRT; and (4) a remote control system that monitor the ORP at different levels/compartments of the side reactor. (Fig 3).

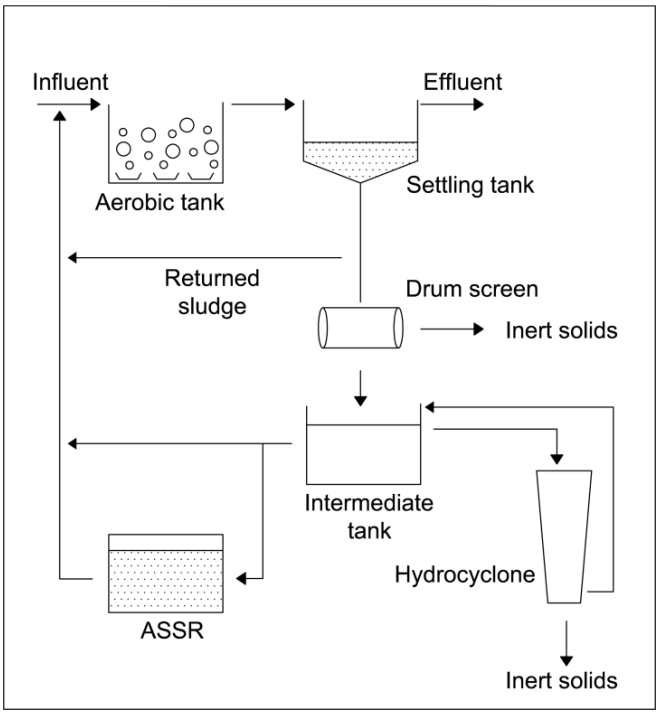


Fig. II.3 Schematic diagrams of Cannibal® system

A portion (90%) of the settled sludge is directly recycled to the CAS system, while the remaining portion (10%) is pumped to the solid separation module which contains a fine drum screen (250 µm) with hydrocyclones to remove trash, grit, and inert material accumulated in the mixed liquor due to their small size. The residue produced by the solids separation module can be compressed and disposed of as screenings waste. After the physical treatment, the returned sludge flow is directed to the ASSR, where particular environmental conditions are provided by the automatic control system. A pH and ORP threshold values are ensured to avoid fermentative phenomena with a consequent emission of odours (Ragazzi et al. 2015). Lab scale reactors have been also used to test the performance of the process. Novak et al. (2007) and Goel and Noguera (2006) simulated the industrial process using a SBR-OSA configuration without the solid separation module to evaluate the biological reduction of excess sludge and the phosphorous removal, respectively. However, both studies employed synthetic wastewater with a low TSS content, thus the results obtained in terms of sludge reduction may differ significantly when real wastewater is used. Johnson et al. (2008) modified the standard ASM 2d model to simulate the effects of the biological sludge reduction in the Cannibal® process. It has been hypothesized that the base biological sludge reduction mechanism is the transformation of a portion of particulate non-biodegradable organic material, entering the bioreactor, to slowly biodegradable particulate.

Recently Chon and Park, (2012) proposed a new patent, called UMass process, characterized by an ASSR with a short SRT (2.5 days). The patent is based on a lab reactor application where a comparison of activated sludge systems with different ASSR schemes is reported. The evaluated

systems were: (1) a SBR coupled with an ASSR with 10 days SRT at 21°C (as in the Cannibal process); (2) a SBR + ASSR-UMass with 2.5 day SRT at 21°C; (3) a SBR + ASSR-UMass with 2.5 day SRT at 37°C; (4) a SBR+ ASSR- UMass with 2.5 day SRT at 50 -55°C; (5) a control SBR without ASSR. In each scheme, 10% volume of settled sludge in SBR was fed to their respective ASSRs. Experiments were performed with real wastewater to investigate the UMass sludge reduction process and to compare its efficiency with that of the Cannibal® solids reduction process. Results clearly indicated that the UMass sludge reduction systems (Systems 2, 3, 4),which employ a short SRT (2.5 days), are very effective in reducing sludge generation, being even more effective than the sludge reduction obtained in the Cannibal-like process (System 1), which is characterized by a longer SRT (10 days). Both the processes require a completed stirred tank reactor as ASSR. However, the reactor of the Cannibal process needs to be huge to sustain a long SRT while the UMass process is characterized by a smaller ASSR, and consequently by lower capital and operating costs.

## 3. Performance of the process

### *3.1 Sludge reduction*

The main goal of the ASSR process is to reduce sludge production. The essential parameter to evaluate the sludge production/reduction is the observed sludge yield (Yobs). The Yobs is basically defined as the amount of sludge formed per the amount of substrate removed (Grady et al. 2011). However this definition, each study follows different ways to express it. Several studies such as Chudoba et al. (1992), Wang et al., (2008) and Torregrossa et al. (2012) evaluated the Yobs using the mass balances tool, considering solids production and substrate consumption for a single day while others, such as Chon et al. (2011a, 2011b), Zhou et al. (2015b) and Coma et al. (2013) evaluated it graphically considering cumulative terms. The latter case allows the Yobs to be more representative of the reality since cumulative terms should be used to take into account the changes in both solids and substrates concentrations during all the experimental period.

The CAS-OSA configuration allows a percentage of sludge reduction varying between 14 -50 % to be obtained (Chudoba et al. 1992; Wang et al. 2008; Ye et al. 2008; Torregrossa et al. 2012). According to Semblante et al. (2014), the observed sludge yield (Yobs) of a lab application of the CAS-OSA configurations ranged from 0.21-0.25 gTSS/gCOD (Chudoba et al. 1992) and 0.53 gMLSS/gCOD (Wang et al. 2008).

Torregrossa et al. (2012) implemented the same configuration as a pilot plant continuously fed with a real municipal wastewater achieving 35% of sludge reduction (Yobs 0.36 gTSS/gCOD), compared to the control system. Ye et al. (2008) had a cumulative sludge production of 1.842,

1.597 and 2.062 g SS/d, depending on the SRT applied, which were 23%, 33% and 14%, respectively, lower than the control system (2.392 g SS/d). The MBR-OSA configuration performed by Saby et al. (2003) achieved a cumulative sludge reduction varying between 28 – 58% with a Yobs 0.17-0.29 gTSS/gCOD, depending on the oxidation potential reduction (ORP) value. Among the different configurations implemented in laboratory studies, the SBR-OSA configurations reported up to 60% sludge reduction, making the best result. Novak et al. (2007) reported a Yobs of 0.11 gVSS/gCOD and a reduction of excess sludge production of 20 – 45%. Similar results were obtained in the studies performed by Chon and Park (2012) and Chon et al. (2011a, 2011b). Zhou et al. (2015b) observed that the A+OSA system had a Yobs of 0.21 gSS/gCOD, 32% lower than a conventional process with anoxic and oxic phases in the water line (A/O) (0.32 gSS/gCOD). The Biminex® had a maximum reduction of 18.3% of the Yobs treating the whole sludge returned line. This value was not in agreement with literature values, but several different operating conditions has to be considered. All the results reported are strictly related to the operating parameters that could significantly affect the performance of the process, which are described more in details in the following paragraphs.

### *3.2 Carbon and nutrients removal*

Despite the aim of the process is to reduce the excess sludge production, it is also extremely important to obtain high percentage of carbon and nutrients removal in order to respect the law limits. One of the main sludge reduction mechanism is the sludge decay that involves the release of soluble chemical oxygen demand (COD) in the ASSR, increasing its concentration. Nevertheless, most of the literature studies reported that the ASSR process is able to achieve high percentages of carbon removal as most of the COD generated in the anaerobic reactor is biodegradable and can be degraded both in the ASSR and in the water line. Thus, the release of soluble COD in the ASSR has a minimal impact on the overall COD removal efficiency of the whole system (Semblante et al. 2014). As show in Table 1, the percentage of COD removal in the ASSR system is often higher than that of the conventional control process or at most equal. In only few cases, the configurations with the ASSR has a slightly lower COD removal efficiency. Ye et al. (2008) reported that the organic removal capacity decreased with increasing SRT in ASSR, which may contribute to the sludge decay. The results obtained by Troiani et al. (2011) could be due to a difference in the influent flow rate, in the organic and nitrogen load and in the mixed liquor concentrations of the oxidation tank and ASSR. Concerning the nitrogen removal, most of the literature studies show that the OSA process does not have negative effect on the nitrogen removal (Ye et al. 2008; Datta et al. 2009; Troiani et al. 2011). On the contrary, Zhou et

al. (2015b) noted a lower nitrification efficiency in the A+OSA process, which could be attribute to the anaerobic decay of nitrifying microorganism in the anaerobic reactor. Nevertheless, the average removal efficiency of the TN was higher in the A+OSA than the AO system due to the availability of a more quantity of carbon source, generated from cell lysis and hydrolysis reactions, which was used for denitrification in the anoxic tank.

The impact of the ASSR on phosphorous removal has been studied by Datta et al. (2009) and Goel and Noguera (2006). In the first study, the SBR-OSA process achieved a lower percentage in P removal than the control process. However, the Authors did not know the reasons of this finding. On the contrary, Goel and Noguera (2006), performing an enhanced biological phosphorus removal (EBPR), reported that the EBPR SBR-OSA had higher P removal (98%) than the control EBPR SBR (84%) and demonstrated that the process was able to reduce simultaneously the excess sludge and the phosphorus concentration. The Authors explained this result as the ability of the ASSR to select fermenting bacteria that contribute to the formation, in the aerobic tank, of volatile fatty acids, which enhance the EBPR metabolism. Other studies show that the ASSR process could enhance the phosphorous removal but different and several explanation were proposed such as the presence of phosphorous accumulating organisms (PAO) (Chudoba et al. 1992; Saby et al. 2003) or the substrate loading mode (Ye et al. 2008). Therefore, according to Semblante et al. (2014), more investigation must be performed to clarify the effect of the ASSR process on phosphorous removal.

Table II-1. COD, nitrogen and phosphorous removal

| References | Configuration | COD removal (%) | | Nitrogen removal (%) | | Phosphorous removal (%) | |
|---|---|---|---|---|---|---|---|
| | | OSA or ASSR | Control | OSA or ASSR | Control | OSA or ASSR | Control |
| **Chudoba et al. (1992)** | CAS-OSA | 82-99 | 83 -95 | | | 19-42[a] | 1-16[a] |
| **Ye et al. (2008)** | CAS-OSA | 91-90 | 93 | 28-30[b] | 30[b] | 48-58[c] | 49[c] |
| **Wang et al. (2008)** | CAS-OSA | - | - | 59[b] | - | 63[c] | - |
| **Troiani et al. (2011)** | CAS-OSA | 93 | 96 | 79[b] | 67[b] | 62[c] | 54[c] |
| **Torregrossa et al. (2012)** | CAS-OSA | 85 | 81 | - | - | - | - |
| **Eom et al. (2013)** | CAS-OSA | 79 | 81 | - | - | - | - |
| **Chen et al. (2003)** | MBR-OSA | 91 | 91 | - | - | - | - |
| **Saby et al. (2003)** | MBR-OSA | 93-98 | 91 | - | - | 28-63[c] | 64[c] |
| **Novak et al. (2007)** | SBR-OSA CANNIBAL® | 96-97 | 96-97 | - | - | - | - |
| **Sun et al. (2010)** | SBR-OSA | 88 - 90 | - | - | - | - | - |
| **Chon et al. (2011)** | SBR-OSA | 91 | 91 | - | - | - | - |
| **Datta et al. (2009)** | SBR-OSA | - | - | 100[d] | 100[d] | 90[a] | 95[a] |
| **Goel and Noguera (2006)** | SBR-OSA | 98 | 97 | | | 98[a] | 84[a] |
| **Coma et al. (2013)** | Biminex™ | 87-93 | - | - | - | - | - |
| **Zhou et al. (2015)** | A+OSA | 85 | 85 | 77[e]; 57[b] | 83[e]; 43[b] | - | - |

**[a] Percentage based on $PO_4^{3-}$P removal**
**[b] Percentage based on TN removal**
**[c] Percentage based on TP removal**
**[d] Percentage based on $NH_3$-N removal**
**[e] Percentage based on $NH_4$-N removal**

## 4. Operating parameters

Looking at the several configurations implemented in literature, some operating parameters, defined as the main ones, could be identified such as SRT, ORP, interchange rates (IR),interchange times (IT) and temperature. Their variation could involve a significant improvement in the sludge reduction. As contrasting results had been reported in literature, the aim of the present paragraph is to present an overview in order to define which is the value of the parameter that allow to obtain the best sludge reduction.

### *4.1 SRT*

The SRT represents the average period of time during which the sludge has remained in the system. As could be expected, the Yobs decreases as the SRT increased, due to biomass loss by a higher endogenous respiration (Tchobanoglus et al. 2003). Nevertheless, several researches confirm the opposite trend. Chudoba et al.(1992) evaluated the CAS-OSA configuration with two distinct value of SRT of the whole system. The system worked at 5 days SRT, with a high organic loading rate, and was able to reduce by half the excess sludge production compared with the CAS system. On the other hand, when the SRT was equal to 12 days, and a lower organic rate was applied, the specific excess sludge production remains the same for both the CAS-OSA and the

control system. However, Torregrossa et al. (2012) implemented a pilot plant with the HRT of the ASSR equal to 9h and the SRT of the whole system was 5 days, as the one proposed by Chudoba et al.(1992). The Authors achieved a worse settleability of sludge, probably due to a high HRT in the anaerobic tank. In the SBR-OSA configurations, the intermittent and low loading rate, usually 10% of the total biomass, lead to long SRT in the whole SBR-OSA system. Novak et al. (2007) operated an SBR-OSA configuration with a SRT almost close to 80 days, achieving 60% of sludge reduction. However, compared the SBR-OSA system with a conventional one, both with the same SRT, the Authors demonstrated that the solid loss in the SBR-OSA system was not the result of the high SRT of the whole system, which cannot be used as a design parameter. Ye et al. (2008) tested three different values of SRT (of 5.5 h, 7.6 h and 11.5 h) in the ASSR and compared the Yobs of the CAS-OSA system with a control CAS process. Results showed the lowest sludge production when the SRT in the ASSR was 7.6 h.

Two more recent studies compared the SBR-OSA process with other sludge treatment configurations serving as controls (Chon et al. 2011b; Kim et al. 2012). In particular, Chon et al. (2011) conducted a parallel comparison between different laboratory systems to better understand the mechanisms of sludge reduction in the SBR-OSA process. The systems studied were: (1) SBR with aerobic side stream reactor treating 10% of the settled sludge, (2) SBR with an ASSR treating 10% of the settled sludge, (3) SBR with aerobic digester, (4) SBR with anaerobic digester, and (5) a no wastage system. The SRT of both of the ASSR in the system 1 and 2 and of the digester in the system 3 and 4 was 10 days, while the SRT of the whole system was 74 days. Comparing all the biological systems implemented, the combination of activated sludge with the ASSR was found to be the most effective in terms of sludge reduction, effluent quality and sludge settleability. In the further study reported in the UMass patent, Chon and Park (2012) showed the performance of a ASSR at low SRT (2.5 days). The lowest Yobs (0.17 gVSS/g COD) was obtained by an SBR + ASSR-UMass configuration with 2.5 days SRT at 37° C, with a reduction of 62% compared to a conventional process and 30% compared to the configuration with a longer SRT (10 days) of ASSR of the Cannibal process. Results clearly indicate that the system that employs a short SRT is able to reduce the sludge generation even more than the system with long SRT.

### *4.2 ORP*

ORP is related to the concentration of oxidizers or reducers present in the water system. It can successfully indicate the oxidative state of the wastewater, providing information on the variations in the dissolved oxygen (DO) concentrations and others oxidized compounds. An anaerobic

reactor is realized when DO and inorganic nitrogen (nitrates and nitrites) are unavailable, and it has an ORP level of less than – 150 mV(Khanal and Huang 2003). Anaerobic conditions allow anaerobic processes to develop, which are characterized by low growth yield, and further enhance the cell lysis and the organic matter solubilisation.

Saby et al. (2003), in a MBR-OSA system, focused the attention on the ORP value in the ASSR that could influence the excess sludge production. MBR-OSA systems were tested with three different ORP values of -250, -100 and +100 mV accompanied to a variation of SRT. The sludge production of the control system was 4.7 gSS/day, while MBR-OSA system achieved a production of 2.3 (-250 mV), 2.7 (-100 mV) and 3.6 gSS/day (+100 mV). A 51% of excess sludge reduction, compared with the control system, was obtained when the ASSR worked with an ORP value equal to -250 mV. Operating at -250 mV, the COD removal efficiency was improved, in particular the COD concentration in the ASSR significantly increased compared with that in phases at higher ORP values.

The study performed by Troiani et al. (2011) is, probably, the only experience where the sludge in the ASSR was subjected to an alternation of oxic and anoxic phases to minimize the waste sludge production. In the ASSR the oxygen supply was alternated with anoxic phases depending on a minimum and a maximum threshold value of the ORP. For the 50% of time the control device operated in the ORP range between -400 and -200 mV, and for the remaining 50% in the ORP range of -200 and +50 mV. Results showed the Yobs was 45% lower than the one found out by Chudoba et al. (1992) and 27% than the Yobs found out by Saby et al. (2003), where the working ORP of the ASSRs was – 250 mV. The lower sludge production is linked to the alternation of oxic and anoxic phases in the sludge line (corresponding at a variable ORP value), that could determine a higher energy uncoupling phenomena than the one achieved with only anaerobic conditions (corresponding at a fixed value of the ORP).

Despite the effect of ORP on excess sludge production has not been widely investigated, literature studies point out that a lower ORP (i.e. -250 mV) resulted in a lowest production of excess sludge as suggest by data reported in Table 2.

### *4.3 Interchange rate*

The interchange rate is defined as the rate of solids passed through the ASSR, expressed as percentage per day of the total biomass in the main activated sludge reactor. In a conventional CAS-OSA process the settled sludge is completely cycled to the ASSR (Chudoba et al. 1992; Saby et al. 2003; Ye and Li 2010) while in the others lab-scale applications only a portion, typically 10%, of the settle sludge is cycled. The importance of the IR was point out, probably for

the first time, by Novak et al. (2007). The Author showed that at low IR (4%) the Yobs was 0.15 gVSS/gCOD, while at higher IR (7%) a value of 0.11 gVSS/gCOD was measured. Referring this results to the one of the control system (0.28 gVSS/gCOD), the SBR-OSA process achieved a sludge reduction of 46 – 60%. Thus, results show that the interchange of sludge between aerobic and anaerobic reactors does not need to be necessary continuous. Based on this evidence, Sun et al. (2010), in a SBR-OSA system, investigates the relationship between the times per day (IT) that the aerobic sludge was interchanged in the ASSR reactor and the excess sludge reduction. The daily IR was 10% of the settled aerobic sludge while the IT investigated were equal to 1 and 4. The Yobs values of the OSA processes with an IT = 1, and IT =4, and the Yobs value of a control SBR process were 0.25 mgTSS/mgCOD, 0.12 mgTSS/mgCOD and 0.53 mgTSS/mgCOD, respectively. Results showed a higher sludge reduction in the OSA-SBR system than in the control SBR process, which increased varying the IT from 1 to 4 times/day.

The influence of the percentage of RAS treated in the ASSR on the excess sludge production was the main objective of the study performed by Coma et al. (2012). The Biminex® process was tested, in an outdoor pilot plant, with four different recirculation ratio at a fixed ORP value: 0%, 10 %, 50 % and 100% of activated sludge treated in the ASSR before being returned to the anaerobic reactor of the water line. The Yobs were normalized at 20°C because results were affected by the seasonal variation of temperature (ranging from 7 to 27 °C) and the percentage of the sludge reduction obtained for 10%, 50% and 100 % of sludge returned ratio were 0.22%, 8.8% and 18.3%, respectively. Thus, the best result was obtained when all the activated sludge was completely treated in the ASSR before being returned to the water line, achieving 0.329 kgVSS/kgCOD.

### *4.4 Sludge loading rate*

Chudoba et al.(1992) verified the sludge production when the CAS-OSA system worked under two different operative conditions of the sludge loading rate (SLR), which represents the daily organic load per whole sludge mass. Results showed 50% of sludge reduction compared to the control CAS system at high SLR (0.92 kgCODin/kgTSStotal sludge d); while no differences were observed between the CAS-OSA and the control system when low SLR (0.33 kgCODin/kgTSStotal sludge d) was tested. Unlike the CAS control system, the sludge production in the CAS-OSA systems appeared to be stable for both high (Yobs 0.21 gTSS/gCOD) and low SLR (Yobs 0.25 gTSS/gCOD).

### *4.5 Temperature*

Because of the great influence of the temperature on biological processes most of the experiments were performed at controlled temperature of about 18 - 20 °C. Most of the kinetic reactions involved in biological treatment are slowed down at low values of temperature while higher values would enhance the auto-digestion of the sludge, decreasing the observed sludge yield. Coma et al. (2012) performed their study at room temperature showing how the seasonal variations of the temperature could influence the sludge yield in the ASSR. The temperature was included in the evaluation of the sludge yield; all the data were normalized at 20°C. The Authors addressed the low sludge reduction obtained in their study to the influence of the variations in the temperature. However, Chon and Park, (2012) showed that conducting the ASSR at temperature higher than 20°C, the sludge reduction process does not increase significantly. The UMass process was tested at different temperature 21, 37 and 50-55°C achieving a Yobs equal to 0.18, 0.17 and 0.19 g VSS/g COD, respectively. Results demonstrated that a significant increase of the temperature does not increase the sludge reduction conducted in the oxidation/nitrification processes.

Temperature could be a limiting parameter when it drops below some degree. However, no applications were performed to evaluate the minimum value of the temperature which could negatively influence the performance of the process.

Table II-2. Main operative parameters

| References | Configuration | Water line process | Wastewater type | SRT in the whole system (d) | SLR in the ASSR ($kgCOD_{in}$/ $kgSST_{total\ sludge}$ d) | HRT in the ASSR | $HRT_{ASSR}$/ $HRT_{CAS}$ | ORP (mV) | T (°C) | Interchange Ratio (%) | $Y_{obs}$ TSS/COD CAS+ASSR | Sludge reduction (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chudoba et al. (1992)** | CAS-OSA | OX | Synthetic wastewater | 5 ($SST_{CAS}$=0.8g/L;$SST_{ASSR}$=2.16g/L[a]) | 0.92 | 3 h | 1.5 [a] | -250 | 18±5 | 100 | 0.21 | 50 % [c] |
| | | | | 12($SST_{CAS}$=2.5g/L;$SST_{ASSR}$=4.3g/L[a]) | 0.33 | | 1.5 [a] | | 20±2 | | 0.25 | No reduction [c] |
| **Ye et al. (2008)** | CAS-OSA | OX + NITR | Synthetic wastewater | n.a. | n.a. | 5.5 h | n.a. | n.a. | 25±1 | 100 | n.a. | 33 % [c] |
| | | | | | | 7.6 h | | | | | | 23 % [c] |
| | | | | | | 11.5 h | | | | | | 14 % [c] |
| **Torregrossa et al. (2012)** | CAS-OSA | OX + NITR | Urban wastewater | 5 ($SST_{CAS}$= $SST_{ASSR}$ =3,5g/L) | 0.3 in terms of $BOD_5$ | 9 h | 2.06 [a] | -180 | - | 30 | 0.36 [a,b] | 35% [c] |
| **Saby et al. (2003)** | MBR-OSA | OX + NITR | Synthetic wastewater | 19.5($SST_{CAS}$=2g/L;$SST_{ASSR}$=8.6g/L[a]) | 0.66 | 11d | 1.7 [a] | +100 | 20 | 100 | 0.29 | 28 % [c] |
| | | | | 25.9($SST_{CAS}$=2g/L;$SST_{ASSR}$=8.6g/L[a]) | | 15d | | -100 | | | 0.21 | 48 % [c] |
| | | | | 30.4($SST_{CAS}$=2g/L;$SST_{ASSR}$=8.6g/L[a]) | | 17d | | -250 | | | 0.17 | 58 % [c] |
| **Novak et al. (2007)** | SBR-OSA CANNIBAL® | OX + NITR | Synthetic wastewater | 80 ($SST_{CAS}$ =2-2.5g/L) | n.a. | 10 d | 5 [a] | n.a. | n.a. | 7 | 0.15 [a,b] | 60 % [d] |
| | | | | | | | | | | 4 | 0.21 [a,b] | 46 % [d] |
| **Sun et al. (2010)** | SBR-OSA | OX + NITR | Synthetic wastewater | n.a. ($SST_{CAS}$=3.6g/L; $SST_{ASSR}$=n.a) | n.a. | 10 d | 5 [a] | n.a. | n.a. | 10 (IT=4) | 0.12 | 77.4% [c] |
| | | | | n.a. ($SST_{CAS}$=2.5g/L; $SST_{ASSR}$=n.a) | | | | | | 10 (IT=1) | 0.25 | 52.8% [c] |
| **Chon et al. (2011)** | SBR-OSA | OX + NITR | Urban wastewater | 74 ($SST_{CAS}$=3g/L; $SST_{ASSR}$=6g/L) | 0.07[a] | 10 d | 10 | n.a. | 19±1.5 | 10 | 0.15 | 49 % [d] 39 % [e] |
| **Chon and Park, 2012** | SBR-OSA | OX + NITR | Urban wastewater | n.a. ($SST_{CAS}$=2g/L;$SST_{ASSR}$=8g/L) | n.a. | 10 d | n.a. | n.a. | 21 | 10 | 0.35 | 43% |
| | | | | n.a. ($SST_{CAS}$=2g/L;$SST_{ASSR}$=10g/L) | | 2.5 d | | | 21 | | 0.25 | 59% |
| | | | | n.a. ($SST_{CAS}$=2g/L;$SST_{ASSR}$=8g/L) | | 2.5 d | | | 37 | | 0.23 | 61% |
| | | | | n.a. ($SST_{CAS}$=2g/L;$SST_{ASSR}$=8g/L) | | 2.5 d | | | 55 | | 0.26 | 57% |
| **Goel and Noguera (2006)** | | OX + P | Synthetic wastewater | n.a. ($SST_{CAS}$=2g/L;$SST_{ASSR}$=8g/L) | 0.08-0.11 [a] | 10 d | 13 | n.a. | n.a. | 10 | 0.19 | 62 - 63 % [c] 21 - 37 % [e] |
| **Datta et al. (2009)** | SBR-OSA | OX + NITR + DEN + P | Synthetic wastewater | 100 ($SST_{CAS}$=6g/L;$SST_{ASSR}$=4g/L) | n.a. | 10 d | 13 | n.a. | n.a. | 10 | 0.17 | 63 % [e] |
| **Coma et al. (2013)** | Biminex™ | OX + NITR + DEN + P | Urban wastewater | 23.3($SST_{CAS}$=4g/L;$SST_{ASSR}$=5g/L) | n.a. | 34.5 h | 1.9 | -150 | 20 | 10 | 0.55 [a,b] | 0.2 % [c] |
| | | | | 23.2($SST_{CAS}$=3g/L;$SST_{ASSR}$=4.2g/L) | | 11.8 h | 0.46 | | | 50 | 0.51 [a,b] | 8.8 % [c] |
| | | | | 26.2($SST_{CAS}$=3g/L;$SST_{ASSR}$=5.3g/L) | | 5.9 h | 0.24 | | | 100 | 0.45 [a,b] | 18.3 % [c] |
| **Zhou et al. (2015)** | A+OSA | OX + NITR+DEN | Urban wastewater | 60 | n.a. | 6 h | n.a. | -150 -100 | - n.a. | 100 | 0.212 | 32% [c] |

**[a] calculated**

**[b] 1gVSS=1.42gCOD; VSS/TSS =0.72 (Novak et al 2003); 1gTSS=1.02gCOD**

**[c] the sludge reduction is calculated compared with a CAS system conducted at the same operative conditions**

**[d] the sludge reduction is calculated compared with a CAS system (conducted at the same operative conditions) with an aerobic post digestion of the sludge**

**[e] the sludge reduction is calculated compared with a CAS system (conducted at the same operative conditions) with an anerobic post digestion of the sludge**

**OX = carbon oxidation; NITR= Nitrification; DEN=Denitrification; P= Phosphorous removal**

**Interchange Rate = % of the raw activated sludge (RAS) sent to the ASSR.**

**IT= number of daily sludge Interchange Times in the anaerobic tank**

## 5. Effects of the water line processes on the sludge reduction

The reason of a different percentage of sludge reduction obtained by using an ASSR, but performing different biological processes in the water line could be related to several mechanisms of reduction and biological interferences, which could be involved in the sludge reduction process.

Chudoba et al (1992) was the unique study reported in litterature on sludge reduction applied to a CAS system, where only carbon oxidation was carried out. The influent of the ASSR was characterized by residual carbon matter and ammonia, while no nitrate and nitrite were present, consequently denitrification process could not take place. The Authors achieved a high sludge reduction efficiency (50%).

When also nitrification is performed in the water line, the influent of the ASSR is characterized by a high nitrite and nitrate concentrations (for urban wastewater treatment usually 20 – 30 mgNOx-N/L are obtained), which increases the ORP level in the ASSR. This could influence the biological mechanisms involved in the sludge reduction process, as also denitrification process could occur. On the contrary, if nitrification- denitrification is performed in the water line, the ASSR influent will be characterized by low organic carbon and total nitrogen content, and it makes possible to achieve easily low ORP levels in the ASSR.

## 6. Sludge reduction mechanisms

Several and conflicting explanations have been provided about the sludge reduction achieved by using the configurations reported above and different mechanisms have been proposed and observed such as uncoupling metabolism, cell lysis and cryptic growth, domination of slow-growing microorganisms and destruction of EPS.

### *6.1 Uncoupling metabolism*

The uncoupling metabolism occurs when the interrelation between catabolic and anabolic reactions is prevent. Their sum made the microbial metabolisms (Liu and Tay 2001). Catabolism reactions are all the metabolic processes that result in the consumption of substrate that lead to the production of simple and poor in energy substances. In this phase, excess energy is produced in the form of Adenosine triphosphate (ATP) and thermal energy, that are used in the anabolic reactions to synthetized new biomass. Thus, catabolism is often preferred to anabolism in order to reduce the production of biomass. The mechanism of uncoupling metabolism could be achieved in different ways such as using chemical uncouples (Low and Chase 1998; Yang et al. 2003; Ye and Li 2010; Feng et al. 2014), biological processes with high ratios of substrate concentration and biomass (Liu 1996, 2000; Chen and Liu 1999) or subjecting the biomass to a metabolic stress due

to cycling passage under aerobic and anaerobic environmental conditions as occurs in the ASSR process (Chen et al. 2003; Jin et al. 2008; Wang et al. 2008). The cycle passage from an anaerobic to an aerobic environment, also defined “sludge fasting/feasting”, submits microorganisms to a stress conditions, promoting the uncoupling between catabolism and anabolism (Chen et al. 2001). This was confirmed by Chudoba et al. (1992), who showed an important ATP consumption after the anaerobic phase. The Authors concluded that in the anaerobic reactor, where food and oxygen are insufficient, microorganisms are subjected to a physiological shock. In these conditions, they spent ATP and polyphosphates to satisfy their maintenance functions and not for the synthesis of new cells. When they are returned in the aerobic reactor, they have enough substrate and rebuilt their energy reserves. On the contrary, Ye and Li (2010, 2005) employed a chemical uncoupler, 3,3', 4', 5-tetrachlorosalicylanilide and OSA combined process, resulting in a significant decrease in sludge production. The Authors showed that for a higher quantity of uncoupler used, a smaller sludge yield could be achieved. However, Wang et al. (2008) reported that energy uncoupling metabolism occurs in the CA-OSA systems even if it was not significant. Chen et al. (2003) explained that the energy uncoupling theory was unable to explain the excess sludge reduction in an MBR-OSA process.

### *6.2 Slow growing microorganisms*

The cycling passage of the sludge under aerobic, anoxic and anaerobic conditions could have effects on bacterial diversity, which plays an important role in biomass growth (Semblante et al. 2014). The idea is that ASSR is able to select slow growing microorganisms, able to degrade organic matter and produce a very small quantity of new biomass.

According to the study of Chudoba et al. (1992), the mechanism of metabolic uncoupling is strictly connected to the selection of particular microorganisms. Therefore the sludge cycling in anaerobic and aerobic phases results in a metabolic selection of those microorganisms able to favour catabolic pathways. The slow growing bacteria present in their study were poly-P bacteria, able to accumulate polyphosphates under aerobic conditions and use them under anaerobic conditions as energy sources. Poly-p were 50-60% of the total bacteria population and the dominant microorganisms of the mixed culture responsible of the enhanced phosphorus removal. An enrichment of slow growing microorganisms, such as PAO, was confirmed by Goel and Noguera (2006), who related their activity to the increase of phosphate concentrations in the anaerobic stage. The mechanism of selection of slow growers was also studied by Chen et al. (2003), who showed that no significant differences between the observed cell growth yield ($Y_{s/x}$) of the MBR-OSA and the CAS systems were recognized, even though microbiological results

indicated a changes in microbial population. On the basis of $Y_{s/x}$ results, Chen et al. (2003) addressed that the excess sludge reduction in the MBR-OSA system could not be attributed to the selection of slow growing microorganism.

### *6.3 Sludge decay*

Sludge decay could be defined as the solubilisation of cellular constituents of microorganisms. Low molecular weight compounds are released into the liquid causing an increase in the concentration of organic matter and nutrients in the supernatant. Furthermore, the released cellular constituents are used by microorganisms for their metabolic function (cryptic growth) (Mason et al. 1986; Chu et al. 2009a).

Wang et al. (2008) investigate the effects of sludge decay, energy uncoupling and low sludge yield of anaerobic oxidation on the minimization of excess sludge. Results showed that the sludge decay is the main cause of sludge reduction in OSA system, accounting for 2/3 reduction. Subjecting the sludge to alternate aerobic and anaerobic conditions, the concentrations of soluble COD, $NH_4^+$-N, TP, protein and carbohydrate in the supernatant gradually increased proving that the sludge lysis and acidogenesis occur and lead to minimization of excess sludge.

Comparing four different excess sludge reduction mechanism (energy uncoupling, domination of slow growers, soluble microbial products (SMPs) effect and sludge decay in the ASSR), Chen et al. (2003) identified the sludge decay as the mechanism underlying the sludge reduction in the MBR-OSA system.

An and Chen (2008) confirmed that the increase of COD concentration into the anaerobic reactors is related to the conversion of biomass COD into soluble COD. Denitrification, sulphate reduction, phosphorous release and methane producing could consume the amount of soluble COD.

### *6.4 EPS destructuration*

The EPS are high molecules weight compounds present outside and inside of the cells (Sheng et al. 2010). They are mainly carbohydrates and proteins generate from the secretion of microorganism, cellular lysis processes and hydrolysis of macromolecules (Nielsen and Jahn 1999; Liu and Fang 2003). The EPS could be divided in bound EPS, closely bound with cells, and soluble EPS, weakly bound with cells or dissolved into the solution (Laspidou and Rittman 2002). The importance of ESP destructuration mechanism was suggested by Novak et al. (2003) who explained that aerobic conditions resulted in the release of calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) into solution in conjunction with volatile solids destruction and accumulation of polysaccharide.

In contrast, a large amount of protein was released during anaerobic digestion, but divalent cations were not released. The large release of protein in anaerobic digestion was assign to the loss of selective binding between protein and iron (FeIII) under Fe-reducing conditions. From these results, the authors proposed that flocs consist of two important biopolymer fractions: divalent cation-bound biopolymer and Fe-associated biopolymer (Park et al. 2006).

Chon et al. (2011) performed the extraction of EPS to test the hypothesis of the digestion mechanism proposed by Novak et al. (2003). Results showed that degradation of EPS, iron and/or aluminium-associated materials occurs under aerobic and anaerobic cycling conditions, while it does not occur in a CAS system even with long SRT. Thus, the EPS destructuration could be responsible of the lower sludge yield in the SBR-OSA system.

Novak et al. (2007) subjected the centrates from the Cannibal® process and from the control CAS to the Oxygen Uptake Rate (OUR) test and to the analysis of protein concentrations. Experiments showed that the OUR of the centrate from the Cannibal® system was higher than that of the control system, due to a higher content of readily biodegradable material.

Soluble EPS were also studied by Chen et al., (2003), referring them to a soluble microbial products (SMP). As no differences in SMPs were found in $Y_{s/x}$ between the MBR-OSA and the CAS systems, the Authors concluded that SMPs should not affect the sludge reduction process.

Furthermore, several studies report that the ASSR process improves sludge settleability, that could be related to the release of intracellular polymers under anoxic conditions, since they can act as floc bridging agents to improve sludge settleability in aerobic conditions (Barker and Stuckey 1999).

## 7. Microbial community

The microbial community structure in the ASSR process differs from that in the CAS system (Semblante et al. 2014). The insertion of an ASSR in the sludge return line promoted the selection of a more abundant microbial community. Chen et al., (2003) and Saby et al. (2003) showed that MBR-OSA configuration has 40-50% more cells that a conventional MBR. Further, they demonstrated that only 7-8% of total cells were respiring, due to the periodic exposure of the biomass to stressful conditions, while the remaining part were active facultative bacteria. . However, after 210 days of operation, Ye et al. (2008) did not identify a distinct shift in the predominant microbial species. On the contrary, Wang and Zhao (2011) reported that most of the bacteria present by dominant denaturing gradient gel electrophoresis (DGGE) bands were phylogenetically related to PAOs, denitrifying bacteria and anaerobes.

The analysis performed by Chon et al. (2011a) showed about 75% similarity for microbial composition between the SBR-OSA system and the anaerobic digester. In a further study, Kim et al., (2012) asserted that the substantial difference between the bacteria population of the SBR-OSA and SBR system was due to the operation mode of feeding. Both systems were connected via continuous sludge recirculation, but the 10% of the interchange rate in the SBR-ASSR system gives time to create a different microbial community that might be significant different if the feeding regime was continuous. Performing a A+OSA system, Zhou et al., (2015a) showed that the insertion of an ASSR resulted in the enrichment of anaerobic bacteria such as fermentative, hydrogenogenic and acidogenic bacteria who allow to obtain sludge reduction through biomass decay and hydrolysis of particulate organic matters. The Authors confirmed also the shift of the microbial population from fast growers to slow growers. However, most of microbial analysis results reported in literature indicate that there is a large number of bacteria detected in the ASSR sludge whose existence and roles is not clearly explained and need further investigations.

## 8. Conclusions

A review of ASSR implemented in a conventional activated sludge process has been proposed to fill the gap of thorough understanding of the mechanisms behind sludge minimisation and the proper operative conditions of the ASSR.

A lot of configurations have been studied at laboratory scale. For its feasibility and low space requirement, the most used configuration is the SBR-ASSR.

The ORP, the SRT, the $HRT_{ASSR}$, and the IR have been defined as the main operative parameters of the process. The optimal ORP value in the ASSR for the sludge reduction is -250 mV.

It is not possible to define a unique value of the SRT of the whole CAS-OSA system, because good sludge reductions were obtained with high as well as with low values of this parameter. Thus, SRT cannot be used as a design parameter. On the other hand, low IR and high HRT in the ASSR allow high sludge reduction efficiency to achieve.

Many mechanisms have been proposed to justify the low sludge production of the OSA process. Nevertheless, up to now is not possible to define a unique reduction mechanism involved. The mechanism which has been proposed frequently is related to the sludge endogenous decay and the EPS destructuration mechanism. The role of slow growing microorganisms is also important, as the review of the literature indicates that some unique anaerobic microorganisms characterize the ASSR system rather than the conventional process.

The mechanism of sludge reduction in the OSA process is thus a synergic effect between the occurrences of sludge decay in the ASSR which correspondingly generates soluble COD, which is mainly depredated by slow growing microorganism.

The biological process that is carried out in the water line can affect the dominant mechanism of the sludge reduction. It has also to be taken into account that the sludge reduction obtained in studies carried out using a synthetic feed could be different, if a real wastewater is used as feed. Sludge generation could be increased by the present of inert compounds which accumulates in the system. Further investigations must be undertaken to completely understand the process and to define which mechanism could be dominant on the sludge reduction using real wastewater.

Further investigations are needed, where not only the sludge reduction is investigated and monitored but also the removal efficiency in terms of pollutants, such as nitrogen compounds, sulphate, orthophosphate.

# III. Chapter Simultaneous biological processes for sludge reduction in Anaerobic Side-Stream Reactor

This chapter was based on:

Ferrentino R., Langone M., Villa R., Andreottola G. (2016). *Simultaneous biological processes for sludge reduction in Anaerobic Side-Stream Reactor*. Submitted

# Simultaneous biological processes for sludge reduction in Anaerobic Side-Stream Reactor

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ABSTRACT

An anaerobic side-stream reactor (ASSR) was operated for 300 days coupled to a conventional activated sludge (CAS) to investigate the impact of SRT in the ASSR and the related sludge interchange ratio (IR) on the sludge reduction process. Different phases varying the SRT and the IR were carried out, revealing that an SRT in the ASSR of 2.5 d and an IR equal to 40% was the most suitable case in terms of sludge reduction, achieving a 66% of sludge reduction as compared to a CAS system.

An increasing release of ammonia, soluble COD and soluble EPS was detected in the ASSR increasing the amount of biomass cycled to the ASSR, showing the importance of the cell lysis process. The release of orthophosphate in the ASSR was also detectable, which was explained by the activity of the total phosphorous accumulating organism (TPAO). Specific batch tests demonstrated that increasing the biomass cycling in the ASSR, the percentage of denitrifying phosphorous accumulating organism (DPAO) over the aerobic phosphorous accumulating organism (PAO) increased. The activity of sulphate reducing bacteria (SRB) was also investigated. All the results observed led to a new explanation of the sludge reduction process achieved by inserting an ASSR in the returned sludge line of a CAS system. This study tried to connect the sludge decay, the cell lysis, the EPS destruction and the presence of slow growing microorganism in order to define a mechanism based on synergy and coexistence of all of them.

*Keywords:* anaerobic side-stream reactor (ASSR), sludge decay, cell lysis, EPS destruction, PAO, DPAO, SRB.

## 1. Introduction

The Conventional Activated Sludge (CAS) treatment is the most used biological process to remove both the organic matter and suspended solids present in municipal and industrial wastewaters. The process has been widely studied in order to enhance the nutrient and phosphorous removal too. Excess sludge production in CAS treatment has a relevant impact on operational cost in WWTPs. Therefore, sludge reduction in wastewater treatment plants is a challenging issue that stimulated the investigation of new technologies.

Up to date, a lot of techniques have been developed and studied for sludge reduction (Foladori et al. 2010) such as biological treatments (Wei et al. 2003; Semblante et al. 2014), thermal (Neyens and Baeyens 2003), thermochemical (Rocher et al. 1999), high temperature oxidation (Hii et al. 2013), mechanical treatments (Weemaes and Verstraete 1998), ultrasonication (Pilli et al. 2011), ozonation (Chu et al. 2009b) and chemical treatments (Liu 2003). However, these sludge reduction technologies are usually costly and could negatively affect the effluent quality of

the process due to the formation of by-products, too. Among the others, biological treatments are a challenging strategy for sludge reduction in WWTPs.

The inserting of an Anaerobic Side-Stream Reactor (ASSR) in the sludge return line of a CAS system, where a portion or, in some cases, all the activated sludge is recycled, is considered a promising biological approach to reduce the sludge production. The process consists of activated sludge cycling between aerobic, anaerobic and anoxic conditions. Over the last two decades, many studies demonstrated the process can reduce the sludge yield ($Y_{obs}$) by up to 40% (Chudoba et al., 1992), 55% (Chen et al., 2003; Saby et al., 2003) and 60% (Novak et al. 2007) as compared to a CAS process.

Chudoba et al. (1992) realized the first laboratory unit called Oxic Settling Anaerobic (OSA) process, composed by a modified activated sludge system with the anaerobic treatment of the whole returned sludge (CAS – OSA). The Authors showed that the periodic passage of facultative aerobic activated sludge microorganisms through the anaerobic zone created conditions of uncoupled growth, due to the anaerobic starvation, indicated by the ATP stock depletion and resulted in a consecutive reduction of the activated sludge production. Saby et al., (2003) showed the importance of the oxidation reduction potential (ORP) level in the anaerobic tank on the production of excess sludge of the system. The Authors reported that operating at an ORP level of – 250 mV could reduce the excess sludge by 51% as compared to a control CAS process. Chen et al. (2003) focused the attention on four possible reduction mechanisms of the sludge production such as the energy uncoupling, the domination of slow growing bacteria, the effect of soluble microbial products and the endogenous sludge decay. The Authors showed that the sludge decay mechanism in the ASSR might involve the reduction of the cell mass. Novak et al. (2007) showed that the CAS-ASSR system generated 60% less solids than the control CAS system, without any negative effect on the effluent quality or the settling characteristics of the activated sludge. The Authors also observed the release of protein in the ASSR and the release of calcium, magnesium, and polysaccharide in the aerobic Sequencing batch Reactor (SBR) in the water line, confirming the hypothesis of the digestion theory reported in his previous work (Novak et al. 2003).

Concerning the sludge reduction mechanisms underlying the process, as recently reviewed by Ferrentino et al. (2016), several explanations have been proposed such as the enhancing of the endogenous decay, the metabolic uncoupling, the feasting/fasting mechanism, the destruction of EPS (extracellular polymeric substances), a domination of slow-growing microorganisms and the predation of bacteria. However, the main mechanisms are not clearly identified.

Several studies reported the efficiency of CAS-ASSR process in carbon and nutrients removal. The percentage of COD removal in the CAS-ASSR system is often higher than that of the

conventional control process or at most equal. Concerning the nitrogen, most of the literature studies have shown that the CAS-OSA process does not have negative effects on the nitrogen removal (Ye et al. 2008; Datta et al. 2009; Troiani et al. 2011). Furthermore, the process efficiency on phosphorous removal has been studied by Datta et al. (2009) and Goel and Noguera (2006) but different results were obtained, requiring other investigations to clarify the effect of the CAS-ASSR process on phosphorous removal, which involves the activity of aerobic phosphorus accumulating organisms (PAO) and denitrifying phosphorus accumulating organisms (DPAO).

The aim of this paper is to present the results, in terms of observed sludge yield ($Y_{obs}$), of a lab scale plant implementation of an ASSR integrated in a SBR-OSA system treating a real urban wastewater. Three configurations were investigated: i) 10% sludge interchange rate and SRT in the ASSR of 10 days; ii) 20% sludge interchange rate and SRT in the ASSR of 5 days and iii) 40% sludge interchange rate and SRT in the ASSR of 2.5 days.

The main mechanisms involved in the sludge reduction were studied, focussing the attention on endogenous decay, destruction of extracellular polymeric substance (EPS) and domination of slow-growing microorganisms.

## 2. Materials and Methods

### *2.1. Experimental set-up and system operation*

The Figure 1 shows the lab-scale system. A SBR was used to simulate the water line. The SBR reactor was a cylindrical tank (190 mm in diameter and 430 mm in height without a cover) with a liquid volume of 10 L. It was made of Pyrex glass and equipped with a mechanical stirrer to ensure a complete mixing of the activated sludge. Air was supplied by a Schego M2K3 350 air pump through an air diffuser located at the bottom of the reactor. The reactor was equipped with a gas-flow to maintain the dissolved oxygen (DO) concentration, during the aeration phase, between 1-2 mg $O_2$/L. The oxygen concentration in the mixed sludge was continuously monitored with a CRISON DO probe. The reactor was also equipped with a set of three peristaltic pumps to introduce the influent, to discharge the effluent and to recycle the settled sludge.

A second cylindrical reactor was used as ASSR for treating the sewage sludge. It had the same dimensional characteristics of the SBR, covered with a plastic plate.

The cylinder and the plate were held together by stainless steel fasteners to ensure anaerobic conditions in the reactor. The ASSR was equipped with a mechanical stirrer and continuously mixed. No sludge discharge was present, thus the Sludge Retention Time (SRT) was equal to the Hydraulic Retention Time (HRT). The pH, ORP and temperature were monitored using two CRISON probes. The pH, ORP were left free to vary.

The system operated at room temperature. Before cycling the settled sludge to the ASSR, it was sent to a thickener of 2 L volume in order to increase the quantity of biomass in the ASSR. All the operational phases of the three units were automated using digital timers.

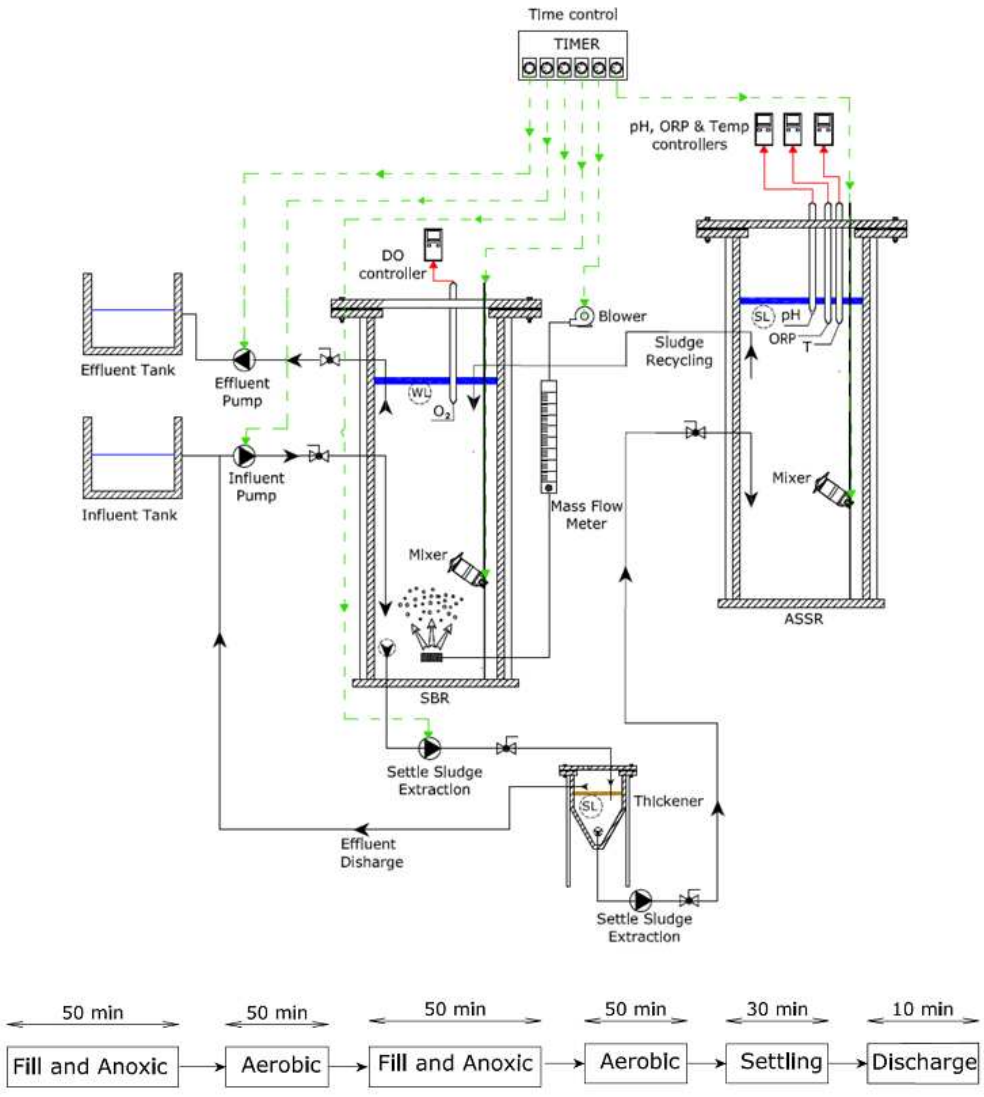



Fig. III.1 Schematic diagrams of the experimental setup and time sequence in each SBR cycle

The SBR operated with six cycles a day in alternate denitrification/nitrification mode. The cycle consisted in 3 h 20 min of reaction period, characterized by aerobic and anoxic phase, 30 min of settling and decanting period, 5 min of supernatant extraction and 5 min of settled sludge extraction. The SBR was fed at the beginning of each anoxic phase for 20 min. The daily influent flow was 18 L/d and the HRT was 0.56 d. Figure 1 outlines the distribution of the denitrification and nitrification periods during each phase.

The settled sludge of the SBR was cycled to the thickener that had a HRT of 3 h. The thickened sludge was finally cycled 6 times/day to the ASSR and an equal amount of biomass was sent back from the ASSR to the SBR.

The entire experimental period consisted of three different phases that lasted for about 90 days each. The experimental lab system was tested under three configurations: i) 10% sludge interchange rate and SRT in the ASSR of 10 days; ii) 20% sludge interchange rate and SRT in the ASSR of 5 days and iii) 40% sludge interchange rate and SRT in the ASSR of 2.5 days. The pH, ORP and temperature, during all the experimental period, ranged between 7±0.5, -400±25 mV and 20±2°C, respectively.

The pH, ORP and DO probes were cleaned and calibrated periodically. Tubes were replaced with new tubes periodically. Furthermore, solids loss from both reactors in terms of sampling or loss with the effluent was evaluated and considered during the biomass yield calculation.

### *2.2. Wastewater*

The system was fed with the primary effluent taken from the municipal wastewater treatment plant (WWTP) of Trento, Italy. The variation of some characteristics of the wastewater taken from Trento's WWTP during 2015 and used in this study is presented in Table 1.

Table III-1. Characteristics of the influent wastewater during the experimental study

| Parameter | Concentration (mg $L^{-1}$) | | |
|---|---|---|---|
| | Average | Max | Min |
| **Total COD** | 244 | 474 | 80 |
| **Soluble COD** | 101 | 252 | 20 |
| **N-$NH_4$** | 46 | 72 | 19 |
| **N-$NO_3$** | 1.18 | 5.4 | 0.1 |
| **P-$PO_4^{3-}$** | 3.6 | 6.0 | 0.8 |
| **S-$SO_4$** | 45 | 58.3 | 28.3 |
| **TSS** | 200 | 530 | 11 |
| **pH** | 7.4 | 8 | 6.9 |

### *2.3. Origin of biomasses*

The SBR was inoculated with the activated sludge (4 ± 0.5 g TSS $L^{-1}$) obtained from the aeration tank of Trento's WWTP, Italy, while the anaerobic sludge obtained from the sludge treatment anaerobic tank of the WWTP of Levico Terme, , Italy, was used as the source for inoculation of the ASSR. The main characteristics of the inoculums of the ASSR are reported in the Table 2.

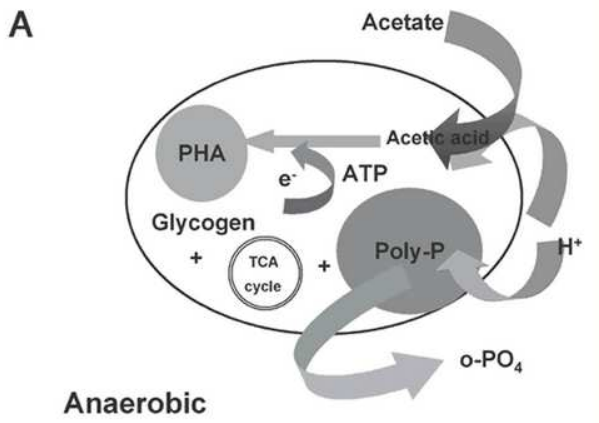
Table III-2. Characteristics of the inoculums of the ASSR

| Parameter | ASSR |
|---|---|
| **Total COD** | 7800 mg $L^{-1}$ |
| **Soluble COD** | 120 ± 15 mg $L^{-1}$ |
| **Ammonium Nitrogen** | 70 ± 10mg N-$NH_4$ $L^{-1}$ |
| **Nitrate** | 1.3 ± 0.5 mg N-$NO_3$ $L^{-1}$ |
| **Soluble phosphorous** | 13 ± 1 mg P-$PO_4^{3-}$ $L^{-1}$ |
| **Sulfate** | 11 ± 0.5 mg S-$SO_4$ $L^{-1}$ |
| **Total suspended solids (TSS)** | 5.5 ± 0.5 g $L^{-1}$ |
| **pH** | 7.7 |

*2.4. Batch tests procedure*

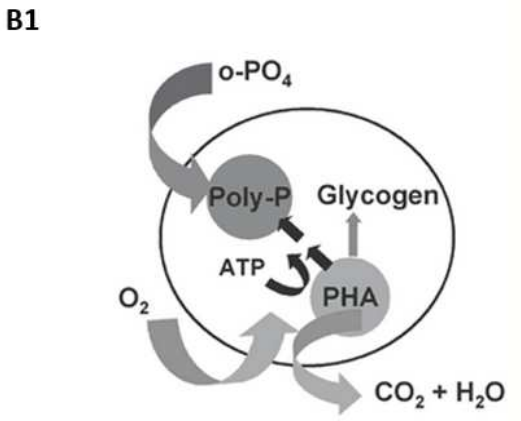
Phosphorus uptake batch tests (PUBT) were conducted for measuring the specific activity of total phosphorus accumulating organisms (TPAOs) and denitrifying phosphorus accumulating organisms (DPAOs) according to the procedure reported in Cyganecka et al. (2011). The main features of TPAOs and DPAOs are given in Figure 2. TPAOs and DPAOs are both phosphorus accumulating organisms. As DPAOs are facultative microorganisms, which use oxygen and nitrate/nitrite as electron acceptor, the TPAOs activity was evaluated under aerobic conditions. The activity of only DPAOs was evaluated under strictly anoxic conditions. During the aerobic and anoxic stage, both of them take up more phosphate from the mixed liquor compared to that anaerobically released, resulting in a net phosphate uptake from the bulk liquid.

The assays were performed in reactors with a total volume of 2L and a volume of sludge of 1.2L, each mixed with a magnetic stirred. The reactors were inoculated with anaerobic sludge from the ASSR. Concentration of the total suspended solid in the batch test reactor was the same of the ASSR sludge, approximately 8 gTSS/L. The temperature of operation was maintained at 20°C by means of a thermostatic jacket. The initial pH value was that of the sludge in the ASSR. The final pH value was always measured in order to check that it was maintained in the optimal range for the biological activity. The batch tests were sampled every 30 minutes. The samples were immediately vacuum filtered on 0.45 µm membrane filters and analyzed.



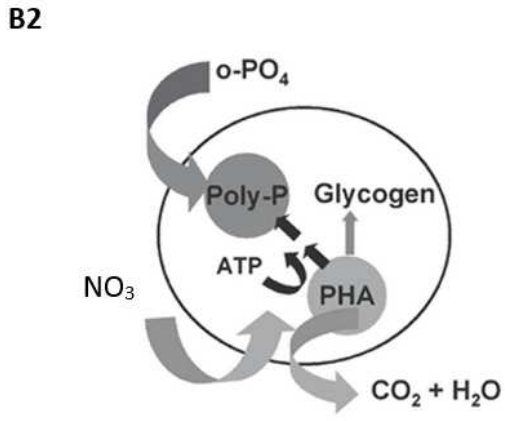

Under anaerobic conditions, TPAOs/DPAOs are thought to rapidly assimilate organic substrates (particularly volatile fatty acids—VFAs) and use these to synthesize poly-b-hydroxyalkanoates (PHA) using intracellularly stored polyphosphate (polyP) as an energy source. Thus, orthophosphates (o-PO4) generated from polyP degradation are released into the bulk liquid.

Electrons required for the synthesis of PHA were derived from the anaerobic operation of the TCA cycle (Wentzel et al. 1991), and from glycogen degradation (Mino et al. 1998), where glycogen is an intracellular storage compound synthesized in the aerobic phase by the TPAOs and DPAOs, respectively.




**Aerobic**

In the subsequent aerobic phase, in the absence of any organic compounds, organisms with stored PHAs are able to use these as carbon and energy sources to grow and to assimilate orthophosphates to synthesize polyP, using oxygen as electron acceptor. In others words, TPAOs take up orthophosphate to recover their intracellular polyP levels by oxidising the stored PHA.




**Anoxic**

At the subsequent anoxic phase, where no extracellular carbon is available, using nitrate or nitrite as electron acceptor, DPAOs degrade stored PHAs, produce glycogen and take up phosphate from the mixed liquor.

Fig. III.2 Summary of the major mechanisms of TPAOs and DPAOs during the anaerobic (A) and aerobic/anoxic (B1/B2) stages. Scheme adapted by Seviour et al., (2003)

### *2.4.1. Total PAOs activity assay*

The specific phosphorous uptake rate of TPAOs ($SPUR_{tot}$) was determined in a 2-stage batch assays, initially under strictly anaerobic conditions and then under aerobic conditions. The sludge was flushed with nitrogen gas for 10 min before use to maintain anaerobic condition in the reactors and the DO was continually monitored. Analysis were performed to ensure that no nitrate were present in the ASSR sludge. No carbon sources were added because already present in the ASSR due to cell lysis mechanisms. Aerobic conditions were ensured by sparging air through the bulk liquid using an aquarium air stones. The resulting DO concentration was higher than 5.5 mg $O_2$/L. The anaerobic phase was maintained in the reactor until a steady state constant concentration of $PO_4^{3-}$-P was attained in the liquid phase and lasted at least for 120 min. The aerobic phases was carried out until no further changes in concentration of $PO_4^{3-}$-P could be observed and lasted at least for 240 min.

Filtered samples were analyzed for $PO_4^{3-}$ - P. The specific TPAOs activity was calculated from the slope of the initial section of $PO_4^{3-}$ - P curves considered linear divided by the biomass concentration in the test.

### *2.4.2. DPAOs activity assay*

The specific phosphorous uptake rate of DPAOs ($SPUR_{DPAO}$) was determined in a 2-stage batch assays, initially under strictly anaerobic conditions and then under anoxic conditions. Anaerobic conditions were guaranteed as reported for the TPAOs activity assays. No carbon sources were added because already present in the ASSR due to cell lysis mechanisms. Anoxic conditions were attained by dosing a nitrate rich solution and monitoring the ORP values. The resulting nitrate concentration was 30 mg $NO_3^-$-N/L. The anaerobic phase was maintained in the reactor until a steady state constant concentration of $PO_4^{3-}$-P was attained in the liquid phase and lasted at least for 120 min. The anoxic phase was carried out until no further changes in concentration of $PO_4^{3-}$-P could be observed and lasted at least for 240 min. Filtered samples were analyzed for $PO_4^{3-}$ - P and $NO_3^-$-N. The specific DPAOs activity was calculated from the slopes of the initial section of $PO_4^{3-}$ - P and $NO_3^-$-N curves considered linear divided by the biomass concentration in the test.

### *2.4.3. DPAO/TPAO ratio*

According to Hu et al. (2003), the fraction of DPAO in TPAO ratio could be determined as the ratio between the total amount of phosphorous ($PO_4^{3-}$ - P/g TSS) taken up by the biomass during the anoxic and aerobic phase within a pre-defined time period. Thus, the percentages of DPAO was evaluated considering the following equation:

$$\%DPAO = \frac{v^{Panoxic}}{v^{Paerobic}} \qquad (1)$$

where $v^{Panoxic}$ and $v^{Paerocic}$ were the anoxic P-uptake rate and the aerobic P-uptake rate, respectively

*2.5. EPS extraction*

Two kinds of EPS were analysed during the present study, namely free and bound fraction. For free fraction (also referred soluble microbial products (SMP)), 200 mL of the ASSR sludge sample was centrifuged at 4000g and room temperature for 20 min, and then the supernatant was filtered through 0.45 µm membrane filters and analysed subsequently.

For a better characterization of bound fraction, combined EPS extractions were performed, following the procedure described by Park and Novak (2007). Both the cation exchange resin (CER), highly selective for $Ca^{2+}$ and $Mg^{2+}$ bound EPS, and the base extraction methods, simultaneously highly selective for Al-associated EPS and weakly selective for Fe-linked EPS, were applied. For CER extraction, the ASSR sludge sample was centrifuged at 4000g and room temperature for 20 min, and then sludge pellet was resuspended with a buffer solution (pH=7; 2 mM $Na_3PO_4 \cdot 12H_2O$; 4 mM $NaH_2PO_4 \cdot 2H_2O$; 9 mM NaCl; 1 mM KCl). Then, the cationic exchange resin extraction was carried out by dosing 70 g resin/g VSS and mixing the sample at 200 rpm for 2 hours at room temperature. A further centrifugation under the above-described conditions produced a supernatant that was filtered through 0.45 µm membrane filters; the filtrate was assumed to contain the bound fraction of EPS. For base extraction, the sludge pellet was resuspended with 10 mM NaCl solution and the pH was adjusted to 10.5 using a 1N NaOH and centrifuged for 1 h at 600 rpm at in the presence of $N_2$ gas. Then, the suspension was centrifuged at 12000 rpm for 15 min at 4°C and filtered through 0.45 µm membrane filters.

Both free and bound forms of EPS were analysed in the spectrophotometer for determining polysaccharide and protein contents. The polysaccharide concentration in the extracted EPS was measured according to DuBois et al. (1956), using glucose as standard. Proteins were measured according to the Frolund et al., (1995) method, utilizing bovine serum albumin as reference protein.

*2.6. Estimation of the observed sludge yield and solid retention time*

As suggested by Chon et al. (2011b), in order to determinate the sludge yield and the SRT, the SBR and the ASSR were considered as a single control unit. The $Y_{obs}$ is the amount of sludge generated per the amount of substrate removed, and could be calculated by the ratio of the

cumulative generated sludge and the cumulative consumed substrate. The cumulative generated sludge consists of the increase in sludge in the control volume itself $\left(\Delta X_{SBR}V_{SBR} + \Delta X_{ASSR}V_{ASSR}\right)$ and cumulative sludge wastage from SBR, ASSR and effluent, including sludge samples collected for analytical determination $\left(\sum (X_{SBR}Q_{SBR,wasste} + X_{ASSR}Q_{ASSR,waste} + X_{eff}Q_{eff}) \cdot \Delta t\right)$.

The cumulative consumed substrate, usually expressed as COD, is evaluated as the difference between the mass of substrate influent and the mass of substrate effluent $\left(\sum (S_{\inf}Q_{\inf} - S_{eff}Q_{eff}) \cdot \Delta t\right)$ (Chon et al. 2011b). Thus, the $Y_{obs}$ in the specific given time can be determined by Eq. (2):

$$Y_{obs} = \frac{\Delta X_{SBR}V_{SBR} + \Delta X_{ASSR}V_{ASSR} + \sum (X_{SBR}Q_{SBR,wasste} + X_{ASSR}Q_{ASSR,waste} + X_{eff}Q_{eff}) \cdot \Delta t}{\sum (S_{\inf}Q_{\inf} - S_{eff}Q_{eff}) \cdot \Delta t} \quad (2)$$

where $S_{inf}$ and $S_{eff}$ are the substrate COD concentration of influent and effluent (mg $L^{-1}$); $Q_{inf}$ and $Q_{eff}$ are the flow rate of the influent and effluent (L $d^{-1}$); $\Delta X_{SBR}$, $\Delta X_{ASSR}$ are the change of sludge (g TSS $L^{-1}$) in SBR and ASSR; $V_{SBR}$, $V_{ASSR}$ are the volumes of SBR and ASSR (L); and $\Delta t$ is the time (d). The $Y_{obs}$ can be graphically evaluated as the slope of the linear regression curve obtained plotting the cumulative sludge generation data against the cumulative consumed substrate data obtained by experimental measurements.

The SRT of the whole system could be defined as the ratio of the total mass of sludge inside the system and the mass rate of sludge leaving out of the system (Eq. 3)

$$SRT = \frac{X_{SBR}V_{SBR} + X_{ASSR}V_{ASSR}}{X_{SBR}Q_{SBR,waste} + X_{ASSR}Q_{ASSR,waste} + X_{eff}Q_{eff}} \quad (3)$$

where $X_{SBR}$ and $X_{ASSR}$ are the sludge concentration in the SBR and in the ASSR (g TSS $L^{-1}$); $V_{SBR}$, $V_{ASSR}$ are the volumes of SBR and ASSR (L); $Q_{SBR,waste}$, $Q_{ASSR,waste\ and}$ $Q_{eff}$ are the flow rate of wastage from SBR, wastage from ASSR and effluent (L $d^{-1}$), respectively.

*2.7. Chemical analysis*

Total suspended solids (TSS), total chemical oxygen demand (COD) were measured according to the Standard Methods (APHA, 2005). The samples were filtered through 0.45 µm membrane filters. The filtrate was analyzed Soluble COD (sCOD), ammonium nitrogen ($N\text{-}NH_4$), nitrate as nitrogen ($N\text{-}NO_3^-$), nitrite as nitrogen ($N\text{-}NO_2$), soluble phosphorous ($P\text{-}PO_4^{3-}$) and Sulfate ($S\ O_4^{2-}$). $N\text{-}NH_4$, $N\text{-}NO_2$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ concentrations were determinate according to APAT CNR IRSA, 2003. Sulfate was analyzed by ion chromatograph (DIONEX ICS-100) equipped with AS9-HC column.

## 3. Results and Discussion

### *3.1 Observed sludge yield and solid retention time*

The $Y_{obs}$ of the SBR-ASSR was determined as the slope of the linear regression curve plotting the cumulative sludge generation data against the cumulative substrate consumption data, based on the Equation (2). The $Y_{obs}$ of the SBR-ASSR under different percentage of interchange rate (volumetric rate) cycled in the ASSR, 10%, 20% and 40% during Phase I, II and III respectively, was evaluated (Fig. 3). The $Y_{obs}$ during Phase I, II and III were 0.21 g TSS/g COD, 0.14 g TSS/g COD and 0.12 g TSS/g COD, respectively. To assess the efficiency of the SBR-ASSR system in terms of sludge reduction, the $Y_{obs}$ values obtained in this study have to be compared to the $Y_{obs}$ of a conventional activated process.

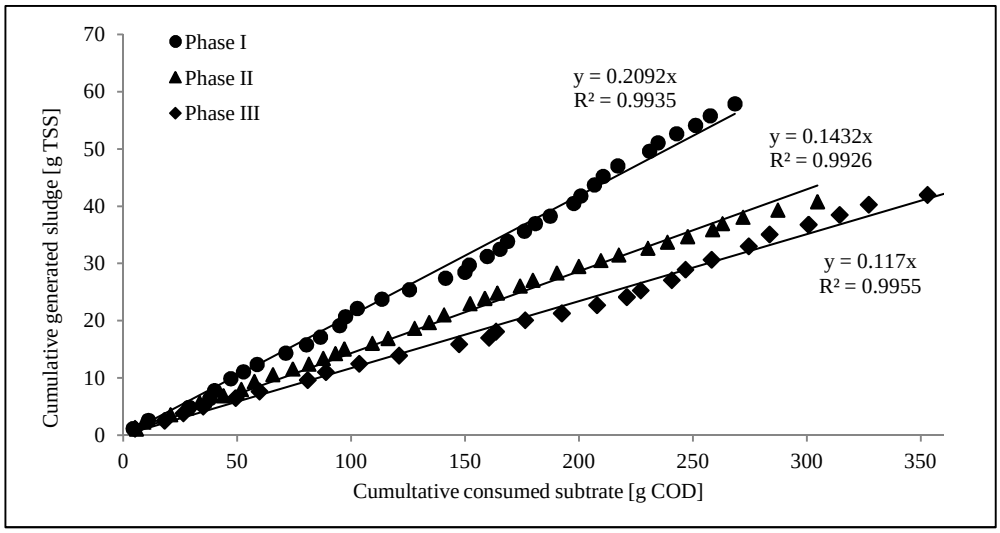



Fig. III.3 Observed sludge yield in phase I, II and III

Thus, a CAS system having as influent wastewater and inoculums the same characteristics of those used for the experimental application, was simulated with the ASM1 Model (WEST® simulation). The $Y_{obs}$ of the reference system was found to be 0.36 g TSS/ g COD. Compared to this value, the $Y_{obs}$ of the SBR-ASSR system was clearly reduced at each IR level. For instance, increasing the IR from 10%, to 20% up to 40%, the sludge yield was 42%, 61% and 66% less than the CAS reference system.

Thus, the best result was obtained when the IR was equal to 40% and the SRT equal to 2.5 d. During all the experimental study there was no sludge withdrawal. The only portion of sludge wasted was due to the amount of sludge taken for analysis, which had been considered in the determination of the $Y_{obs}$. Results obtained were compared to those reported in literature. The sludge reduction obtained during Phase I, performed with an IR of 10% and 10 d of HRT in the ASSR, was quite similar to those reported in previous studies. The study performed by Chon and

Park (2012) showed 43% of sludge reduction treating urban wastewater. Chon et al. (2011a), Goel and Noguera (2006) and Sun et al. (2010) obtained similar percentage, 49%, 37% and 53%, respectively, treating synthetic wastewaters. While no studies had been performed with an IR equal to 20% and 5 d of HRT in the ASSR (Phase II), the last phase (IR= 40% and $HRT_{ASSR}$=2.5 d) could be compared to the study of Chon and Park, (2012). The Authors performed the ASSR process with 2.5 d HRT in the ASSR and 10% of IR. A sludge reduction of 61% was obtained increasing the temperature from 21 °C to 37°C. The high sludge reduction was attribute to the high temperature. In our study the ASSR operated at 20°C. The obtained high sludge reduction was related to the high percentage of biomass cycled to the ASSR, that could enhance the biological activity as much as an increase of the temperature.

The SRTs in the three Phases that were determined using Equation (3). The SRT of Phase I, II and III were found to be 47, 60 and 83 days, respectively. These values are quite high because in all phases there was not a regular wastage. The system was operated with minimized solids wastage except for sampling.

### *3.2 Sludge decay: cryptic growth and organic matter accumulation*

As recently reviewed by Ferrentino et al. (2016), the sludge decay is one of the sludge reduction mechanisms that has been quite frequently proposed in the ASSR processes . Sludge decay could be defined as the solubilisation of cellular constituents of microorganisms. Low molecular weight compounds are released into the liquid causing an increase in the concentration of organic matter and nutrients in the supernatant (organic matter accumulation). Furthermore, the released cellular constituents are used by microorganisms for their metabolic function (cryptic growth) (Mason et al. 1986; Chu et al. 2009a). To test this hypothesis, the concentrations of ammonium nitrogen (Fig. 4a) and soluble COD (Fig. 4b) of the influent and the effluent of the ASSR were monitored during all the experimental study.

In our study, the concentration of the influent and effluent $NH_4^+$-N in the ASSR reactor was monitored. $NH_4^+$-N is a conservative parameter in anaerobic conditions, thus, the $NH_4^+$-N released from the solubilisation of organic nitrogen will be accumulated in the anaerobic reactor and measured in its effluent. Figure 4a shows an increasing solubilisation of organic nitrogen to $NH_4^+$-N during the entire period of the research with increasing of the IR from 10% to 40%. In Phase I starting from a $NH_4^+$-N concentration in the influent of about 10 mg $NH_4^+$-N/L, a value of 50 mg $NH_4^+$-N/L had been reached at the end of Phase I. During Phase II, the effluent concentration of $NH_4^+$-N increased again up to 70mg $NH_4^+$-N/L. A same increasing trend was repeated in Phase III, reaching a value of 90 mg $NH_4^+$-N/L.

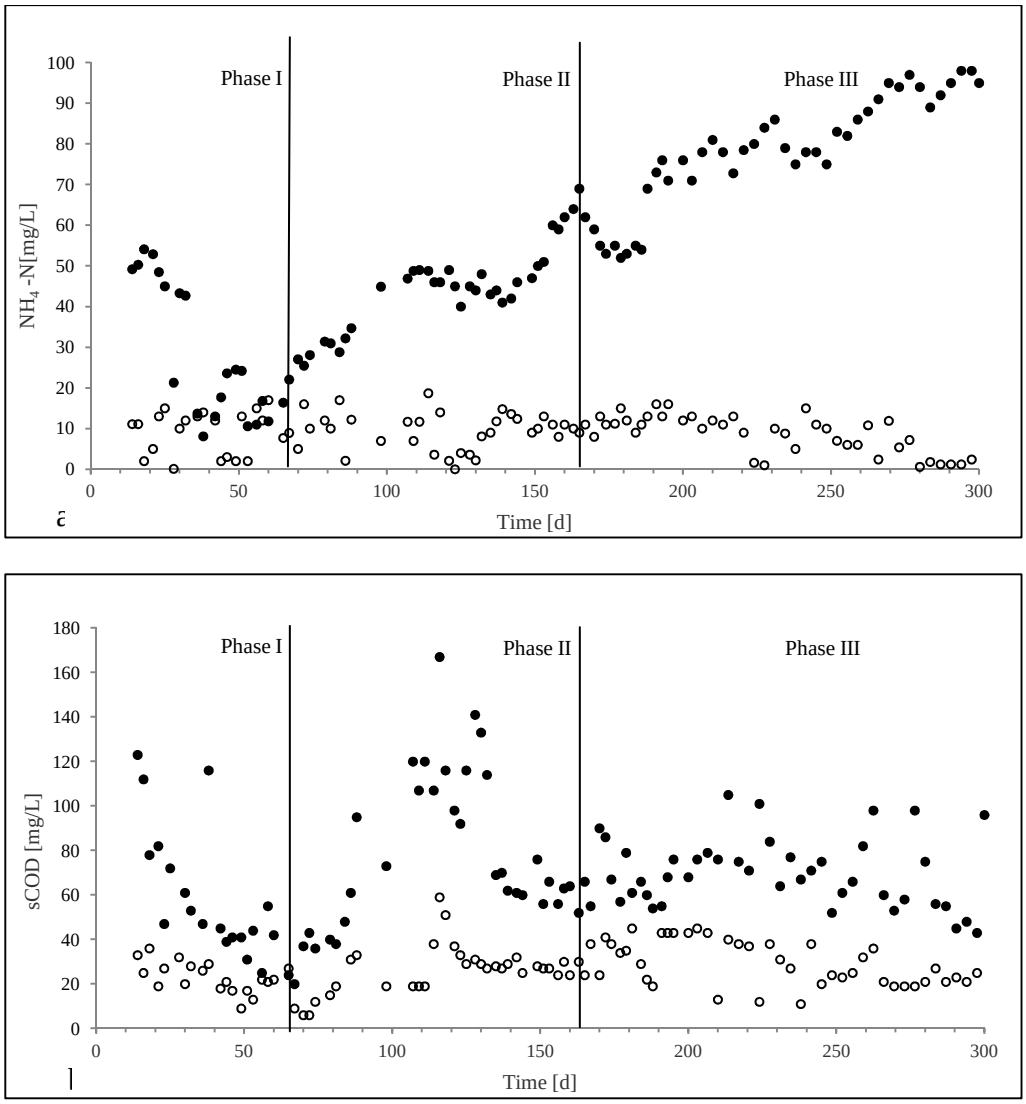



Fig. III.4 Profile of a) $NH_4$-N and b) soluble COD during the experimental study. The open and solid symbols represent the influent and effluent values of the ASSR, respectively.

Concerning the sCOD profile, it is not possible to observe a net and clear increase of solubilisation from phase I to III (Fig. 4b). It is important to underline that the released sCOD could be both used by several microorganisms for their metabolic functions (cryptic growth) and accumulated by some particular microorganisms which are continuously subject to aerobic/anaerobic conditions, such as TPAOs and DPAOs. Those processes could lead to underestimate the COD solubilisation evaluated by mean of the sCOD analysis.

The COD solubilisation measured was maximum at the beginning of each phase. In the ASSR, the average influent concentrations of soluble COD during Phase I, II and III were 21, 31 and 28 mg sCOD/L, respectively, that reached an average effluent value of 55, 80 and 71 mg sCOD/L. Even if it is no possible to observe a net increase of solubilisation from phase I to III, in Phase II and III a slowly higher solubilisation of COD has been achieved.

Both the profile of sCOD and $NH_4$ confirmed that under anaerobic conditions in the ASSR the cell lysis mechanism occurred. Sludge could be hydrolyzed, enhancing the solubilisation and disintegration of the organic matter and nutrients. Biodegradable compounds, released in the ASSR, are substrate available for degradation, both in the ASSR and in the water line where aerobic and anoxic condition are carried out, enhancing the cryptic growth. The presence of the ASSR integrated in the sludge recycle line allows a sludge cycling under anaerobic/anoxic/aerobic conditions. These mechanisms could enhance the sludge decay and cell lysis, as also reported by several studies, such as Wang et al. (2008), Chon et al. (2011), Novak et al. (2003), contributing to sludge reduction process

*3.3 Quantitative analysis of EPS*

The increase of $NH_4^+$-N and sCOD could be associated to the release of proteins and polysaccharide, which are the major components of EPS, and could be used by bacteria as source of carbon and energy for cell growth (Sheng et al. 2010). In our study, both the free-EPS (SMP) and the bound – EPS (attached to the sludge flocs) had been analysed for proteins and polysaccharides at the end of each phases.

Concerning the concentration of SMP, our results showed a strong increasing concentration of both protein (Fig. 6 a) and polysaccharides (Fig. 6 b), passing from Phase I to Phase III, possibly because of the higher IR in the ASSR. This evidence is consistent with the increase of $NH_4$-N and $_S$COD concentrations detected in the ASSR effluent. These data support the observation already reported in other studies (Novak el al 2007), that one of the mechanisms for solids reduction by the ASSR is the release of protein and polysaccharides matter in liquid bulk of the anaerobic bioreactor. In all the three Phases, the EPS of ASSR sludge contained more proteins than polysaccharides similar to previous work on ASSR (Chon et al. 2011a).

However, as expected, the bound EPS in the ASSR, both CER and BASE extracted, decreased from Phase I to III with the increasing of SMP concentration in the bulk liquid from Phase I to III. The increasing percentage of biomass cycled to the ASSR caused the bound EPS destruction (Fig. 5).

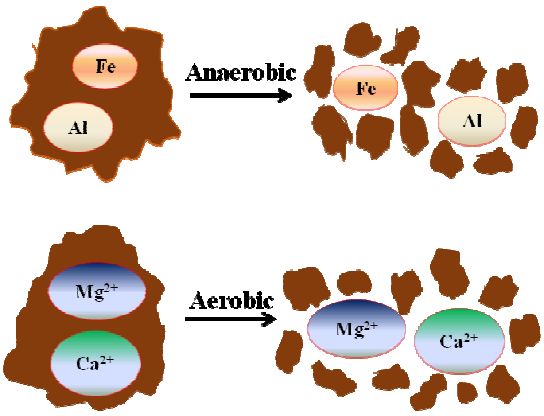



Fig. III.5 EPS destruction –The destruction of Fe-bound and Al-bound floc and Ca2+ and Mg2+ - bound floc mechanisms as reported in literature.

According to Novak et al. (2003), different extracellular biopolymer fractions exist in floc: (a) lectin-like proteins that are linked to polysaccharides and bridged by $Ca^{2+}$ and Mg $^{2+}$, (b) biopolymers that are bound to Fe and Al. Cations function as a "bridge" that connects negatively-charged EPS and cells. In this study, CER and Base extraction were employed to target and extract the divalent cations-bound EPS and aluminium and/or iron-bound EPS, respectively. CER mainly removes divalent cation ($Ca^{2+}$ and $Mg^{2+}$) from sludge and extracts divalent cation-associated EPS, while base extraction hydrolyzes a large amount of aluminium and iron, releasing aluminium and iron-bound floc materials (Chon et al. 2011a). The concentrations of proteins and polysaccharides extracted from each phase are shown in Figure 6.

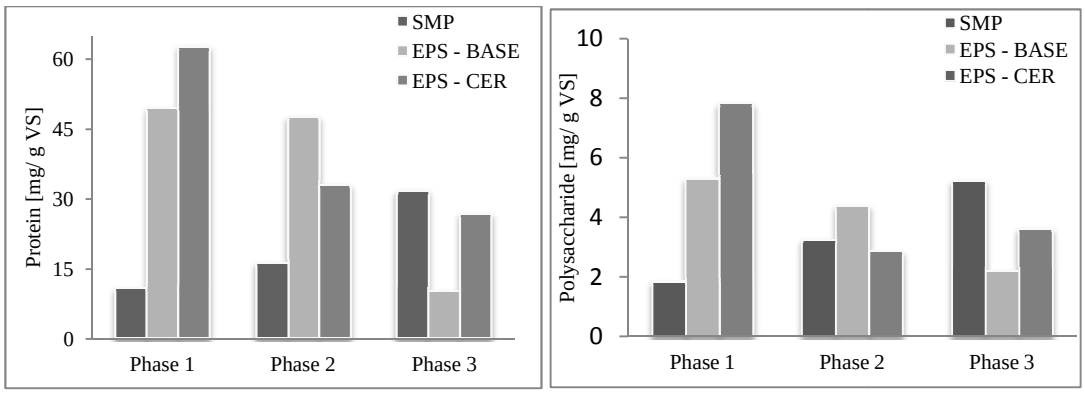



Fig. III.6 a) Proteins and b) Polysaccharides extracted by CER and Base methods for each experimental phase

During Phase I, proteins and polysaccharides are closely linked within the sludge floc, as can be seen from the high values of Base- and CER-extractable EPS. In Phase II, the amount of proteins EPS-Base extracted is quite similar to that of the previous Phase I, while the EPS-CER decreased.

Thus, the increase in the amount of SMP in Phase II could be associated to a decrease of EPS-CER and, consequently to the release of divalent cations, $Ca^{2+}$ and $Mg^{2+}$, in solution. Concerning the amount of polysaccharides in Phase II, a decrease in both EPS-Base and EPS-CER extracted is shown. However, the decrease of the EPS-CER is still higher than the EPS-Base, confirming that the release of divalent cations is the main EPS destruction mechanisms in Phase II (IR=20% in the ASSR) as compared to the release of Fe-and Al-associated EPS. This finding is in contrast with literature, where usually this EPS destruction mechanism has been observed in aerobic digestion, rather than anaerobic digestion.

On the contrary, during Phase III, when the maximum IR was applied to the ASSR, while the EPS-CER was almost similar to that of the previous Phase II, the EPS – Base decreased, achieving the lowest proteins and polysaccharides concentration in the flocs. Thus, in Phase III, the iron and aluminum associated materials mainly accounted for the increase of SMP. This means that the degradation of iron and/or aluminium associated materials could be somehow predominant than the release of divalent cations in Phase III. Similar results had been achieved by Chon et al. (2011a), who showed that degradation of base-extractable EPS accounts for the lower sludge yield in AS+ASSR.

### *3.4 The influence of slow growing bacteria*

Several literature studies reported that sludge cycling between different environmental conditions could enhance the selection of slow growing microorganism. The aim of this paragraph is to focus the attention on phosphate accumulating organisms (TPAO and DPAO) and sulphate reducing bacteria (SRB).

#### *3.4.1. Phosphate accumulating organisms (TPAO)*

The $PO^{3-}_4$ –P concentrations in the influent and effluent of the ASSR were monitored (Fig. 7). Results showed a gradually increasing of the phosphorus concentration in the ASSR effluent. The average influent concentrations during Phase I, II and III were 2.7, 3.3, 2.0 P- $PO^{3-}_4$/L, respectively. The effluent concentrations reached values of 4.2, 15.4, 32.8 P- $PO^{3-}_4$/L.

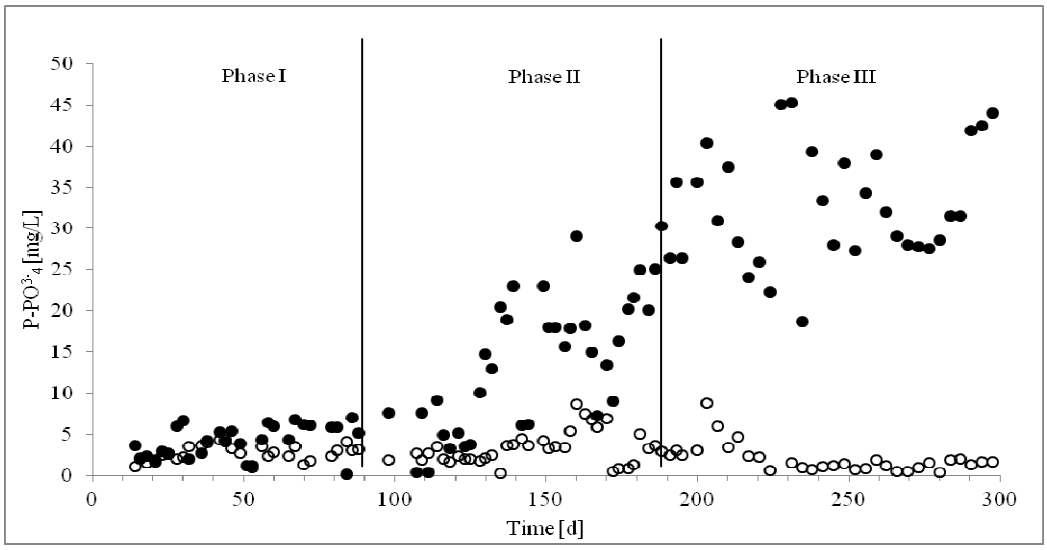



Fig. III.7 Profile of P-PO3-4 removal. The open and solid symbols represent the influent and effluent values, respectively.

As data shows, during the three experimental phases the release of phosphate slowly increased. This evidence suggests that the increasing percentage of biomass cycled thought the ASSR could enhance a microbial activity causing the selection of phosphate accumulating organisms. These bacteria are able to accumulate polyphosphates (Poly-P) under aerobic and anoxic conditions and release them under anaerobic condition. The presence of Poly-P bacteria in configurations that involved an ASSR has been noted in previous studies (Chudoba et al. 1992; Goel and Noguera 2006). In a CAS-OSA system Chudoba et al. (1992) reported that Poly-P bacteria were 50-60% of the total bacteria population. Results reported also an increase of orthophosphates concentration in the biomass and an enhance in phosphorus removal. Goel and Noguera (2006), performing an EBPR-SBR system also noted an enrichment of slow growing bacteria such as PAO and fermenters due to sludge cycling under aerobic and anaerobic conditions.

Due to the cycling between aerobic, anoxic and anaerobic conditions in the SBR-ASSR carried out in our study, both the presence of TPAO and DPAO had been supposed. Thus, the increasing microbial activity of TPAO and DPAO during all the experimental study was evaluated with batch experiments performed at the end of each phase.

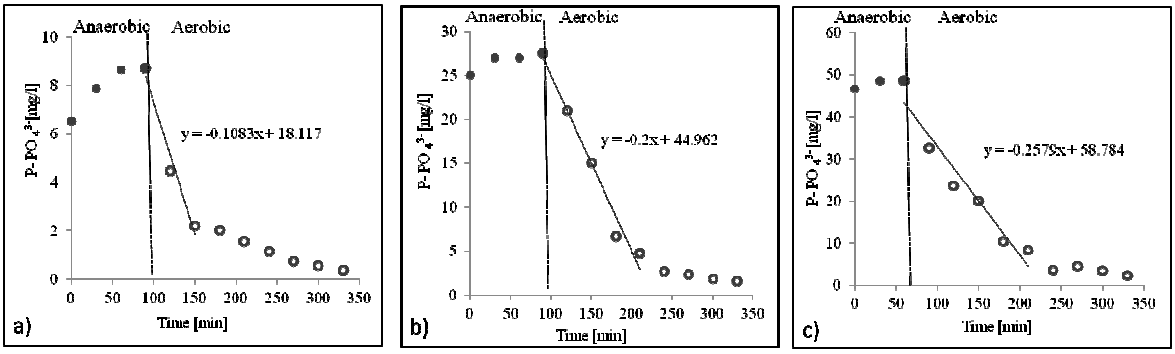



Fig. III.8 Batch test under anaerobic-aerobic condition a) Phase I; b) Phase II and c) Phase III. The close and open symbols are the P-PO43-concentration during anaerobic and aerobic phase, respectively.

Figure 8 shows the batch test results performed in order to evaluate the specific phosphorous uptake rate ($SPUR_{tot}$) of TPAO. Before to determine the $SPUR_{tot}$, phosphate was induced to be released under anaerobic condition for about 2 hr. Results showed that $SPUR_{tot}$ increased from an initial value of 0.77 mg $PO_4^{3-}$-P/ (g TSS h) in Phase I to 1.41 mg $PO_4^{3-}$-P/ (g TSS h) in Phase II and up to 1.75 mg $PO_4^{3-}$-P/ (g TSS h) in Phase III. This results confirmed the growing activity and the selection of TPAO in the ASSR process.

In our study, we further evaluated the specific phosphorous uptake rate ($SPUR_{DPAO}$) of DPAO, performing batch tests at the end of each phases. As in the $SPUR_{tot}$ tests, before determining the $SPUR_{DPAO}$ phosphate was induced to be released under anaerobic condition. According to Lee and Yun (2014), the major advantage of DPAO is the anoxic reduction of nitrate without the additional of external carbon energy for denitrification.

In our study, the ability of the ASSR to select DPAO was confirmed. The $SPUR_{DPAO}$ increased from 0.11 mg $PO_4^{3-}$-P/ (g TSS h) in Phase I, to 0.89 mg $PO_4^{3-}$-P / (g TSS h) in Phase II and to 1.64 mg $PO_4^{3-}$-P / (g TSS h) in Phase III. In terms of nitrate, the specific denitrification rate (SDNR) of DPAO ranges between 0.34 mg $NO_3^-$-N/ (g TSS h) in Phase I to 0.62 mg $NO_3^-$-N / (g TSS h) in Phase III.

Figure 9 shows the results of the $SPUR_{DPAO}$ batch test. It is clearly obvious that in anoxic conditions the decrease of phosphate concentration is closely related to the presence of nitrate in the solution. Moreover, when all the phosphate is almost removed, the nitrate concentration did not decrease further. During each test, sCOD was evaluated and no differences between the beginning and the end of each test was found (data not show) confirming that nitrate reduction could be ascribed to DPAO activity.

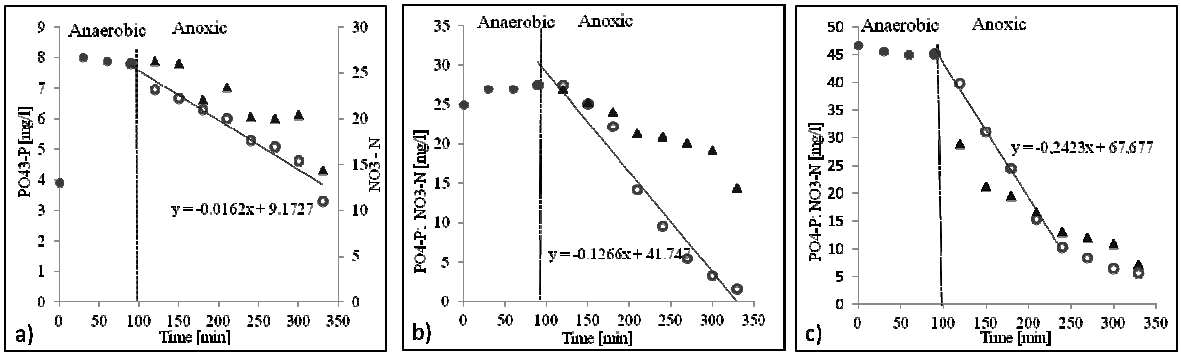



Fig. III.9 Batch test under anaerobic-anoxic condition a) Phase I; b) Phase II and c) Phase III. The close and open circles are P-PO43- concentration during the anaerobic and anoxic phase, respectively; the triangle represent the nitrate concentration.

Data collected by aerobic and anoxic batch tests allowed the percentage of DPAO over the TPAO to be calculated following the Equation (1). Table 3 indicates that at the beginning of the experimental study, the DPAO accounted for only 15% of the TPAO, thus the IR equal to 10% did not affect significantly their selection. During Phase II the percentage of DPAO increased up to 63%. In Phase III the fraction of DPAO present in the sludge was approximately equal to 94% of the TPAO.

Table III-3. Fraction of DPAO and PAO during batch tests

| | | Aerobic conditions | | Anoxic conditions | | |
|---|---|---|---|---|---|---|
| | **TSS [g/L]** | **Direction parameter** | **P uptake rate [mg P/g TSS h]** | **Direction parameter** | **P uptake rate [mg P/g TSS h]** | **DPAO [%]** |
| **Phase I** | 8.48 | -0.1083 | 0.013 | -0.0162 | 0.002 | 15 |
| **Phase II** | 8.52 | -0.2000 | 0.020 | -0.1266 | 0.015 | 63 |
| **Phase III** | 8.86 | -0.2579 | 0.031 | -0.2423 | 0.027 | 94 |

While the biomass yield of TPAO is 0.45 gVSS/g COD (Henze et al. 2008), the growth yield of DPAO is about 70% lower than that of PAO (Hu et al. 2002), reaching 0.13 gVSS/g COD. Thus, in our study, the selection of DPAO over the TPAO obtained by increasing the biomass cycling in the ASSR, justified the low sludge production measured in Phase III.

### *3.4.2. Sulphate reducing bacteria (SRB)*

The influent and effluent $SO^{2-}_4$–S concentrations of the ASSR were also monitored (Fig. 10). The average sulphate influent during Phase I, II, and III were 40.9, 45.8 and 48.2 mg $SO^{2-}_4$-S/L, respectively. The effluent concentrations decreased, achieving value of 28.3, 8.6 and 9.8 mg $SO^{2-}_4$-S /L

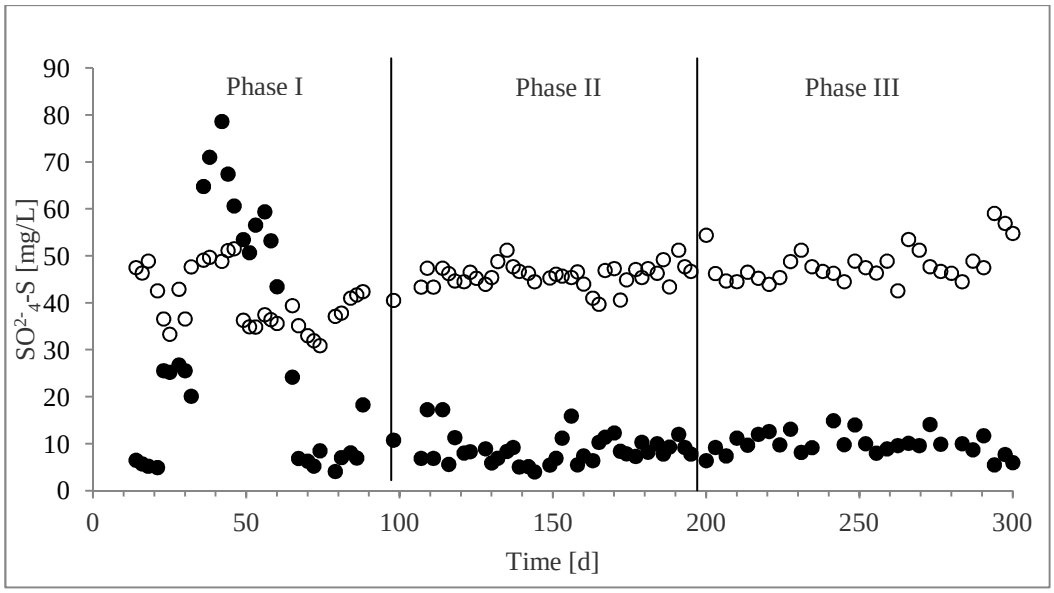



Fig. III.10 Profile of $SO_2^{-4}$-S removal. The solid and open symbols represent the influent and effluent values, respectively.

As Figure 10 shows, the reduction of the sulphate concentration in the ASSR is quite stable during all phases, achieved up to 80% sulfate removal efficiency. However, almost in correspondence to day 40, sulphate in the ASSR was no more removed, because of an accumulation of nitrate in the water line (average value 20 mg $NO_3$-N/l) and thus in the sludge cycled in the ASSR from the SBR. The biomass with a high nitrate concentration cycled to the ASSR inhibited the activity of sulphate reducing bacteria (SRB), because the anaerobic environment changed in anoxic. When this problem was solved, the sulphate reduction restarted again. In a CAS-ASSR configuration, the profile of sulphate concentration could be considered as a marker both of the microbiological activity carried out by SRB and of the performance of the water line. These microorganisms obtain energy for growth by oxidation of organic substrates, by removing hydrogen atoms from organic molecule, and by using sulphate, naturally present in urban wastewaters, as the terminal electron acceptor (Hao et al. 1996). In the presence of sulphate some SRB may break down volatile fatty acids (VFA) incompletely to acetate (Lens et al. 1998). Their role could be extremely important for other microorganism such as TPAOs and DPAOs that could use acetate for their metabolic functions.

The growth yield of SRB is very low, about 0.2 g VSS/g reduced sulphate or 0.1 g VSS/g COD removed (Kleerebezem and Mendez, 2002; van den Bosch et al., 2007), leading to a low sludge yield. Furthermore, it has been reported that the sulphide produced by SRB can led to a change of sludge floc structure due to specific reduction of $Fe^{3+}$ to FeS, contributing to cell lysis mechanisms and dissolved extracellular polymeric substances (EPS) release (Nielsen and Keiding 1998).

SRB has been widely studied for the treatment and bioremediation of acid mine drainage (AMD) (Bai et al. 2013). However, the development of a recently innovative process, called SANI® (Sulfate reduction, Autotrophic denitrification and Nitrification Integrated), has opened the possibility of using SRB to remove organic matter and to realize the autotrophic denitrification using the dissolved sulphide generated from sulfate reduction. This process lead to low energy consumption, small oxygen demand for COD removal and zero sludge withdrawal (Lu et al. 2009).

## 4. Conclusions

The use of an ASSR involved in the returned sludge line of a CAS system has been widely studied in literature. All the reduction mechanisms investigated in our study have already been presented in previously. The novelty of this paper is to propose a new sludge reduction mechanism which connects all the mechanisms proposed so far as expressed as follow.

By acting on the operative parameters IR and the related SRT of the ASSR, the solubilisation of organic matter could be enhanced and a particular microbial community structure could be selected. Some microorganisms, such as SRB, improve the sludge disintegration thanks to the sulphide production. They further degrade the VFA incompletely to acetate that could taken up and internally stored by TPAO and DPAO in anaerobic conditions releasing orto-phosphate. The internally stored acetate can form long chain carbons molecules of PHA that could be used for TPAO and DPAO energy generation and for the growth of new cell.

Other heterotrophic microorganisms are not able to utilize the acetate because of the absence of an external electron acceptor donor (nitrate or oxygen). Thus adequate environmental conditions of the ASSR has to be ensured. Furthermore, the overall SRT of the system has to be high enough to ensure the selection and growth of such microorganism. Results of the present study show that the DPAO population could significantly increase over the TPAO, reaching half of the TPAO population. When the sludge from ASSR is recirculated to the CAS system, the DPAO contribute to the denitrification process, with low specific growth yield as compared to the heterotrophic microorganisms. One of main point of the process is the selection of DPAO and SRB due to their very slow growth yield, obtaining a substantial reduction in the sludge production. This mechanism is however related to the cell lysis and EPS destructuration. We concluded that it is not possible to define a unique sludge reduction mechanism as tried to do so far because several mechanisms are involved and are all linked so closely each other in order to reach the final aim.

# IV. Chapter

# Shift in microbial community structure of anaerobic side-stream reactor in response in changes to solid retention time and interchange ratio

This chapter was based on:

Ferrentino R., Langone M., Gandolfi I., Bertoldini V., Franzetti A., Andreottola G., 2016. *Shift in microbial community structure of anaerobic side-stream reactor in response in changes to solid retention time and interchange ratio.* Submitted

# Shift in microbial community structure of anaerobic side-stream reactor in response in changes to solid retention time and interchange ratio

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ABSTRACT

A laboratory scale sequencing batch reactor (SBR) - anaerobic side-stream reactor (ASSR) system was operated for 300 days under three different values of anaerobic solid retention time ($SRT_{ASSR}$) and interchange ratio (IR) to investigate carbon and nutrient removal efficiencies and sludge reduction as compared with the microbial community structure of the ASSR. Under each experimental phase, the SBR-ASSR system was effective in the removal of COD, ammonia nitrogen and phosphorous. The best carbon and nutrient removal efficiencies were obtained under the last experimental phase when the $SRT_{ASSR}$ was 2.5 days and IR equal to 40% (Phase III). In this phase, the highest reduction of sludge production was also observed (66%). Quantitative PCR analyses encoding 16 rRNA gene revealed a wide diversity of phylogenetic groups in each phase. However, an increasing selection of fermenting bacteria able to release EPS, denitrifying phosphate accumulating bacteria (DPAOs) and heterotrophic denitrifying bacteria was observed from Phase I to Phase III. Further, specific qPCR analyses targeted *apsA* gene showed an increase of sulphate reducing bacteria (SRBs) in Phase III. The total number of Archaea was almost the same for each experimental Phase. However, a shift from hydrogenotrophic methanogens to methylotrophic and acetoclastic methanogens was detected.

*Keywords:* Anaerobic side-stream reactor, microbial community, removal efficiency, q-PCR, taxonomic identification

## 1. Introduction

The more stringent regulatory limits imposed to guarantee high environmental standards caused an inevitable increasing of the production of excess sludge. The EU, the USA, and China each produce 6–11 million tons of sludge as dry solids per year; Australia produces 0.3 million tons each year (Semblante et al. 2016). The handling, treatment and disposal of sewage sludge are challenging waste management problems common to many countries that could account for 25 - 65% of the total plant operating costs (Chon et al., 2011). In this view, the development of technologies able to reduce the sludge production within the activated sludge (AS) process inspired an overall rising interest, representing nowadays one of the first priorities in waste management hierarchy. Among all, the insertion of an anaerobic side-stream reactor (ASSR) in the return sludge line of a conventional activated process (CAS) could significantly enhance the sludge reduction. Over the last two decades, many studies demonstrated that the process can

reduce the sludge yield ($Y_{obs}$) by up to 40% (Chudoba et al., 1992), 55% (Chen et al. 2003; Saby et al. 2003), 60% (Novak et al. 2007), 66% (Ferrentino et al. 2016c) as compared to a CAS process.

In spite of the abundant literature on sludge reduction, the complexity of the processes and mechanisms leading to sludge reduction has not yet been fully understood. As reviewed by (Ferrentino et al. 2016b), different mechanisms are involved in sludge reduction in an ASSR. In our previous study, we demonstrated that key operative parameters are the anaerobic solid retention time ($SRT_{ASSR}$) and the interchange ratio (IR) (Ferrentino et al. 2016c). Results further showed the sludge reduction mechanism is based on the simultaneous occurrence of sludge decay, cell lysis, EPS destructuration and selection of slow growing bacteria such as sulphate reducing bacteria (SRB), total phosphorous accumulating organisms (TPAOs) and denitrifying phosphate accumulating organisms (DPAOs), which are a fraction of TPAOs (Zhou et al. 2010). In particular, the development of a specific microbial community in the ASSR seems to be of prime importance for sludge reduction process (Chon et al. 2011a).

In the literature, several papers investigated microbial community in ASSR. . Chudoba et al. (1992) was probably the first to find that 60% of the microbial populations could be classified as PAOs, contrary to 10% in the activated sludge from the CAS system. The Authors showed that the periodic passageway of facultative aerobic activated sludge microorganisms through the anaerobic zone created conditions of uncoupled growth. First under anaerobic conditions and in absence of substrate, PAOs use ATP and polyphosphates as a source of energy, then under aerobic conditions and in presence of exogenous substrate, they rebuild their energy reserves at the expense of growth, resulting in a consecutive reduction of activated sludge production. This phenomenon may be explained by the presence of uncoupling of catabolism and anabolism. Wang and Zhao (2011) reported that most bacteria were phylogenetically related to PAOs, denitrifying bacteria and anaerobes. Chon et al. (2011a) showed about 75% similarity for microbial composition between the SBR-ASSR system and an anaerobic digester. Recently, Zhou et al., (2015a) showed that the insertion of an ASSR could enhance the selection of anaerobic bacteria such as fermentative, hydrogenogenic and acidogenic bacteria that are able to improve the biomass decay and hydrolysis of particulate organic matters. The Authors confirmed also the shift of the main microbial populations from fast growers to slow growers.

However, there is still insufficient literature on the correlation between the microbial community in the ASSR and the different mechanisms involved in the sludge reduction process. Further, up to now there is no information about how a change of two main operative parameters,

such as the $SRT_{ASSR}$ and the IR, could affect the microbial community structure of the ASSR process.

Therefore, in this study a laboratory scale SBR-ASSR reactor was operated for 9 months under different $SRT_{ASSR}$ and IR. Both the performances of the whole process, in terms of carbon and nutrient removal, the sludge reduction efficiencies and the microbial community structure were analysed.

## 2. Materials and Methods

### *2.1. System operation*

The lab-scale system consisted of a sequencing batch reactor (SBR), as water line of a wastewater treatment plant (WWTP), an anaerobic side-stream reactor (ASSR) where the settled sludge was treated and a thickener (Fig. 1). This last unit allowed both increasing biomass to cycled back to the ASSR and completing nitrate removal in order to ensure a tightly anaerobic environment in the ASSR. Reactors were made of Plexiglas. Both the SBR and the ASSR had a working liquid volume of 10 L, while the thickener 2 L. The SBR was equipped with a mechanical stirrer and air sparging system. It operated with six cycles per day in alternate nitrification/denitrification mode. The schematic operating strategy is depicted in Fig. 1. The dissolved oxygen (DO) in the aerobic phase was maintained between 1-2 mg $O_2$/L. The ASSR was covered with a plastic plate. It was equipped with a mechanical stirrer, and it was continuously mixed. ORP and pH in the ASSR were monitored and left free to vary. The lab-scale system was equipped with peristaltic pumps and digital timers. The influent was first pumped into the SBR from a storage tank for simultaneous carbon and nutrient removal. Every SBR cycle, the settled sludge was pumped to the thickener and after 3 h the thickened sludge was cycled to the ASSR. At the same time, an equal volume of sludge was cycled from the ASSR to the SBR. The entire experimental period consisted of three different phases that lasted for about 90 days each: i) 10% sludge interchange rate and SRT in the ASSR of 10 days; ii) 20% sludge interchange rate and SRT in the ASSR of 5 days and iii) 40% sludge interchange rate and SRT in the ASSR of 2.5 days.

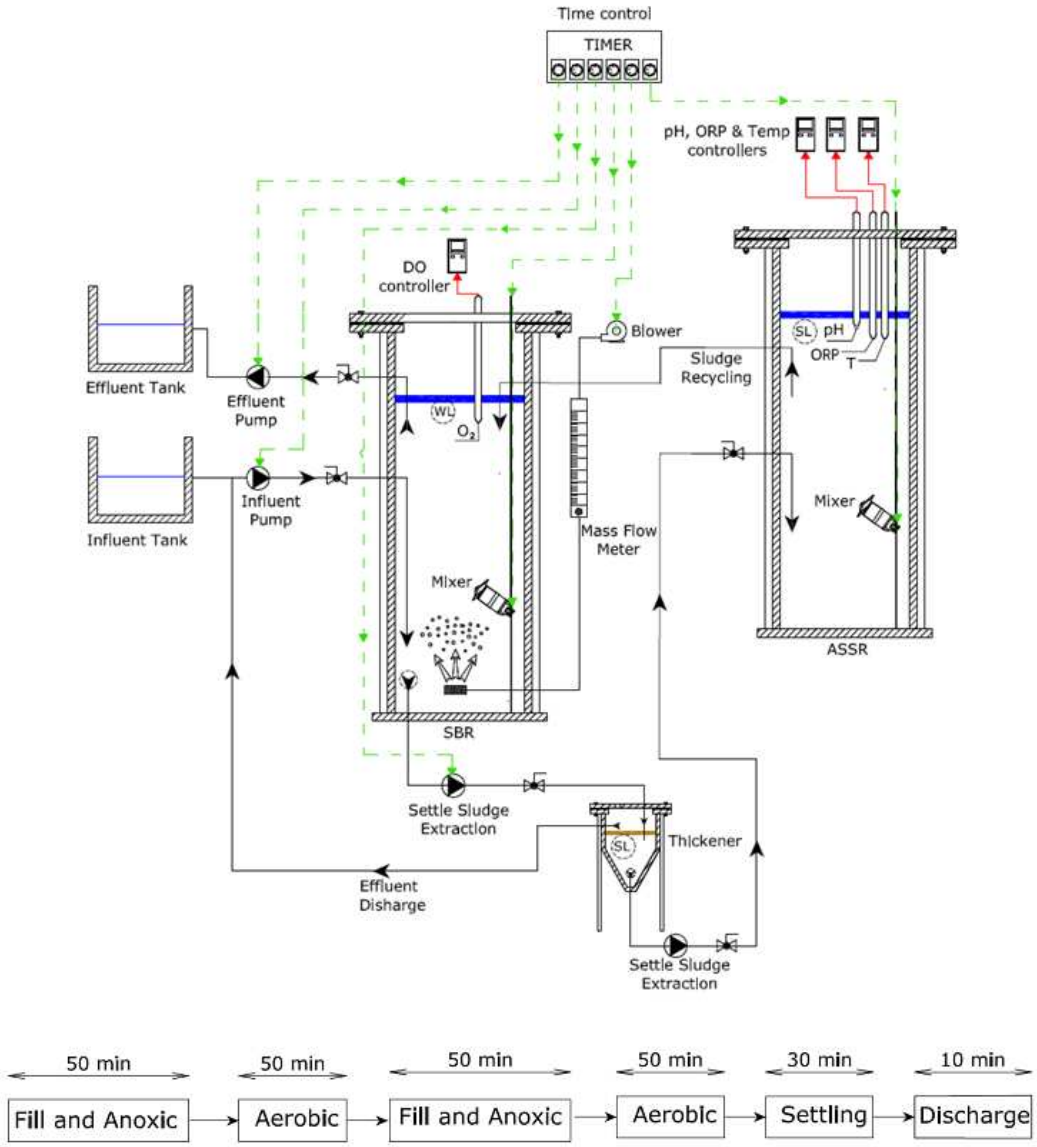



Fig. IV.1 Schematic diagrams of the experimental setup and time sequence in each SBR cycle

The SBR was inoculated with the activated sludge obtained from the CAS of the WWTP of Trento Nord (Italy) operated for nitrogen removal. The ASSR was inoculated with the anaerobic sludge obtained from the anaerobic sewage treatment tank of the WWTP of Levico Terme (Italy).

*2.2. Characteristics of wastewater*

The SBR was fed with the effluent of the primary sedimentation tank of Trento Nord WWTP, Italy. The characteristics and the chemical composition of the influent are described as follows: pH, 7.4; COD, 244 mg/L; sCOD, 100 mg/L; $NH_4^+$-N, 46 mg/L; $NO_3^-$-N 1.18 mg/L; $PO_4^{3-}$-P 3.6 mg/L; TSS, 200 mg/L.

*2.3. Chemical analysis*

Samples were taken three times a week. Total suspended solids (TSS), total chemical oxygen demand (COD) and nitrate as nitrogen ($NO_3^-$-N) were measured according to the Standard Methods (APHA, 2005). Soluble COD (sCOD) was measured after filtration on a 0.45-μm membrane. Ammonium nitrogen ($NH_4^+$-N) and soluble phosphorous ($PO_4^{3-}$-P) were analyzed according to APAT CNR IRSA, 2003. DO, pH and ORP were measured with three different probes connected to a multimeter (Multimeter 44, Crison).

*2.4. Microbial community structure assessment*

Samples of sludge for microbiological analyses were collected in duplicate at the end of each experimental phase. Total DNA was extracted from 0.5 mL of sludge using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The V5-V6 hypervariable regions of the bacterial 16S rRNA gene were PCR-amplified using 783F and 1046R primers (Huber et al. 2007; Wang and Qian 2009), while the IA_349F-IA_571R primer pair was used for the amplification of a fragment of the archaeal 16S rRNA gene (Gagliano et al. 2015). At the 5' end of each primer, a 6-bp barcode was also included to allow sample pooling and subsequent sequence sorting. All amplicons were sequenced by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) with a 250 bp × 2 paired-end protocol.

For Bacteria, 2 × 50 μL volume PCR reactions were performed with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 μM of each primer. The cycling conditions were: initial denaturation at 94 °C for 4 min; 28 cycles at 94 °C for 50 s, 47 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. For Archaea, 2 × 50 μL volume PCR reactions were performed with Phusion® High-Fidelity DNA Polymerase (NEB Inc., Ipswich, MA, USA), 1x HF Buffer, 0.2 mM dNTP mix, 1.5 U of Phusion DNA Polymerase, and 0.5 μM of each primer. The cycling conditions were: initial denaturation at 98 °C for 30 s; 10 cycles at 98 °C for 10 s, 68 °C for 20 s, and 72 °C for 15 s; 30 cycles at 98 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s and a final extension at 72 °C for 2 min (Gagliano et al. 2015). The amplicons were purified with the Wizard® SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI, USA) and purified DNA was quantified using Qubit® (Life Technologies, Carlsbad, CA, USA). Groups of 9 amplicons bearing different barcode pairs were pooled together to build a single library. Further library preparation with the addition of standard Nextera indexes (Illumina, Inc., San Diego, CA, USA) and sequencing were carried out at Parco Tecnologico Padano (Lodi, Italy).

Reads from sequencing were demultiplexed according to the indexes and the barcodes.

Uparse pipeline was used for the following elaborations (Edgar 2013). Forward and reverse reads were merged with perfect overlapping and quality filtered with default parameters. For Archaea the final 50 bp were removed from each read to allow using both reads. Suspected chimeras and singletons sequences (i.e. sequences appearing only once in the whole data set) were removed. OTUs were defined on the whole data set clustering the sequences at > 97% of similarity and defining a representative sequence for each cluster. A subset of 5000 random sequences for Bacteria and 10000 for Archaea was chosen from each sample and the abundance of each OTU was estimated by mapping the sequences of each sample against the representative sequence of each OTU at 97% of similarity. Taxonomic classification of the OTU representative sequences was obtained by RDP classifier (Wang et al., 2007).

*2.5. Quantification of total Bacteria, total Archaea and Sulphate Reducing Bacteria (SRB)*

Quantitative PCR (qPCR) was used to assess the abundance of total Bacteria, total Archaea in each experimental phase. Among Bacteria, specific qPCR - analyses were performed to estimate SRBs. For total Bacteria, a 466-bp fragment of the bacterial 16S rRNA gene (331–797 according to *Escherichia coli* position) was PCR-amplified with a universal primer set (Nadkarni et al. 2002). For total Archaea, a 417-bp fragment of the archaeal 16S rRNA gene was amplified with the primer pair Arch349F-Arch 806R (Takai and Horikoshi 2000). For SRB, a 396-bp fragment of the adenosine 5'-phosphosulphate reductase (*aps*A) gene was amplified with the primer pair aps3F-aps2R (Christophersen et al. 2011). All PCR reactions were performed in a total volume of 10 μL using the FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy) with 0.3 μM of each primer, and an Eco Illumina thermocycler (Illumina, Inc., San Diego, CA, USA). The amplifications were carried out under the following conditions: 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s, annealing temperature for 30 s and 72 °C for 30 s, with acquisition of the fluorescence at the end of each 72 °C elongation step. The annealing temperatures were set as 60°C, 54 °C and 55 °C for Bacteria, Archaea and SRB, respectively. The fragments of interest were amplified from reference strains (*Escherichia coli* K-12 substr. DH10B for bacterial 16S rRNA gene, *Methanosarcina acetivorans* C2A for archaeal 16S rRNA gene, and *Desulfovibrio vulgaris* subsp. *vulgaris* DSM 644 for *apr*A) and cloned into the plasmid pGEM®-T (Promega Corporation, Madison, WI, USA) to prepare standards for calibration curves. Plasmids were extracted from fresh cultures, and the concentration of plasmidic DNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Serial dilutions of the plasmidic DNA were included in triplicate in each run together with triplicates of the samples. For calculation, the average number of 16S rRNA gene copies (Bertolini et al. 2013) were used to

estimate the abundances of total Bacteria and total Archaea, while the average number of *apr*A copies, was used to estimate the abundance of SRB.

## 3. Results

### *3.1 Performance of the system*

The efficiency of the SBR-ASSR process in terms of sludge reduction, sCOD, ammonia nitrogen ($NH_4^+$-N), total nitrogen (TN) and orthophosphate ($PO_4^{3-}$-P) removal in each experimental phase was investigated.

#### *3.1.1 Sludge reduction*

In this study the observed sludge yield ($Y_{obs}$) was calculated to evaluate the sludge production, considering the SBR - ASSR system as a single control unit (Chon et al. (2011b). The $Y_{obs}$ is the amount of sludge generated per the amount of substrate removed, and could be calculated by the ratio of the cumulative generated sludge and the cumulative consumed substrate Eq. (1).

$$Y_{obs} = \frac{\Delta X_{SBR} V_{SBR} + \Delta X_{ASSR} V_{ASSR} + \sum (X_{SBR} Q_{SBR,wasste} + X_{ASSR} Q_{ASSR,waste} + X_{eff} Q_{eff}) \cdot \Delta t}{\sum (S_{\inf} Q_{\inf} - S_{eff} Q_{eff}) \cdot \Delta t} \quad (1)$$

where $S_{inf}$ and $S_{eff}$ are the substrate COD concentration of influent and effluent (mg $L^{-1}$); $Q_{inf}$ and $Q_{eff}$ are the flow rate of the influent and effluent (L $d^{-1}$); $\Delta X_{SBR}$, $\Delta X_{ASSR}$ are the change of sludge (g TSS $L^{-1}$) in SBR and ASSR; $V_{SBR}$, $V_{ASSR}$ are the volumes of SBR and ASSR (L); and $\Delta t$ is the time (d). The $Y_{obs}$ can be graphically evaluated as the slope of the linear regression curve obtained plotting the cumulative sludge generation data against the cumulative consumed substrate data obtained by experimental measurements.

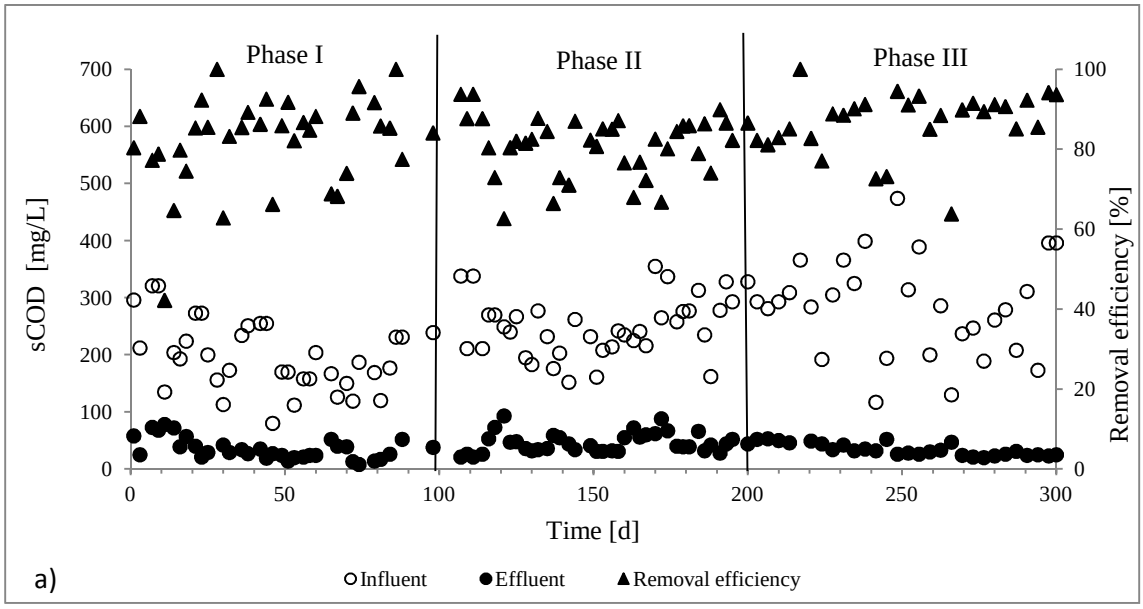
The decrease of the $SRT_{ASSR}$ and the increase of the IR in the SBR-ASSR caused a reduction of the sludge production. The observed sludge yields ($Y_{obs}$) of Phase I (10 d $SRT_{ASSR}$; IR 10%), Phase II (5 d $SRT_{ASSR}$; IR 20%), and Phase III (2.5 d $SRT_{ASSR}$; IR 40%) were 0.21 g TSS/g COD, 0.14 g TSS/g COD and 0.12 g TSS/g COD, respectively. Compared to the $Y_{obs}$ of a CAS process (0.36 g TSS/ g COD), the SBR-ASSR system reduced the sludge production in each experimental phase. A 66% percentage of sludge reduction was achieved in Phase III.

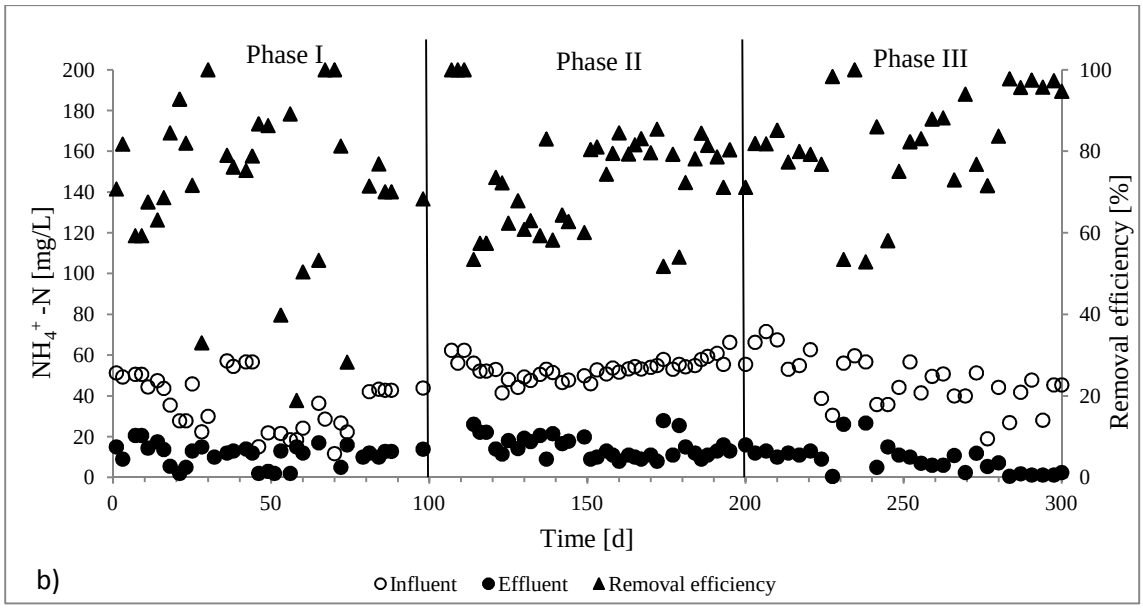
#### *3.1.2 Removal of COD, ammonia nitrogen and orthophosphates*

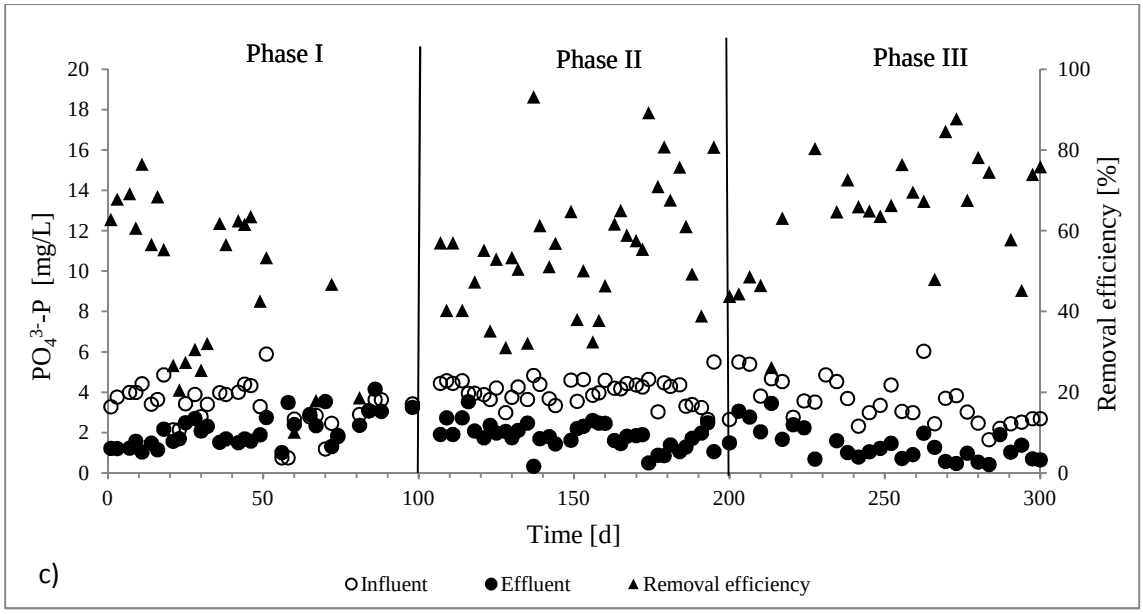
The experimental period covers 300 days of the SBR-ASSR operation. During this period, concentration of sCOD, $NH_4^+$-N, $PO_4^{3-}$-P in the influent ranged in a wide range due to the seasonal variability of the municipal WWTP. The influent and effluent profiles of sCOD, $NH_4^+$-N, TN, $PO_4^{3-}$-P in the SBR-ASSR and removal efficiencies of the process, during each phase, are reported in Figure 2.

In the Phase I (10 d $SRT_{ASSR}$; IR 10%), a consistent average sCOD removal efficiency of 82.5% was observed. However, $NH_4^+$-N, TN and $PO_4^{3-}$-P average concentrations in the SBR displayed very poor ammonia (61%), total nitrogen (69%), and orthophosphate (41%) removal efficiencies. The lowest ammonia, total nitrogen and orthophosphate removal efficiencies were measured when low sCOD concentration was detected in the influent.

With the aim of improving sludge production, we decreased the $SRT_{ASSR}$ from 10 to 5 d in Phase II, increasing the IR from 10 to 20%. The response of the system was positive both in terms of sludge reduction an effluent quality. The system maintained a good removal percentage of organic matter (sCOD, 80%) while removed up to 71%, 55% and 73% of ammonia, orthophosphate and total nitrogen, respectively. When the $SRT_{ASSR}$ was further decreased to 2.5 d, corresponding to IR of 40%, sCOD removal efficiency increased up to 86%. An increase of ammonia, orthophosphate and total nitrogen removal efficiencies were measured, achieving average values of 83%, and 63%, and 82% respectively. From Figure 2b, 2c, 2d, it can be clearly seen that increasing $NH_4^+$-N, $PO_4^{3-}$-P and TN removal efficiencies were achieved after acclimation of the biomass to the operating conditions. Furthermore, the TSS effluent concentration and the sludge settling proprieties improved switching from Phase I to Phase III (data not showed).




Phase I
Phase II
Phase III
$NH_4^+$-N [mg/L]
Removal efficiency [%]
Time [d]
b)
Influent
Effluent
Removal efficiency


Phase I
Phase II
Phase III
$PO_4^{3-}$-P [mg/L]
Removal efficiency [%]
Time [d]
c)
Influent
Effluent
Removal efficiency

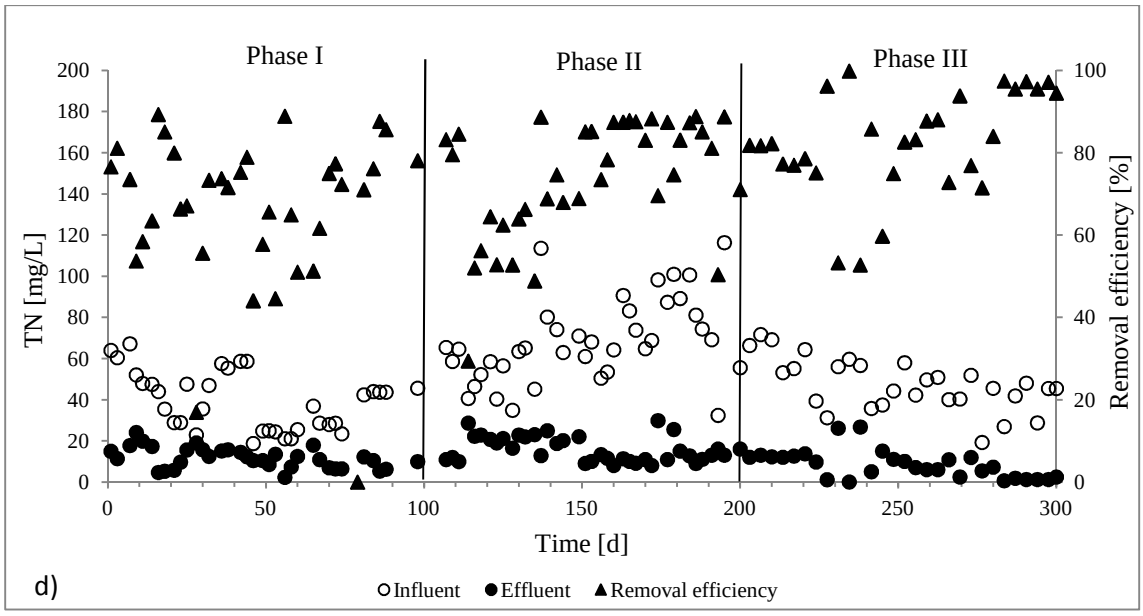



Fig. IV.2 Performance of the process in terms of a) COD, b) $NH_4^+$-N c) $PO_4^{3-}$-P d) TN removal

### *3.2 Bacteria, Archae and SRB total quantification*

The qPCR results showed the abundance of total Bacteria increased in each experimental phase. The average number of bacterial ribosomal operons in Phase I, II and III was $4.82 \cdot 10^8 \pm 0.71 \cdot 10^8$ copies/mL, $1.22 \cdot 10^9 \pm 0.17 \cdot 10^9$ copies/mL, and $2.37 \cdot 10^9 \pm 0.16 \cdot 10^9$ copies/mL, respectively. Among bacteria, the abundance of SRB increased as well. The copy numbers of *aprA* gene of SRB in the biomass enrichment were quantified as $1.71 \cdot 10^7 \pm 0.06 \cdot 10^7$ copies/mL, $3.59 \cdot 10^7 \pm 0.19 \cdot 10^7$ copies/mL, and $1.61 \cdot 10^8 \pm 0.03 \cdot 10^8$ copies/mL in Phase I, II and III, respectively. Finally, the qPCR results showed the abundance of total Archaea was almost constant. The average number of archaeal ribosomal operons in Phase I, II and III was $4.93 \cdot 10^8 \pm 0.12 \cdot 10^8$ copies/mL, $6.75 \cdot 10^8 \pm 0.13 \cdot 10^8$ copies/mL, and $5.32 \cdot 10^8 \pm 0.16 \cdot 10^9$ copies/mL, respectively.

### *3.3 Bacterial taxonomic identification*

The phylogenetic and taxonomic diversity of bacterial communities in Phase I, II and III was assessed through sequencing of the 16S rRNA gene. The sequenced 16S rRNA gene fragments were classified up to the lowest taxonomic level possible. The values of relative abundance indicate the percentage of one specific group of cells in relation to the total bacterial 16 rRNA gene copies.

***Phylum level***

Our results showed 20 bacterial phyla in the ASSR biomass. The phylum *Proteobacteria* was the most abundant in all samples, accounting for 42.8%, 40.2% and 43.9% of the total bacterial

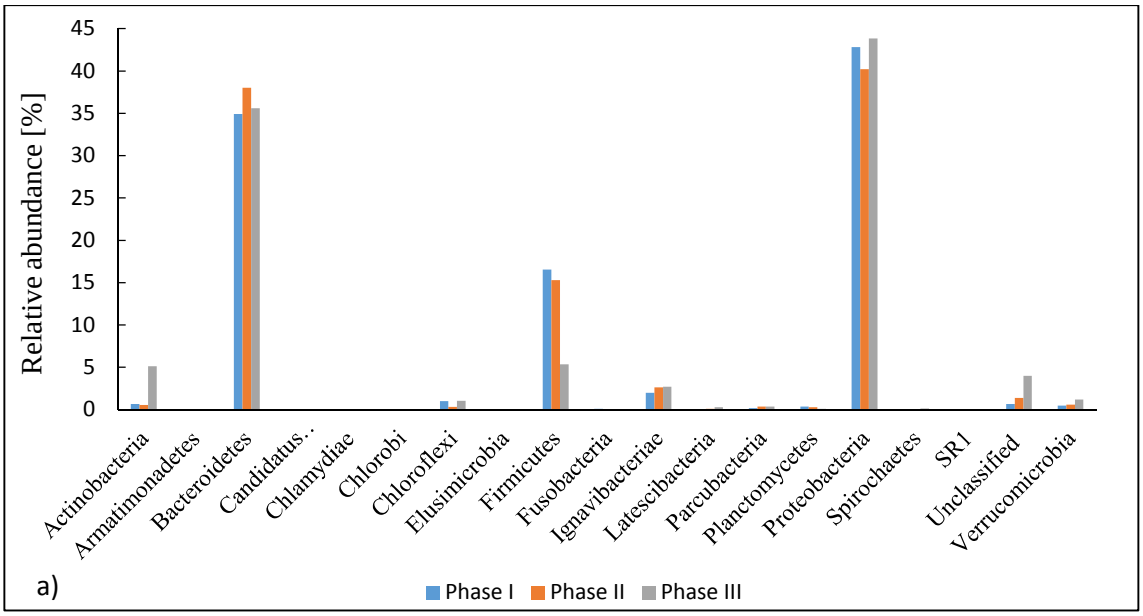
sequences, in Phase I, II and III, respectively. The second dominant phylum was *Bacteroidetes* accounting for 34.9% (Phase I), 38.0% (Phase II) and 35.6% (Phase III) of the total bacterial sequences. Other abundant phyla (abundance > 1% in at least one sample) were *Firmicutes* (5.4 – 16.5%), *Actinobacteria* (0.7 – 5.1%), *Chloroflexi* (0.3 – 1.1%), *Ignavibacteriae* (2.0 – 2.7%) and *Verrucomicrobia* (0.5 – 1.2%). In particular, *Actinobacteria* and *Verrucomicrobia* were the major phyla in the third phase, with low abundances in the first and second phases. Others phyla detected had lower relative abundances (< 0.5%).

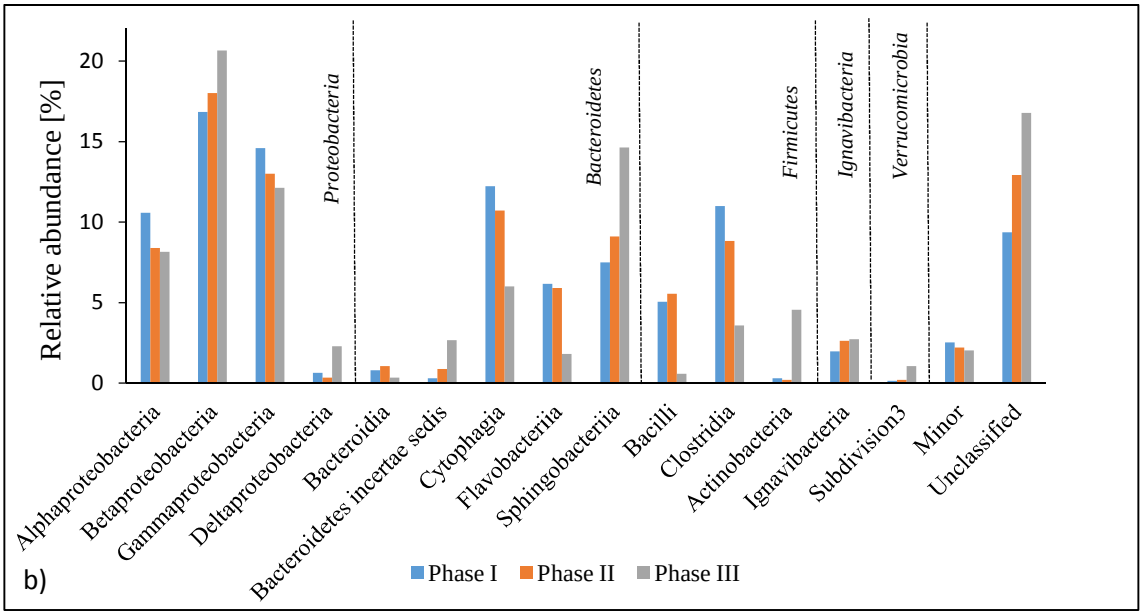
***Class level***

Within *Proteobacteria* phylum, *Betaproteobacteria* was the most abundant class in all the sample accounting for 16.8% (Phase I), 18.0% (Phase II) and 20.7% (Phase III) of total bacterial sequences. Other dominant classes were *Gammaproteobacteria* (12.1 – 14.6%), *Alphaproteobacteria* (8.2 – 10.6%) and *Deltaproteobacteria* (0.4 – 2.3%). The relative abundance of *Betaproteobacteria* and *Deltaproteobacteria* increased from the first to the third phase while the relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria* decreased.

In the phylum *Bacteroidetes*, the two most abundant classes were *Sphingobacteriia* and *Cytophagia*. Data showed that the first increased from 7.5%, to 9.1% and to 14.6% of the total bacterial sequences in Phase I, II and III, respectively. The latter class decreased from 12.2% (Phase I), to 10.7% (Phase II) and to 6.0% (Phase III) of the total reads. Moreover, the relative abundance of *Flavobacteriia* decreased from the first phase to the last one, accounting for 6.2% in Phase I, 5.9% in Phase II and only 1.8% in Phase III.

Within the *Firmicutes* phylum, the dominant class was *Clostridia*, which decreased from 11% (Phase I), 8.8% (Phase II) and 3.6% (Phase III) of the total bacterial sequences. Furthermore, the relative abundance of class *Actinobacteria* increased significantly from 0.3% in Phase I to 4.6% in Phase III. Other abundant classes were *Ignavibacteria* (2.0 – 2.7%) and *Subdivision 3* which also increased from Phase I to Phase III(0.2 – 1.1%).


Relative abundance [%]
45
40
35
30
25
20
15
10
5
0
Actinobacteria
Armatimonadetes
Bacteroidetes
Candidatus..
Chlamydiae
Chlorobi
Chloroflexi
Elusimicrobia
Firmicutes
Fusobacteria
Ignavibacteriae
Latescibacteria
Parcubacteria
Planctomycetes
Proteobacteria
Spirochaetes
SR1
Unclassified
Verrucomicrobia
a)
Phase I
Phase II
Phase III


Relative abundance [%]
20
15
10
5
0
Proteobacteria
Bacteroidetes
Firmicutes
Ignavibacteria
Verrucomicrobia
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Bacteroidia
Bacteroidetes incertae sedis
Cytophagia
Flavobacteriia
Sphingobacteriia
Bacilli
Clostridia
Actinobacteria
Ignavibacteria
Subdivision3
Minor
Unclassified
b)
Phase I
Phase II
Phase III

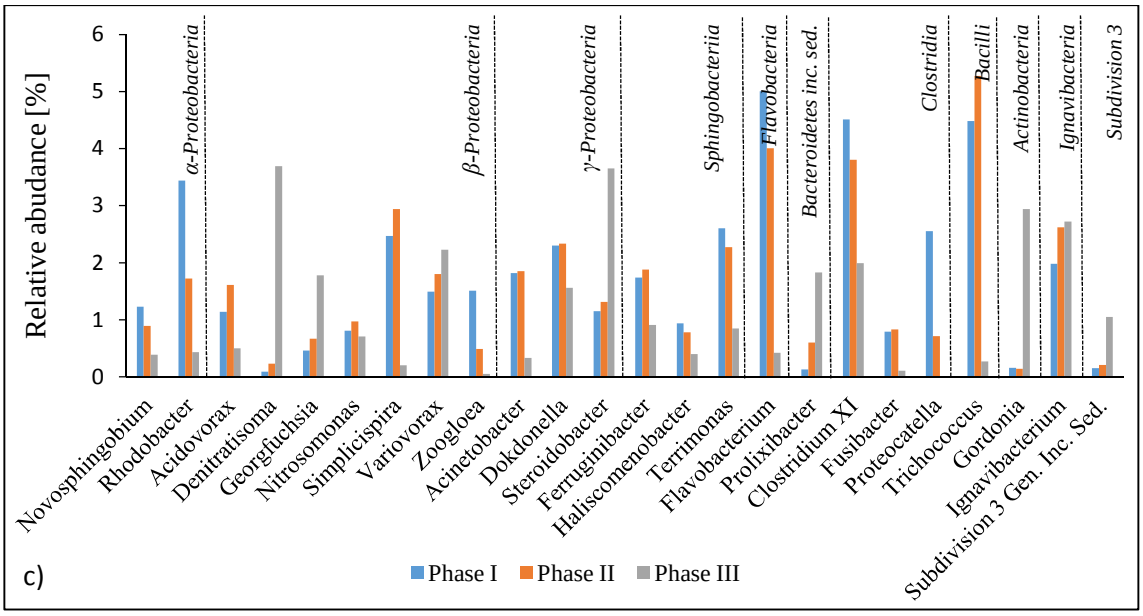



Fig. IV.3 The relative abundance of the predominant bacterial phylogenetic group at a) phylum b) class and c) genus level

***Genus level***

Analyses based on genus level allowed obtaining more detailed information about the changes in bacterial community structure along the different experimental phases. Among the 202 genera detected, 24 genera with a relative abundance above 1% in at least one sample were identified and compared based on their relative abundance.

Two genera were detected belonging to the *Alphaproteobacteria* class, *Novosphingobium* and *Rhodobacter*. The first genus had a relative abundance accounting for 1.23% of the total bacterial sequences in Phase I that decreased to 0.89% and 0.39% in Phase II and III, respectively. *Rhodobacter* was more abundant in Phase I (3.44%) and decreased in Phase II (1.72%) and in Phase III (0.43%).

Within the class *Betaproteobacteria*, *Denitratisoma* and *Georgfuchsia* were almost absent in Phase I and II while they reached a relative abundance of 3.69% and 1.78%, respectively, in Phase III. *Variovorax* also increased from the Phase I to Phase III. On the contrary, the relative abundance of *Simplicispira, Zoogloea and Acidovorax* strongly decreased from Phase I to Phase III. Other relevant genera in the class *Betaproteobacteria* were *Nitrosomonas* which did not show significant variation during the whole experimental period.

Within the class *Gammaproteobacteria, Steroidobacte,* was increased significantly, reaching a relative abundance of 3.65% in Phase III. *Acinetobacter* and *Dokdonella* were other two abundant genera that decreased from the Phase I to Phase III.

Among the genera belonging to the *Sphingobacteriia class*, *Ferruginibacter*, *Haliscomenobacter*, *Terrimonas* were relevant in Phase I, while decreasing in Phase III.

*Flavobacterium*, was the most abundant genera of *Flavobacteria* class in Phase I (5.00%) and in Phase II (4.00%), while strongly decreased in the last Phase (0.42%). On the contrary, among the genera belonging to the *Bacteroidetes* phylum, *Prolixibacter* showed an increasing trend from Phase I to Phase III.

Within the class *Clostridia*, the genus *Clostridium XI* accounted for 4.51%, 3.80% and 1.99% of the total bacterial reads in Phase I, II and III, respectively. Similar to *Clostridium XI*, other genera belonging to the class *Clostridia*, *Fusibacter* and *Proteocatella*, decreased as well from Phase I to Phase III.

Among the genera belonging to the class *Bacilli*, only *Trichococcus* was abundant. *Trichococcus* was one of the most abundant genera in Phase I and II during the whole experiment with a relative abundance of 4.48% and 5.28%, respectively. However, its relative abundance strongly decreased in Phase III reaching a value of 0.27%.

The genus *Gordonia*, was the only one abundant in the class *Actinobacteria*. Its abundance increased from 0.16% in Phase I, to 0.14% in Phase II and to 2.94% in Phase III.

*Ignavibacterium*, belonging to the class *Ignavibacteria*, accounted for 1.98%, 2.62% and 2.72% of the total reads in Phase I, II and III, respectively. Finally, the genus *Subdivision 3*, belonging to the class *Verrucomicrobia*, was also detected, accounting for a low percentage of total bacterial sequences in Phase I and II, and increasing in Phase III.

### *3.4 Archaeal taxonomic identification*

#### ***Phylum level***

At the phylum level, the *Euryarchaeota*, accounting for 94.2%, 98.9% and 99.6% of the total archaeal community in Phase I, II and III, respectively, consistently dominated the archaeal community. Other archaeal phyla were detected with a very low relative abundance, such as *Crenarchaeota* (0 – 1.3%) and *Thaumarchaeota* (0.2 – 0.3%).

#### ***Class level***

Within the phylum *Euryarchaeota*, *Methanobacteria* was the most abundant class, but it decreased from 98.0%, to 82.8% and to 67.3% of the total reads in Phase I, II and III, respectively. On the contrary, an increase of the abundant relevance of the class *Methanomicrobia* was measured, increasing from 1.3% (Phase I) to 16.3% (Phase III) of the total archaeal community. The other classes detected had a lower relative abundance: *Thermoprotei* (0 – 1.3%) and *Nitrososphaerales* (0.1 – 0.3%).

***Genus level***

Among *Methanobacteria* class, the genus *Methanobrevibacter* was the most abundant in all phases, although it decreased from 95.0% in Phase I to 45.7%, in Phase III. On the contrary, the relative abundance of the genus *Methanosphaera* increased from 1.6% and 1.9% of Phase I and II, respectively, to 17.7% in Phase III. *Methanobacterium* was also detected, accounting for only 1.4 – 2.7%; a slight increase from Phase I to Phase III was observed.

Among *Methanomicrobia* class, low relative abundances of *Methanosarcina* and *Methanosphaerula* were detected in Phase I, 0.4% and 0.5%, respectively. They increased from Phase I to Phase II, while slightly decreased in Phase III, reaching 7.0% and 4.0%, respectively. Other detected genera were *Methanolinea* (0.2 – 1.5%), *Methanosaeta* (0.0 – 1.4%) and *Methanospirillum* (0.1 – 2.1%), which slightly increased from Phase I to Phase III.

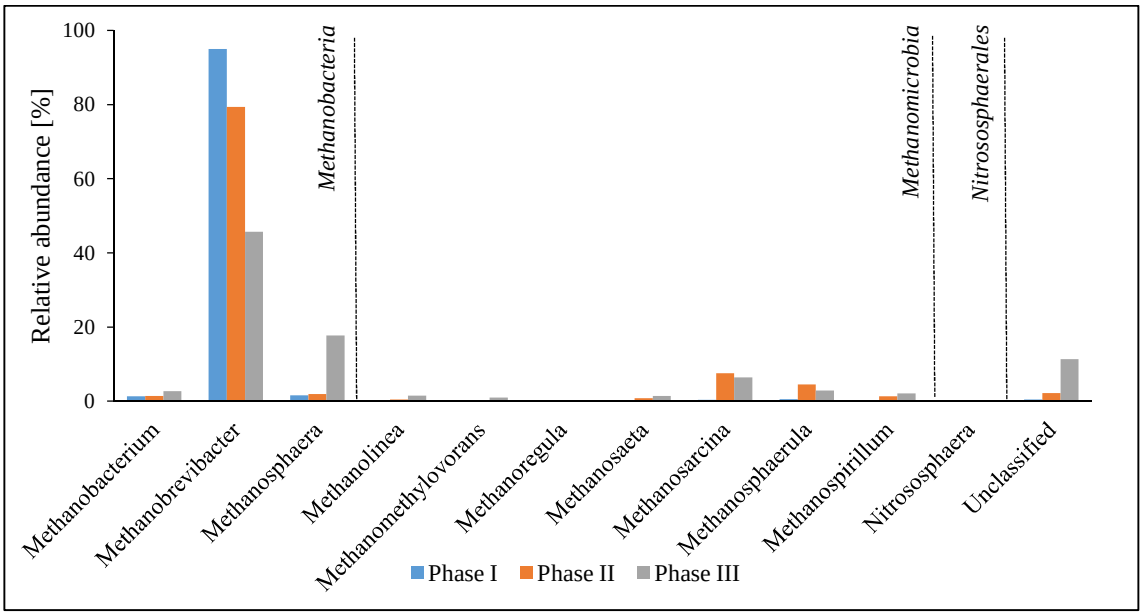



Fig. IV.4 The relative abundance of the predominant Archaeal phylogenetic group at genus level

## 4. Discussion

### *4.1 Performance of the system*

The general aim of the SBR-ASSR application in this study is the reduction of sewage sludge production. However, the primary goal of a wastewater treatment is to ensure that effluent wastewater meets the regulatory limits. For this reasons the performance of the process in terms of sCOD, $NH_4^+$-N, TN, and $PO_4^{3-}$-P removal is here investigated.

In this study, short $SRT_{ASSR}$ and high IR resulted in a high reduction of sludge production. As reported in our previous research, a decrease of $SRT_{ASSR,}$ and thus an increase of IR, caused an

increasing solubilisation of organic matter in the ASSR, related to sludge decay, cell lysis, EPS destructuration, and a selection of specific slow growing microorganisms, such as SRBs and DPAOs over TPAOs (Ferrentino et al. 2016c). DPAOs are important both for sludge reduction, because of their low growth yield, which is 70% lower than TPAO (Hu et al. 2002), and for biological phosphate and nitrate removal process, because of DPAOs utilized $NO_3^-$-N as electron acceptor for $PO_4^{3-}$-P removal (Lee and Yun 2014). SRBs have also a function of primary importance for sludge reduction due to their low sludge yield (Kleerebezem and Mendez, 2002; van den Bosch et al., 2007), contributing to cell lysis mechanisms and dissolved EPS release (Nielsen and Keiding 1998), and further incompletely degrading complex carbon compounds to acetate (Muyzer and Stams 2008a) which may be used by TPAOs. Thus, in the ASSR, under anoxic conditions, hydrolysis and fermentation processes occur, while SRBs incompletely degrade complex carbon compounds to acetate. Acetate may easily be taken up and stored by TPAOs, releasing orthophosphate in the solution. Then, in aerobic and anoxic conditions of the SBR, the internally stored acetate is used while consuming nitrate or oxygen for TPAOs maintenance functions and for the growth of new cells. Heterotrophic carbon oxidation, nitrification and denitrification processes further occur in the SBR.

The best results in terms of carbon and nutrient removal were obtained in Phase III, when the IR was the maximum (40%) and the $SRT_{ASSR}$ was the minimum (2.5 d). In Phase III, sCOD, $NH_4^+$-N, $PO_4^{3-}$-P and TN, removal efficiencies were equal to 86%, 71%, 63%, and 83% respectively.

In this study, the higher COD efficiencies in the Phase III have been related to the improvement of the hydrolysis and fermentation processes in the ASSR, sludge decay, cell lysis and EPS destructuration (Ferrentino et al. 2016c). Indeed, increasing the IR, a higher biomass quantity was subjected to anaerobic - aerobic/anoxic alternate conditions.

The higher hydrolyzed and partially fermented sludge obtained in the ASSR in Phase III could be easily degraded when it comes back to the SBR, by means of both aerobic heterotrophs and denitrifiers microorganisms. Considering the ammonium nitrogen, decreasing the $SRT_{ASSR}$ the removal efficiency in the SBR-ASSR system slowly increased from 61% in Phase I, to 71% in Phase II, and to 83% in Phase III. Different explanations could be addressed about the increasing ammonium nitrogen removal. A higher removal percentage in Phase III could be due to the lower inhibition effect of particulate organic matter on nitrification (Figueroa and Silverstein, (1992). On the contrary a higher ammonium removal percentage in Phase III could be related to the lower decay rate of nitrifying biomass under anaerobic conditions as compared to the values under aerobic and anoxic conditions (Zhou et al. 2015a). Finally, the higher SRT of the whole SBR-

ASSR system in Phase III (83 d) than Phase I (47 d) and Phase II (60 d) could be also responsible for the improvement of the $NH_4^+$-N removal efficiency.

With reference to the orthophosphate, results showed very low removal efficiency in Phase I, which increased in Phase II and III, reaching a value of 63%. The increasing phosphorous removal efficiency is concordant with the increasing selection of DPAOs over TPAOs.

Finally, the average removal efficiency of TN increased during the experimental period, reaching 83% in Phase III. The higher TN removal efficiency in Phase III was related to the higher denitrification contribution in the SBR, due to both heterotrophic denitrifiers, which had a higher available sCOD from cell lysis and hydrolysis of particulate organic substances in the ASSR, and selected DPAOs, which contribute for nitrate removal using the internal stored acetate.

Nevertheless, it should be noted that the sludge cycled from the ASSR to the SBR brought back to the water line a considerable amount of sCOD,$NH_4^+$-N and $PO_4^{3-}$ -P as consequence of fermentation and cell lysis processes and TPAOs activities, contributing to a sCOD, $NH_4^+$-N and $PO_4^{3-}$-P overloading, which increased from Phase I to Phase III.

The sCOD overloading was difficult to estimate, as COD is not a conservative parameter. Indeed, sCOD is consumed by several biological processes both in the ASSR and in the SBR, e.g. aerobic heterotrophs, denitrifiers microorganisms, TPAO, DPAO and SRB.

On the contrary, the $NH_4^+$-N and $PO_4^{3-}$ -P and TN were not consumed in ASSR, and supplementary loads to the water line were estimated. The supplementary $NH_4^+$-N load accounted for 4.5%, 10% and 29% in Phase I, II, and III, respectively. The supplementary $PO_4^{3-}$ -P load was 6.5%, 29% and 38% in Phase I, II, and III, respectively. The supplementary TN load was 4.5%, 5.6% and 8% was in Phase I, II, and III, respectively.

Considering the $NH_4^+$-N overloading, the real removal efficiencies increased up to 74%, 76% and 90% in Phase I, II and III, respectively. Similarly, the real $PO_4^{3-}$ -P removal efficiencies increased up to 44%, 70% and 87% in Phase I, II and III, respectively. The removal efficiencies of TN increased up to 71%, 76% and 88% in Phase I, II and III, respectively.

### *4.2 Microbial community*

qPCR results showed significant increase in total bacteria abundance from Phase I to Phase II, while a slight increase has been also detected in Phase III. Results of the bacterial taxonomic identification reported that *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were the most abundant phyla in all experimental Phases. Previous literature studies reported that these phylogenetic groups were ubiquitous in both laboratory-scale and pilot-scale anaerobic bioreactors (Gao et al. 2010). *Proteobacteria* are listed by several studies as the most abundant phylum in the community

structure of several WWTPs (Wagner and Loy 2002; Satoh et al. 2012; Zhang et al. 2012). In this study, within the phylum *Proteobacteria*, the most abundant class was *Betaproteobacteria* with a relative abundance that increased from Phase I to Phase III. Several studies reported *Betaproteobacteria* as the major class in conventional anaerobic digester (AD), membrane bioreactor (MBR) and anaerobic dynamic membrane bioreactor (AnDMBR) systems (Ma et al., 2013; Nelson et al., 2011; Zhang et al., 2011). Furthermore *Betaproteobacteria*, together with *Gammaproteobacteria*, abundant in each phase, were also able to cycle phosphorous for energy in enhanced biological phosphorous removal (EBPR) system (Lv et al. 2014).

On the other hand, *Bacteroidetes* are considered to potentially release EPS (Han et al. 2015) in the solutions. Within the phylum *Bacteroidetes*, the relative abundance of *Sphingobacteria* significantly increased from Phase I to Phase III. These bacteria have the capability to produce extracellular proteases (Zhou et al. 2015b) increasing the release of soluble microbial products (SMP) in the solutions. This result confirms the relevant role of EPS destructuration, which has been measured in our previous work (Ferrentino et al. 2006b). The relative abundance of *Cytophagia*, that use protein, N-acetylglucosamine, and chitin, and proficient in degrading part of the high molecular mass fraction of organic matter, was observed to decrease from Phase I to Phase III.

While the relative abundances of *Proteobacteria* and *Bacteroidetes* were quite stable during all the experimental Phases, the bacteria belonging to the phylum *Firmicutes* decreased.

*Firmicutes* generally includes a great number of fatty acids-oxidizing bacteria capable of degrading complex organic matter (Narihiro et al. 2012). In our study the class *Clostridia*, which included numerous bacteria that can efficiently degrade complex organic matter and ferment lactic or acetic acid to $H_2$ and $CO_2$ (Nelson et al., 2011), significantly decreased as well. On the contrary, *Actinobacteria*, that were strongly related to the production of propionic acid in anaerobic environments (Jang et al., 2014), strongly increased in the third Phase. According to Zhou et al. (2015), *Actinobacteria* class could be responsible for the process of hydrolysis and fermentation of organic matter in the anaerobic tank and probably played an important role in sludge reduction of the ASSR system. Indeed, propionic acid is volatile fatty acids (VFA) that may be incompletely degraded by SRB to acetate, which may be subsequently used by phosphorous accumulating organisms.

The classification at genus level clearly showed an increase of relative abundance from Phase I to Phase III of *Prolixibacter* that consists of facultative anaerobes that can ferment sugars by a mixed acid fermentation pathway. On the contrary other genera responsible of hydrolysis and fermentation decreased in Phase III, such as *Flavobacterium, Clostridia* and *Trichococcus.*

*Flavobacterium* are strictly aerobic bacteria but can also grow anaerobically when some growth factors are provided and is able to hydrolyse various polysaccharides (Bernardet et al. 1996). *Clostridia* are versatile microorganisms capable of fermenting complex biopolymers such as cellulose and various carbohydrates into acetate, butyrate, carbon dioxide and hydrogen (Ghasimi et al., 2015). *Trichococcus* are facultative anaerobic (Liu et al. 2002), slow growing microorganisms found in anaerobic fermentation system (Zhou et al. 2015b).

The genus *Variovorax* sp. increased from Phase I to Phase III. It is known to have the ability to metabolize sulphur compounds producing sulphite (Han et al. 2011). It is capable of using glucose, benzoate, acetate, and salicylate as carbon sources if sulfate and vitamin B12 are supplied (Young et al. 2006). Moreover, specific qPCR analyses targeting the *apsA* gene were performed showing an increase of SRBs abundance from Phase I to Phase III. The *apsA* gene is involved in the energy metabolism of SRBs and has been identified as reliable gene markers for SRB (Wagner et al. 2005). SRB are anaerobic microorganisms that use sulphate as a terminal electron acceptor for the degradation of complex organic compounds. Results showed that SRB abundance in Phase III was about 10-fold higher than that at the beginning in Phase I, which indicates the important role of SRB bacteria in enhancing the simultaneous biological processes for sludge reduction in ASSR.

Phosphate accumulating bacteria abundance increased in this study. They may consist of several diverse phylogenetic groups that vary among different treatment systems (Seviour and Blackall 1999). In this study, *Gordonia* was detected that are bacteria specialized in lipid degradation in EBPR ecosystems (Krageland et al., 2007), and further in poly-P granules accumulation (Wong et al. 2005; Beer et al. 2006). *Gordonia* are characterized by a mycelial growth and foaming abilities, causing settleability problems; several studies showed that its abundance was slightly higher in denitrifying than in aerobic phosphorus removal sludge (Lv et al. 2014). Nevertheless, in our study, despite the increasing relative abundance of *Gordonia* in Phase III, this genus did not induce problems in sludge settleability. Furthermore, the relative abundance of two main genera able to accumulate phosphate, such as *Accumulibacter* and *Tetrasphera*, was also detected (data not showed). The betaproteobacterial Rhodocyclus-related *Candidatus* 'Accumulibacter phosphatis' (hereafter, *Accumulibacter*) and the *Actinobacterial* genus are important PAOs in EBPR systems (Kong et al. 2005; Kim et al. 2010). However, both some *Accumulibacter* and *Tetrasphera* are also believed to be capable of denitrification, using nitrate as an electron acceptor for phosphorus uptake (Flowers et al. 2009; Kristiansen et al. 2012). Thus, both *Accumulibacter* and *Tetrasphera* can be classified as DPAOs. Despite the low relative abundance, that was lower than 1%, results showed their increase in the Phase III confirming an

improvement of their selection and growing. Further we observed that *Simplicispira* genus, which was isolated from activated sludge that performed enhanced biological phosphorus removal in a sequencing batch reactor with sodium acetate as the sole carbon source (Lu et al. 2007), decreased in our study.

Furthermore, our results showed an increasing selection of denitrifying bacteria from Phase I to Phase III. Denitrifying bacteria such as *Denitratisoma, Georgfuchsia* and *Steroidobacter* were detected and significantly increased from Phase I to Phase III. The first represented a group of denitrifying bacteria that were firstly isolated from activated sludge of WWTPs (Fahrbach 2006). *Denitratisoma* and *Georgfuchsia* were both classified as chemoorganoheterotrophic denitrifying bacteria that can oxide different carbon sources such as exogenous organic materials, cell debris, and metabolites to $CO_2$, and reduce nitrate to nitrogen (Wang et al. 2015). *Steroidobacter* is a genus of denitrifying bacteria that exhibited a reduction of nitrate to dinitrogen monoxide and further to dinitrogen without any intermediate accumulation of nitrite (Fahrbach et al., 2008). On the contrary, *Rhodobacter*, that is known to be capable of $N_2O$ reduction (Song et al. 2014), and *Zoogloea*, able to to utilize organic electron donors and reduce nitrate or nitrite to nitrogen (Han et al. 2015), decreased from Phase I to Phase III. However, *Zoogloea* is a genus of bacteria that are commonly known for excreting high levels of EPS in wastewater treatment systems (Norberg and Enfors 1982). The decrease of *Zoogloea* abundance from Phase I to Phase III is in line with the lower EPS content measured in the Phase III of the ASSR in our previous study (Ferrentino et al. 2016c). Furthermore, *Ignavibacteria* which play a role in AnAOB (Anaerobic Ammonium Oxidation Bacteria) (Zhang et al., 2014) slightly increased in Phase III.

The total number of Archaea was almost the same for each experimental Phase. However, a shift from hydrogenotrophic methanogens to methylotrophic and acetoclastic methanogens was detected. *Methanobrevibacter* that is a hydrogenotrophic methanogen already found by several studies in anaerobic digesters (Liu & Whitman, 2008), was the most relevant genus and had a decreasing trend from Phase I to III. On the contrary, *Methanosphera, Methanosarcina* and *Methanosaeta* increased from Phase I to III. *Methanosphera* genus is composed by obligate methylotrophic and hydrogenotrophic methanogens specialized to reduce methyl groups with $H_2$. Their metabolism is restricted to methanol (Liu & Whitman, 2008). The genus *Methanosarcina* is frequently present in methanogenic sludge. They are, together with *Methanosaeta*, the only two genera known to use acetate for methanogenesis. However, *Methanosarcina* can also use methanol, methylamine or $H_2$ instead of acetate. *Methanosaeta* uses only acetate. They carry out an aceticlastic reaction that splits acetate, oxidizing the carboxyl-group to $CO_2$ and reducing the methyl group to $CH_4$ (Liu & Whitman, 2008).

## 5. Conclusions

In this study, the performances, in terms of COD and nutrient removals, and the microbial community structure of an ASSR in three different experimental phases have been discussed. Results showed that increasing $SRT_{ASSR}$ and IR had positive effect on both sludge reduction and carbon and nutrients removal processes. Working at $SRT_{ASSR}$ of 2.5 d and IR of 40%, a maximum sludge reduction of 66% was achieved together with high COD, ammonia nitrogen phosphorous and total nitrogen removal efficiencies, 88%, 90%, 87%, and 88% respectively. Concerning the microbial community structure, a large diversity of microbial populations was detected. In particular, our results showed an increasing selection of fermenting bacteria able to release EPS, sulphate-reducing bacteria, denitrifying phosphate accumulating bacteria and denitrifying bacteria as $SRT_{ASSR}$ decreased from 10d to 2.5 d and the IR increased from 10% to 40%.

# V. Chapter

# Temperature effects on PAO and DPAO activity in Anaerobic Side-Stream Reactor

# Temperature effects on PAO and DPAO activity in Anaerobic Side-Stream Reactor

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ABSTRACT

In this study, the effect of the temperature on total phosphorous accumulating organisms (TPAOs), both aerobic phosphorous accumulating organisms (PAOs) and anoxic denitrifying phosphorous accumulating organisms (DPAOs) were investigated. Four different temperatures, 5, 10, 15 and 20°C were tested in batch assays using a selected biomass from an ASSR process performed at lab-scale at room temperature. Batch tests were carried out in anaerobic, aerobic and anoxic conditions to evaluate the phosphorous release of TPAOs, the uptake of PAOs and the uptake of DPAOs, respectively. Results showed that the phosphorous release and uptake kinetics were influenced from the variation of the temperature, while temperature did not influence significantly the anaerobic and the anoxic processes stoichiometry. In general, decreasing the temperature, a decreasing in the P-uptake and release rates was observed.

In anaerobic conditions, the P-release rate was 0.06, 0.08, 0.20 and 0.30 mg $PO_4^{3-}$-P/ (g TSS h) at 5, 10, 15 and 20 °C, respectively. Under aerobic conditions the P -uptake was 0.95, 1.47, 2.41 and 4.53 mg $PO_4^{3-}$-P/ (g TSS h) at 5, 10, 15 and 20 °C, respectively. In anoxic conditions the P- uptake was 0.24, 0.53, 1.55 and 3.01 mg $PO_4^{3-}$-P/ (g TSS h) at 5, 10, 15 and 20 °C, respectively. Arrhenius temperature coefficients θ for anaerobic, aerobic and anoxic metabolism were found to be 1.114, 1.121 and 1.165, respectively. Results revealed that DPAO activity was more affected by a lower temperature than PAO activity as a higher Arrhenius coefficient was estimated.

*Keywords:* Anaerobic side-stream reactor, temperature, PAO, DPAO.

## 1. Introduction

Temperature is a very important parameter in wastewater treatment because of its effect on chemical reactions, on the physicochemical properties of wastewater compounds and on the reaction rates of biological processes such as for phosphorous and nitrogen removal. Temperature exerts several effects on biological reactions, by influencing the rates of enzymatically catalyzed reactions and by affecting the rate of diffusion of substrate to the cells (Grady et al. 1999). The dependence of microbial activity on temperature may be strongly affected by the kind of bacteria used. Any species' response to temperature is characterized by upper and lower limits of temperature for growth (Morita 1975). In general, it is expected that reaction rate coefficients of biological process have temperature dependencies which can be expressed by a simplified Arrhenius equation (Gujer et al. 1995), increasing with temperature up to an optimum. Temperatures below the optimum typically have a more significant effect on growth rate than temperatures above the optimum (Mulkerrins et al. 2004). The reason for the upper limit of temperature is relatively well understood, and it is related to the increasing rate of denaturation of particular cell components as temperature rise, with consequent disruption of cellular function.

However, some degrees of adaptation are possible, but limited. The lower temperature limit for growth is linked to a loss of efficiency of transport proteins embedded in the membrane, and thus to a loss of affinity for substrates (Nedwell 1999).

The influence of the temperature on the biological phosphorus removal (BPR) process has been studied in several publications since the 1980s, but there are conflicting reports that describe its effects (Brdjanovic et al. 1997). Bacteria able to take up and store polyphosphate are predominant microorganisms in an efficient enhanced biological phosphorus removal (EBPR) process (Welles et al. 2015). The EBPR system is based on enrichment of total polyphosphate-accumulating organisms (TPAOs) through recycling of the sludge between anaerobic and aerobic zones (Toerien et al. 1990; Kortstee et al. 1994; Lee et al. 2003). Usually, aerobic PAOs have been identified in EBPR systems, capable to release phosphate under anaerobic condition, while consuming it under aerobic conditions. Recently, it was demonstrated that, not only under aerobic conditions but also under anoxic conditions, i.e., with nitrate as the electron acceptor, denitrifying phosphorous accumulating organism (DPAO) are capable of polyphosphate accumulation (Jørgensen and Pauli 1995).

Thus, the following metabolic pathways have been identified (Mino et al. 1998): (i) under anaerobic conditions, acetate or other low-molecular-weight organic compounds are converted to polyhydroxyalkanoates (PHAs), while intracellular stored polyphosphate (Poly-P) and glycogen are degraded, and phosphate, generated from Poly-P degradation, is released into the bulk liquid; (ii) under aerobic or anoxic conditions, the stored PHAs are converted to glycogen, phosphate is assimilated, and Poly-P is intracellular produced using oxygen and nitrate/nitrite as electron acceptor, respectively. Under aerobic or anoxic conditions, bacterial growth and phosphate uptake are regulated by the energy released from the breakdown of PHAs.

Several studies reported that high temperatures, in the range between 20 – 37°C, could improve the BPR efficiency (Yeoman et al. 1988; McClintock et al. 1993; Converti et al. 1995). Others reported that good or even comparatively better P-removal efficiency could be reached at lower temperature in the range between 5-15 °C (Florentz et al. 1987). However, the effects of temperature on the stoichiometry and kinetics of the BPR processes had not been studied in great detail. To the best of our knowledge, up to now results on temperature effect on BPR are referred the phosphorous release process in anaerobic condition and the phosphorous uptake process in aerobic conditions. On the contrary, studies on the effect of temperature on DPAOs activity are missing.

Brdjanovic et al. (1997, 1998) suggested that the contrasting results obtained so far on the temperature effects on BPR could be explained by the use of different substances, activated sludge

and measurements methods. The Authors, probably for the first time, studied the temperature effects (over a range 5 – 30 °C) on stoichiometry and kinetics of the process in the anaerobic and aerobic phase of the BPR process under definite laboratory conditions. Studying the convention of phosphate, acetate and polyhydroxybutyrate (PHB, the most common type of PHA), they founded that the stoichiometry of the anaerobic process, relate to the P-release, was insensitive to temperature changes while some effects on aerobic stoichiometry, relate to the P-uptake, were observed. On the contrary, temperature had a strong influence on the kinetics of the processes under anaerobic as well as under aerobic conditions At 5 and 10°C an incomplete P-uptake was observed in the aerobic phase, while a complete P-uptake at 20 and 30°C was measured. Under anaerobic conditions, phosphorous release activity increased from 5°C to 20°C, reaching a maximum at 20°C. Based on these results, the Authors calculated the anaerobic and aerobic temperature coefficients θ from short-term steady state experiments that were found to be 1.055 and 1.065, respectively (Brdjanovic et al. 1998)..

Temperature coefficients obtained in the study performed by Brdjanovic et al. (1998) were used by Meijer (2004), in the range between 5 and 30°C, to create a new extended model for BPR, that was a combination of the activated sludge model no. 1 (ASM1) and the Delft University of Technology EBPR model (TUDP). As reported by Henze et al. (2008), this model was successfully applied for simulation of EBPRs systems (Brdjanovic et al. 2007; Pinzon et al. 2007) and for developing an anaerobic and aerobic metabolic models that incorporated the carbon source, temperature and pH dependences of TPAOs and (glycogen-accumulating organisms) GAOs (Lopez-Vazquez et al., 2009a,). Results of this study showed that for high pH (7.5), and temperature lower than 20°C TPAOs tended to be the dominant microorganisms and, therefore, beneficial for the BPR. Krishna and Van Loosdrecht (1999) investigated the effects of temperature on the kinetics of PHB formation and consumption in a sequencing batch reactor (SBR) with alternate anaerobic/aerobic phases. The authors showed that the accumulation of storage polymers strongly depended on temperature, with less PHB formation at higher temperatures. According to Mulkerrins et al. (2004), all these investigations allow to define clearly that phosphorous removal could be negatively affected by low temperatures.

As recently demonstrated in (Ferrentino et al., 2016 c), BPR also plays an in important role in the biological reduction of sludge production in the activated sludge - anaerobic side-stream reactor (AS –ASSR) system, where a high percentage of sludge reduction (66%) has been obtained by the implementation of a lab-scale ASSR at room temperature (20°C). A new sludge reduction mechanism based on simultaneous biological processes such as sludge decay, cell lysis, EPS destructuration and the selection of a particular microorganisms such as sulphate reducing

bacteria (SRBs), total phosphate accumulating organisms (TPAOs) and denitrifying phosphate accumulating organisms (DPAOs), has been proposed (Ferrentino et al. 2016c). Even if the high reduction of excess sludge was obtained as a combination of mechanisms, the selection of TPAOs and in particular of DPAOs, characterized by low growth yield and involved in phosphorus and nitrogen removal processes, is of primary importance.

As recently reviewed by (Ferrentino et al. 2016b), most of the literature studies on ASSR were performed at controlled temperature ranging between 18 – 20°C, and effects of temperature on the ASSR has never been investigated. Thus, as one of the key parameters is the biological activity of TPAOs and DPAOs, one of the main questions that may rise is: how the temperature could affect the activity of TPAOs and DPAOs? In this light, the main goal of this study was to establish the short term temperature effects on anaerobic, aerobic and anoxic metabolisms of polyphosphate-accumulating organisms. Four different temperatures, 5, 10, 15 and 20°C were tested in batch assays using a selected biomass from an ASSR process performed at lab-scale at room temperature.

## 2. Materials and methods

### 3.1 *Phosphate accumulating bacteria culture*

A phosphate accumulating bacteria culture was developed in an ASSR, previously described by the Authors (Ferrentino et al., 2016 a, 2016 c). The solid retention time of the ASSR ($SRT_{ASSR}$) was 2.5 d and the interchange ratio (IR) between the SBR and the ASSR was 40%.

The ASSR having a working liquid volume of 10 liters was completely mixed and equipped with a mechanical stirrer. To ensure anaerobic conditions the ASSR was covered with a plastic plate. The lab-scale system operated at room temperature ranged between 18 and 21°C. ORP and pH in the ASSR were monitored and left free to vary. The characteristics of the ASSR biomass are reported in Table 1.

Table 0-1. Characteristics of the biomass used as culture

| Parameter | ASSR |
|---|---|
| **Soluble COD** | 43 mg $L^{-1}$ |
| **Ammonium Nitrogen** | 30 mg $N\text{-}NH_4$ $L^{-1}$ |
| **Nitrate** | 0.1 mg $N\text{-}NO_3$ $L^{-1}$ |
| **Soluble phosphorous** | 32 mg $P\text{-}PO_4^{3-}$ $L^{-1}$ |
| **Sulfate** | 13 mg $SO_4$ $L^{-1}$ |
| **Total suspended solids (TSS)** | 8.5 g $L^{-1}$ |
| **pH** | 7.4 |

### 3.2 *Batch phosphate accumulating bacteria assay*

The assays were performed in a double jacked laboratory reactor with a maximal operating volume of 2.0 L and a working volume of sludge of 1.5L, each mixed with a magnetic stirred (150 rpm). The reactors were inoculated with the anaerobic sludge from an ASSR lab-scale plant where phosphate accumulating organisms were previously detected (Ferrentino et al., 2016c). Concentration of the total suspended solid (TSS) in the batch test reactor was approximately 8.5 gTSS/L. Each test lasted at least 12 hours, and it was composed by three different phases: anaerobic (240 min), aerobic (at least 240 min), and anoxic (at least for 240 min). The initial pH value was that of the sludge in the ASSR, and equal to 7.4. The final pH value of each phase was always measured in order to check that it was maintained in the optimal range for the biological activity (7.0 - 7.5). The batch tests were sampled every 30 minutes. The samples were immediately vacuum filtered on 0.45 µm membrane filters and analyzed. A summary of operative conditions is reported in Table 2.

#### *2.2.1. Anaerobic phase*

The anaerobic phase was performed to evaluate the total release of phosphate by TPAOs, both PAOs and DPAOs. $N_2$ gas was introduced to the reactor at the beginning of the anaerobic experiments. DO and ORP were continually monitored. Nitrate was also monitored during the experiment to ensure that anaerobic conditions were present. Working temperatures in the batch reactor (5, 10, 15 or 20 °C) were set 1 h before the beginning of the test, in order to acclimatize the biomass. After this period, acetate was added to the batch reactor as substrate for TPAOs. In accordance to the procedure proposed by Brdjanovic et al. (1997), the amount of acetate added to the batch reactor was less, but not limiting, at lower temperature (from 350 mg COD/L at 20°C to 250 mg COD/L at 5°C) in order to obtain a similar acetate uptake at each temperature without changing the duration of the anaerobic phase. At the beginning of the anaerobic phase a concentration of 50 mg/L of sulphate was further added in order to enhance the activity of SRBs. Anaerobic conditions were maintained for 240 min. For each temperature, the anaerobic phase was double performed in two reactors (test A, B).

#### *2.2.2. Aerobic phase*

In the Test A, the aerobic phase was performed to evaluate the aerobic total phosphorous uptake rate of TPAOs. Aerobic conditions were ensured by sparging air through the bulk liquid using an aquarium air stones. The resulting DO concentration was about 5.5 mg $O_2$/L. The aerobic

phases was carried out until no further changes in concentration of $PO_4^{3-}$-P could be observed and lasted at least for 240 min.

*2.2.3. Anoxic phase*

In the Test B, the anoxic phase was performed in order to evaluate the denitrifying phosphorous uptake rate of DPAOs. Anoxic conditions were obtained by adding 30 mg $NO_3$-N/L to the bulk solution. DO and ORP were continually monitored during the entire experimental test. The anoxic phase was carried out until no further changes in concentration of $PO_4^{3-}$-P could be observed and lasted at least for 240 min. other anaerobic reactor, at the end of the anaerobic phase, the anoxic phase was performed in order to evaluate the denitrifying phosphorous uptake rate of DPAO. Anoxic conditions were obtained adding 30 mg $NO_3$-N/L to the bulk solution. DO and ORP were continually monitored during the entire experimental test. The anoxic phases was carried out until no further changes in concentration of $PO_4^{3-}$-P could be observed and lasted at least for 240 min.

Table 0-2. Batch tests: operative parameters

| **Test** | **Phases** | *5°C* | *10°C* | *15°C* | *20°C* |
|---|---|---|---|---|---|
| **Test A (TPAO)** | *Anaerobic* | 250 mg COD/L*<br>50 mg $SO_4$-S/L* | 300 mg COD/L*<br>50 mg $SO_4$-S/L* | 325 mg COD/L*<br>50 mg $SO_4$-S/L* | 350 mg COD/L*<br>50 mg $SO_4$-S/L* |
| | *Aerobic* | 5.5 mg $O_2$/L | 5.5 mg $O_2$/L | 5.5 mg $O_2$/L | 5.5 mg $O_2$/L |
| **Test B (DPAOs)** | *Anaerobic* | 250 mg COD/L*<br>50 mg $SO_4$-S/L* | 300 mg COD/L*<br>50 mg $SO_4$-S/L* | 325 mg COD/L*<br>50 mg $SO_4$-S/L* | 350 mg COD/L*<br>50 mg $SO_4$-S/L* |
| | *Anoxic* | 30 mg $NO_3$-N/L | 30 mg $NO_3$-N/L | 30 mg $NO_3$-N/L | 30 mg $NO_3$-N/L |

* After the first 60 min

## 3.3 *Chemical analysis*

Total suspended solids (TSS), total chemical oxygen demand (COD) were measured according to the Standard Methods (APHA, 2005). The samples were filtered through 0.45 µm membrane filters. The filtrate was analyzed for Soluble COD (sCOD), ammonium nitrogen ($NH_4^+$-N), nitrate as nitrogen ($NO_3^-$ -N), soluble phosphorous ($PO_4^{3-}$-P) and Sulfate ($SO_4^{2-}$-S). $NH_4^+$-N, $NO_3^-$-N and $PO_4^{3-}$-P concentrations were determinate according to (APAT CNR IRSA, 2003). Sulfate were analyzed by ion chromatograph (DIONEX ICS-100) equipped with AS9-HC column. COD in the form of sodium Acetate ($CH_3COONa$), Nitrate in the form of sodium nitrate ($NaNO_3$) and Sulphate in the form of Sulphuric Acid ($H_2SO_4$) were added at the required final concentration.

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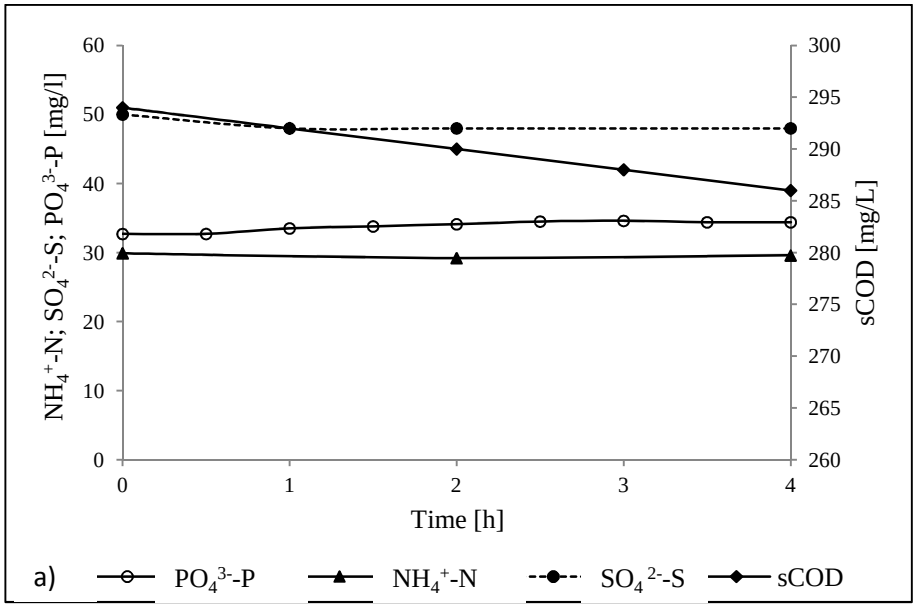
## 3. Results and discussion

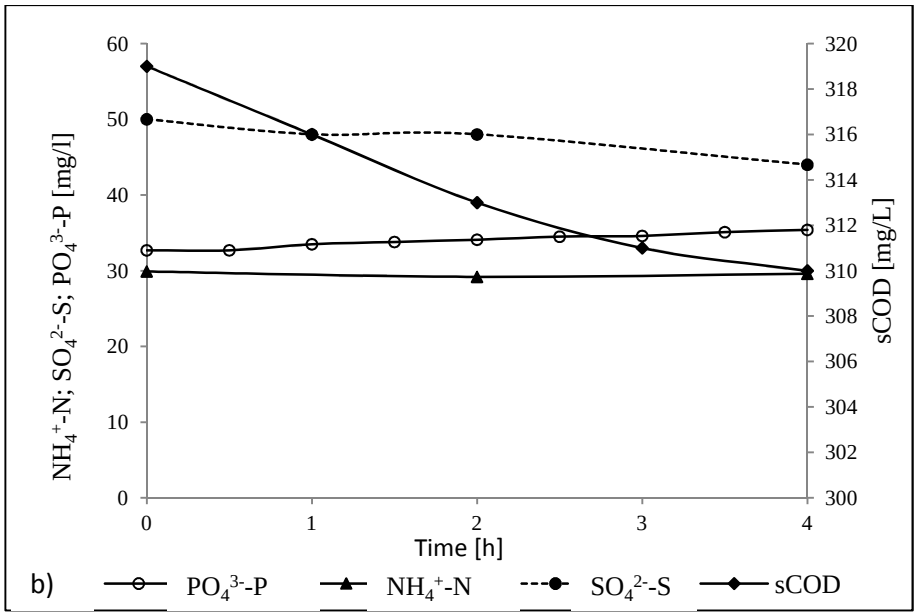
### 3.1 *Effect of temperature on P release*

Under anaerobic conditions, TPAOs do not grow, but store acetic acids as PHB through the cleavage of Poly-P with the associated release of phosphate in the bulk solution (Grady et al. 1999). Thus, the orthophosphates profiles in the anaerobic phase have been related to the anaerobic metabolism of TPAOs. The assays testing the effect of temperature on P- release lasted for 5 hours in anaerobic conditions.

The final TSS of the biomass at 5, 10, 15 or 20 °C was 8.5, 8.4, 8.5 and 8.4 g/L, respectively. Fig. 1 shows the distribution of orthophosphate, sCOD during the anaerobic period for all culture series. Nitrate concentration was monitored during the entire experimental period and its value was always equal to zero.

Looking at the graphs it becomes clear that both the P- release and the COD uptake changed largely with a different temperature. In each assay, the added acetate was partially consumed, corresponding to sCOD removal efficiencies of 2%, 3%, 9% and 10% at 5, 10, 15 and 20°C, respectively.

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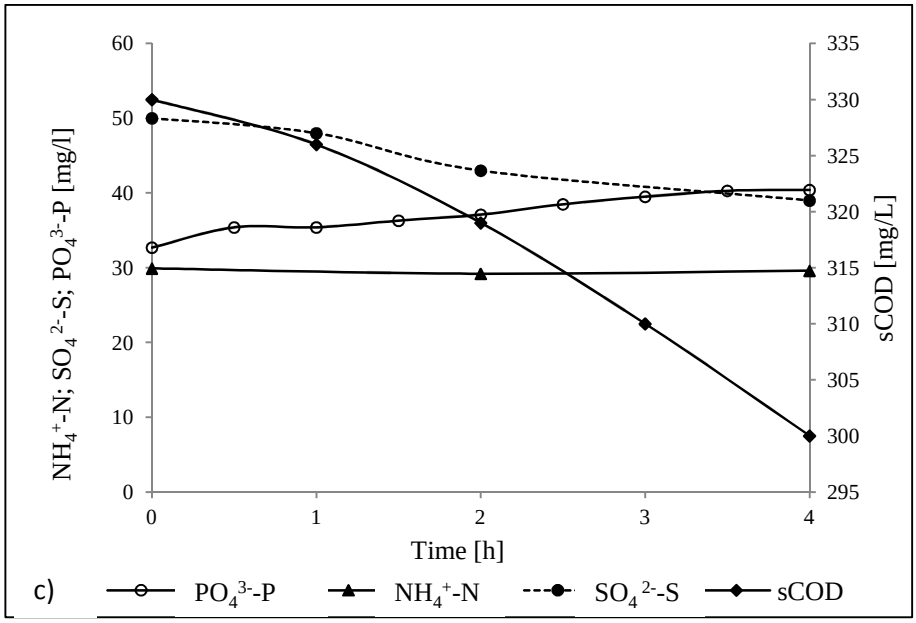
318
316
314
312
310
308
306
304
302
300
0
1
2
3
4
Time [h]
NH4+-N; SO4 2--S; PO4 3--P [mg/l]
sCOD [mg/L]
b)
PO4 3--P
NH4+-N
SO4 2--S
sCOD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30
20
10
0
335
330
325
320
315
310
305
300
295
0
1
2
3
4
Time [h]
NH4+-N; SO4 2--S; PO4 3--P [mg/l]
sCOD [mg/L]
c)
PO4 3--P
NH4+-N
SO4 2--S
sCOD

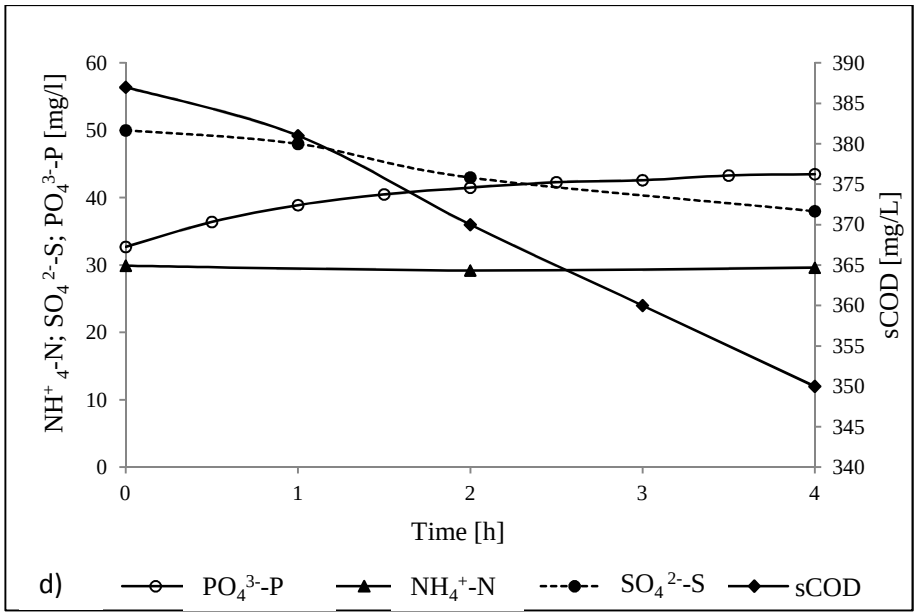



Fig. 0.1 Temperature effects on P-release in anaerobic phase: a) 5°C; b) 10°C; c) 15°C; d) 20°C

At 5°C (Fig. 1 a) and 10°C (Fig. 1 b) and the specific P- release rates were very low and equal to 0.06 mg $PO_4^{3-}$-P/(g TSS h) and 0.08 mg $PO_4^{3-}$-P/(g TSS h), respectively. At 15°C (Fig. 1 c) the specific P release rate increased up to 0.20 mg $PO_4^{3-}$-P/ (g TSS h), reaching the maximum value of 0.30 mg $PO_4^{3-}$-P/ (g TSS h) at 20°C. Thus, data showed that the P release rate at 15, 10 and 5°C was 33, 73 and 81% lower than that measured at 20°C, respectively.

Similarly, the rate of sCOD uptake was very low both at 5 and 10°C, accounting for 0.23 mg sCOD/ (gTSS h) and 0.27 mg sCOD/ (gTSS h), respectively. The rate of sCOD uptake increased at 15 and 20°C, reaching values of 0.89 and 1.15 mg sCOD/ (gTSS h). The sCOD uptake rate at 15, 10 and 5°C was 23, 77 and 80% lower than that measured at 20°C, respectively.

COD consumption took into account both SRBs and TPAOs activities. The stoichiometric ratios between the $PO_4^{3-}$-P released and the sCOD consumed at each experimental temperature were listed in Table 3.

Table 0-3. Stoichiometric ratio $PO_4^{3-}$-P release/sCOD uptake

| **Temperature [°C]** | 5 | 10 | 15 | 20 |
|---|---|---|---|---|
| **$PO_4^{3-}$-P release/sCOD uptake** | 0.26 | 0.29 | 0.22 | 0.26 |

Smolders et al., (1994) obtained similar results for a sludge from a MBR, where for 1 C-mol of acetate 0.5 P-mol phosphate was released in anaerobic conditions, obtaining a $PO_4^{3-}$-P/acetate ratios of about 0.25. According to Brdjanovic el at (1998), our results from short-term test showed that the stoichiometry of the anaerobic phase was relatively insensitive to temperature changes. However, the stoichiometric ratios measured in this study were lower than values reported by

Brdjanovic el at. (1998) who measured $PO_4^{3-}$-P/acetate ratios of about 0.4. The lower ratios measured in our tests may be related to the additional COD consumption by SRB.

Thus, sulphate concentration was monitored during each batch test as a marker of the SRBs activity. Results from the short term experiments showed that low temperatures affected also SRBs activity. The sulphate uptake rate was 0.05, 0.17, 0.33 and 0.38 mg $SO_4^{2-}$-S/ (gTSS h) at 5, 10, 15 and 20°C. For temperature equal to 20 and 15°C, the sulphate uptake differed only for 13%. However, decreasing the temperature to 10 and 5°, the percentage increased significantly accounting for 55% and 87%, respectively.

Comparing these results of sulphate uptake with those directly measured in the lab-scale ASSR at 20°C (Ferrentino et al. 2016a, 2016c), a higher value (0.74 mg $SO_4^{2-}$-S/ (gTSS h)) was found in the ASSR than the value obtained in the present study at 20°C.

Currently, SRBs can be divided into two main groups: those that degrade organic compounds incompletely to acetate and those that degrade organic compounds completely to carbon dioxide, which commonly also use acetate as a growth substrate (Muyzer and Stams 2008b). In this study, sodium acetate was selected as a carbonaceous substrate because it is readily biodegradable by most heterotrophic populations, among them TPAOs. Nevertheless, when the settled sludge is recycled from the AS to the ASSR in the lab scale plant, more complex volatile fatty acids could be present, thus enhancing the activity of SRBs and justifying the higher sulphate uptake rate (Ferrentino et al. 2016a, 2016c). Ammonia concentration was further monitored and was always constant during the anaerobic phase in all batch assays. Thus, unlike in the ASSR, these results indicated that the sludge decay and EPS destructuration processes were negligible during the short term experiments.

### 3.2 *Effect of temperature on P uptake in aerobic phase*

In the aerobic assays the orthophosphates profiles can be related to the aerobic metabolism of TPAO. Under aerobic conditions, PAOs use the stored PHBs as carbon and energy sources to grow and to assimilate orthophosphates to synthesize poly-P, using oxygen as electron acceptor. In the aerobic conditions, our results showed that TPAO activity was influenced by the temperature as well as in anaerobic conditions but in a less significant way.

Figure 2 shows the aerobic phosphorous uptake at 5, 10, 15 and 20°C.

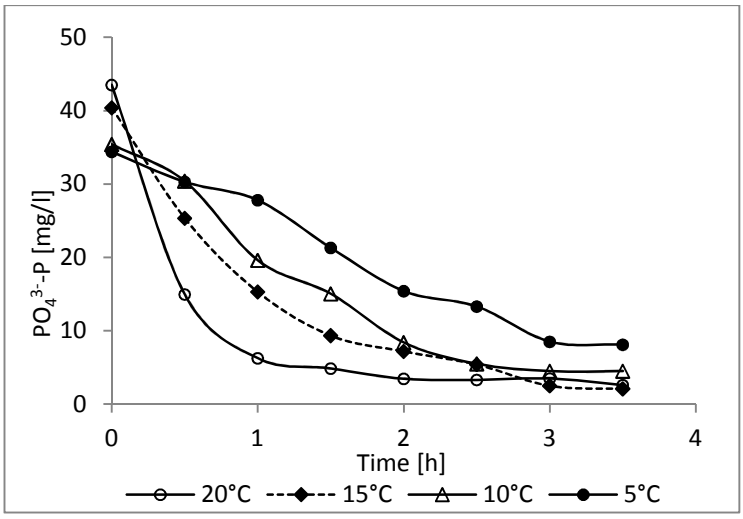
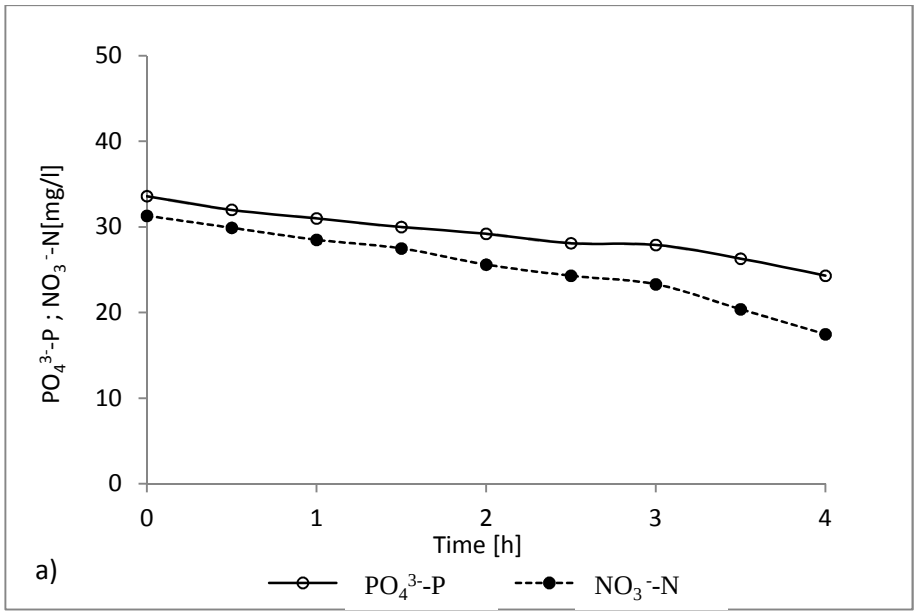



Fig. 0.2 Temperature effects in aerobic phase at 5, 10, 15 and 20 °C

The phosphorous uptake at 20 and 15°C had an exponential trend. On the contrary, at 10 and 5°C the phosphorous uptake had a fairly linear trend. The specific P uptake rate was 4.53 mg $PO_4^{3-}$-P/ (g TSS h) at 20°C. At 15°C, the P uptake rate decreased down to 2.41 mg $PO_4^{3-}$-P/ (g TSS h). At 10°C and 5°C, the P uptake was 1.47 mg $PO_4^{3-}$-P/ (g TSS h) and 0.95 mg $PO_4^{3-}$-P/ (g TSS h), respectively. A reduction of 48%, 67% and 79% was observed at 15, 10 and 5°C, respectively. After 3.5 h the same $PO_4^{3-}$-P concentration of 3.0 mg $PO_4^{3-}$-P /L was reached both at 20 and 15°C. On the contrary, at 10°C and 5°C, the final $PO_4^{3-}$-P concentrations were higher, and equal to 5.0 and 10.0 mg $PO_4^{3-}$-P /L, respectively.

### 3.3 *Effect of temperature on P uptake in anoxic phase*

In anoxic environment the orthophosphates profile could be associated to the anoxic metabolism of DPAOs. Under anoxic conditions DPAOs degrade stored PHB accumulated in the previous anaerobic phase using nitrate, or eventually nitrite, as electron acceptor. DPAOs produce glycogen and take up phosphate from the mixed liquor. The trends of the phosphorus uptake at different temperatures showed that the anoxic DPAOs metabolisms could be negatively affected by the low temperature, more than the aerobic TPAO metabolism. Figure 3 shows the anoxic phosphorous uptake at 5, 10, 15 and 20°C. The added nitrate was totally consumed only at 20°C. At the other temperatures, the added nitrate was partially consumed up to 44%, 68% and 84% at 5, 10 and 15°C, respectively.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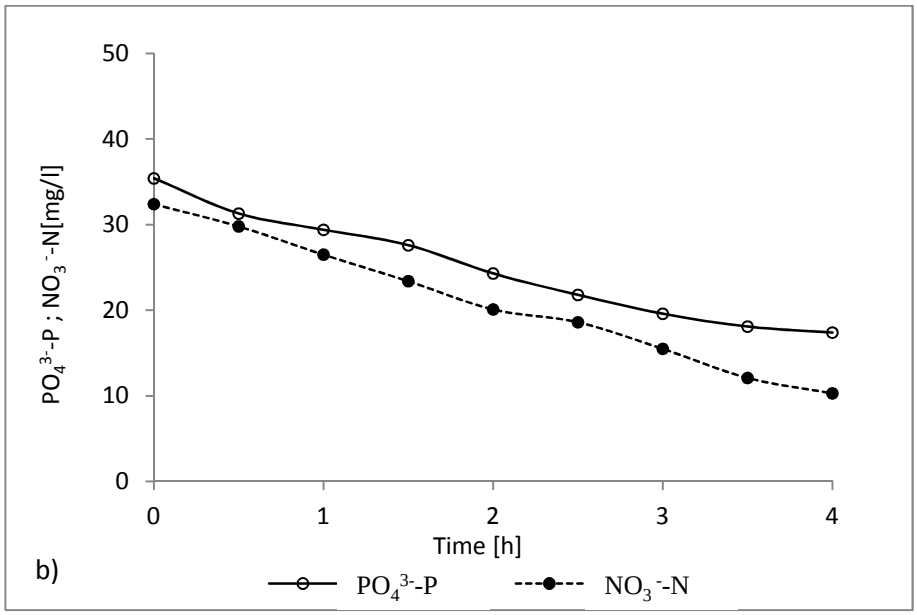
Time [h]
$PO_4^{3-}$-P ; $NO_3^-$-N[mg/l]
a)
$PO_4^{3-}$-P
$NO_3^-$-N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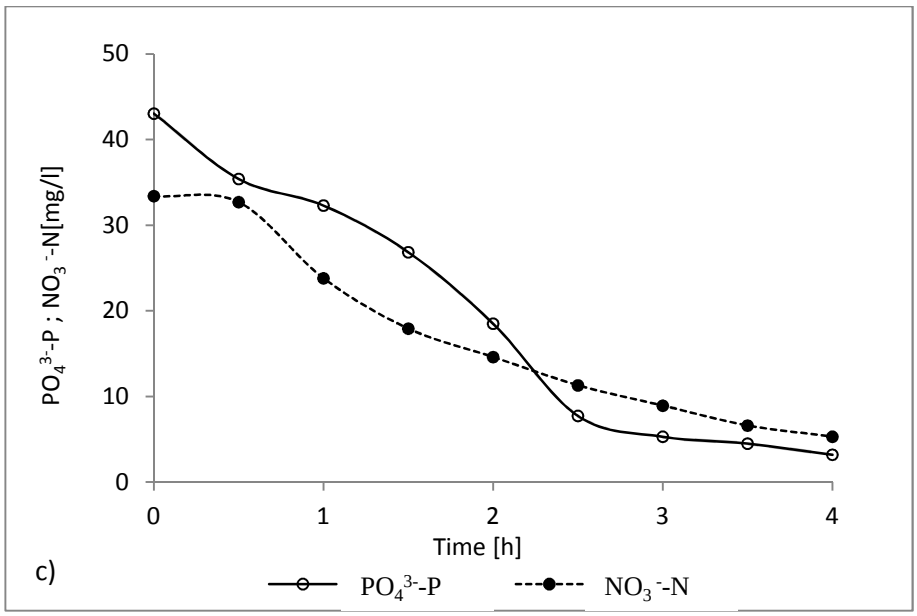
Time [h]
$PO_4^{3-}$-P ; $NO_3^-$-N[mg/l]
b)
$PO_4^{3-}$-P
$NO_3^-$-N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Time [h]
$PO_4^{3-}$-P ; $NO_3^-$-N[mg/l]
c)
$PO_4^{3-}$-P
$NO_3^-$-N

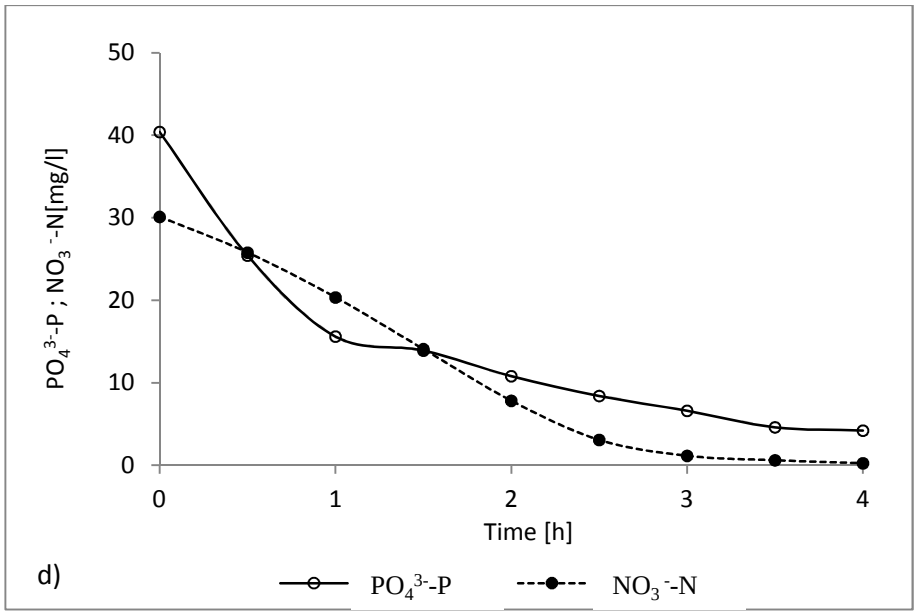


Fig. 0.3 Temperature effects in anoxic phase at a) 5 °C; b) 10 °C; c) 15 °C; d) 20 °C

Our results showed that the anoxic P- uptake kinetic was influenced by the temperature. At 5 and 10°C the P- uptake was equal to 0.24 mg $PO_4^{3-}$-P/ (g TSS h) (Fig. 3 a) and 0.53 mg $PO_4^{3-}$-P/ (g TSS h) (Fig. 3 b), respectively. At 15°C the P -uptake rate was 1.55 mg $PO_4^{3-}$-P/ (g TSS h) (Fig. 3 c), increasing up to 3.01 mg $PO_4^{3-}$-P/ (g TSS h) at 20°C. Thus the P -uptake rate was 48, 82 an 92% lower than the value measured at 20°C. As for the aerobic phase, the phosphorous uptake at 20 and 15°C had an exponential trend which became linear at 10 and 5°C.

The stoichiometric ratios between the $PO_4^{3-}$-P uptake and the $NO_3^-$-N consumed at each experimental temperature were listed in Table 4.

Table 0-4. Stoichiometric ratio $PO_4^{3-}$-P uptake/ $NO_3^-$-N consumed

| **Temperature [°C]** | 5 | 10 | 15 | 20 |
|---|---|---|---|---|
| **$PO_4^{3-}$-P uptake/ $NO_3^-$-N consumed** | 0.07 | 0.10 | 0.19 | 0.29 |

On the contrary of the anaerobic phase, our results showed that the stoichiometry of the anoxic metabolism was strongly influenced by temperature changes. In particular, he $PO_4^{3-}$-P uptake/ $NO_3^-$-N consumed ratio became greater as the temperature increases.

### 3.4 *Temperature coefficients*

The temperature coefficient θ was calculated for each reaction rate using the simplified Arrhenius equation for the temperature dependency. The simplified Arrhenius expression was used to describe the effect of the temperature both on phosphorus release and on sulphate consumption in anaerobic tests, and on phosphorus uptake in aerobic and anoxic tests:

$$r_T = r_{20} \cdot \theta^{(T-T_{20})} \quad (1)$$

where T is the temperature in °C, $r_T$ is the kinetic parameter (P release rate, P uptake rate or S uptake rate) at temperature T, T20 is the reference temperature (20°C), $r_{20}$ is the kinetic parameter at temperature equal to 20°C, and $\theta$ is the Arrhenius temperature coefficient.

From short term experiments, calculated temperature coefficients for the anaerobic, aerobic and anoxic metabolisms of TPAOs were equal to 1.114, 1.121 and 1.165, respectively. Brdjanovic et al. (1997, 1998) for short-term steady state experiments, showed that temperature had a moderate impact on the anaerobic P-release process rate (θ=1.071) and on the aerobic P-up-take process rate (θ=1.032). A strong temperature effect in this study has been evaluated on both the anaerobic P-release process rate (θ=1.114) and on the aerobic P-up-take process rate (θ=1.121). However, similarly to Brdjanovic et al. (1997, 1998), the aerobic metabolisms of PAO was higher influenced by temperature as compared to the anaerobic metabolism of TPAOs. Concerning the anoxic metabolism of DPAOs, for the first time, our data showed that the DPAOs are more sensitive to the temperature than PAO. Our results showed that temperature has a high impact on the biological phosphorus removal.

On the contrary, temperature had a lower effect on SRBs activity. The calculated temperature coefficient for the sulphate reduction process in the anaerobic phase was 1.087.

In the present study, in anaerobic, aerobic and anoxic conditions lower temperature coefficients have been founded than those of other biological processes involved in an AS-ASSR system. For instance, the fermentation process and the sulphate reduction temperature coefficients were 1.070 (Henze et al. 2000) and 1.087, respectively. Thus, it can be hypothesized the biological phosphorus removal process as the limiting step of the sludge reduction process at low temperature.

## 4. Conclusions

Batch tests under anaerobic, anoxic and aerobic phase were performed at 20, 15, 10 and 5°C to evaluate the stoichiometric and kinetic effects of the temperature on phosphorous release and uptake. The stoichiometry of the processes was evaluated under anaerobic and anoxic conditions. Results showed that the stoichiometry of the anoxic metabolism of DPAOs was strongly sensitive to the temperature; on the contrary temperature changes seem to have no negative effects on the anaerobic metabolism of TPAOs. Concerning the kinetic aspects, anaerobic, aerobic and anoxic metabolisms of phosphorous accumulating organisms seem to be significantly affected by low temperatures. In particular, for temperature lower than 10°C the P- release rate in anaerobic

condition and the uptake in aerobic and anoxic condition was 73%, 67% and 82%, respectively, lower than those at 20°C. These percentages increased even more with decreasing temperature down to 5°C. Above all, the anoxic metabolism of DPAOs highlighted the highest sensitivity to the lowering temperature. Temperature had a lower effect on SRBs activity.

Given these results, the decrease in temperatures mainly compromised the activities of TPAOs, both PAOs and DPAOs and supposedly the efficiencies of the reduction of sludge in a AS-ASSR process. However, the consequent negative effect due to low temperatures in an ASSR could be exceeded by increasing the concentration of biomass in the ASSR, thus improving the P -release and uptake. By performing a simple mathematical simulation, results showed that down to15 °C it could be actually possible to enhance the P-release and uptake of each phase by increasing the biomass in the ASSR to about 16 g/L. At temperatures lower than 15°C, too high solid concentrations should be needed, making the solution not easily applicable. Future studies directly performed running an AS-ASSR at lower temperatures are necessary to evaluate the long term effect of temperature on TPAOs, both PAOs and DPAOs, SRBs, EPS destructuration and cell lysis and thus in the sludge reduction process.

# VI. Chapter
# Conclusions

# Conclusions

The aim of the thesis was to fill the gap of understanding between the mechanisms behind sludge minimisation and the proper operative conditions of the ASSR. An SBR-ASSR lab-scale system was implemented and tested for about 300 days in three different conditions varying the solid retention time (SRT) of the ASSR and the interchange rate (IR). Thus, three different phases were considered: 10% sludge interchange rate and SRT in the ASSR of 10 days; ii) 20% sludge interchange rate and SRT in the ASSR of 5 days and iii) 40% sludge interchange rate and SRT in the ASSR of 2.5 days.

The $Y_{obs}$ calculated in Phase I, II and III were 0.21 g TSS/g COD, 0.14 g TSS/g COD and 0.12 g TSS/g COD, respectively. Comparing these values to those of a reference system, the $Y_{obs}$ of the SBR-ASSR system was clearly reduced at each IR level. For instance, increasing the IR from 10%, to 20% up to 40%, the sludge yield was 42%, 61% and 66% less than the CAS reference system. The best result was obtained when the IR was equal to 40% and the SRT equal to 2.5 d. To explain this good results, several mechanisms were considered such as the sludge decay, the EPS destructuration and the selection of slow growing microorganism.

The profile of sCOD and $NH_4^+$-N in the ASSR confirmed that under anaerobic conditions the cell lysis mechanism occurred. Sludge could be hydrolyzed, enhancing the solubilisation and disintegration of the organic matter and nutrients. Biodegradable compounds, released in the ASSR, are substrate available for degradation, both in the ASSR and in the water line where aerobic and anoxic conditions are carried out, enhancing the cryptic growth. The increase of $NH_4^+$-N and sCOD could be associated to the release of proteins and polysaccharide, which are the major components of EPS, and could be used by bacteria as source of carbon and energy for cell growth. Both free-EPS (SMP) and bound – EPS (attached to the sludge flocs) had been analysed for proteins and polysaccharides at the end of each phase. Concerning the concentration of SMP, our results showed a strong increasing concentration of both protein and polysaccharides passing from Phase I to Phase III, possibly because of the higher IR in the ASSR. On the contrary, the bound EPS in the ASSR decreased from Phase I to III with the increasing of SMP concentration in the bulk liquid from Phase I to III. Thus, the increasing percentage of biomass cycled to the ASSR caused the bound EPS destruction. Concerning the role of slow growing microorganism, total phosphate accumulating organisms (TPAOs), denitrifying phosphate accumulating organisms (DPAOs) and sulphate reducing bacteria (SRBs) were investigated. During the three experimental phases the release of phosphate slowly increased.

This evidence suggests that the increasing percentage of biomass cycled thought the ASSR could enhance a microbial activity causing the selection of phosphate accumulating organisms.

These bacteria are able to accumulate polyphosphates (Poly-P) under aerobic and anoxic conditions and release them under anaerobic condition. Due to the cycling between aerobic, anoxic and anaerobic conditions in the SBR-ASSR carried out in our study, both the presence of TPAOs and DPAOs had been supposed. Thus, the increasing microbial activity of TPAOs and DPAOs during all the experimental study was evaluated with batch experiments performed at the end of each phase. Results of the batch tests revealed the ability of the ASSR to select DPAOs. Passing from the first to the third Phase the percentage of DPAOs over the TPAOs population strongly increased, reaching in the last phase up to 80% of TPAOs. Furthermore, the influent and effluent $SO^{2-}_4$–S concentrations of the ASSR were also monitored and a reduction of the sulphate concentration quite stable during all phases was observed. This evidence was probably related to a microbial activity of SRB.

All these results have led to propose a new mechanism of sludge reduction which connects all the mechanisms proposed so far as expressed as follow. The anaerobic SRT and the IR could be defined as two main operative parameters. Acting on them, the solubilization of the organic matter could be enhanced and a particular microbial community structure could be selected. In the ASSR several microorganisms were responsible of hydrolysis and fermentation reactions that could be addressed to the enhance of the sludge decay or of the EPS destruction. Others microorganisms such as sulphate reducing bacteria (SRBs) could be responsible for the incomplete degradation of complex organic molecules to acetate. This last organic by-product could be taken up by the phosphate accumulating organism (TPAOs) and denitrifying phosphate accumulating organisms (DPAO) in anaerobic conditions causing a release of phosphate in the solution. Acetate could be internally stored by TPAOs and DPAOs as a long chain of carbon molecules of polyhydroxyalkanoates (PHAs) and further used under aerobic and anoxic conditions, respectively, for their maintenance functions or for the growth of new cells.

Our results showed that the variation of the anaerobic SRT and IR could significantly affect the selection of both TPAO and DPAO in the ASSR. At low anaerobic SRT and high IR, DPAOs could reach up to 80-90% of the TPAOs population. The importance of the selection of DPAOs has always been linked to the possibility to carry out both the biological removal of phosphorous and nitrate because they use nitrate as electron acceptor. Furthermore, they play a fundamental role in the sludge reduction due to their low growth yield that is about 70% lower than TPAOs. Thus, enhancing the selection of DPAO could significantly increase the percentage of sludge reduction.

Even if the high reduction of excess sludge was obtained as a combination of mechanisms, the selection of DPAOs over the TPAOs population is of primary importance. One of the main questions that may arise is: how the temperature could affect the activity of TPAOs and DPAOs? Four different temperatures, 5, 10, 15 and 20°C were tested, Results showed that the stoichiometry of the anoxic metabolism of DPAOs was strongly sensitive to the temperature; on the contrary temperature changes seem to have no negative effects on the anaerobic metabolism of TPAOs. Concerning the kinetic aspects, anaerobic, aerobic and anoxic metabolisms of phosphorous accumulating organisms seem to be significantly affected by low temperatures. In particular, for temperature lower than 10°C the P- release rate in anaerobic conditions and the uptake in aerobic and anoxic condition were 73%, 67% and 82%, respectively, lower than values measured at 20°C. These percentages increased even more with decreasing temperature down to 5°C. Above all, the anoxic metabolism of DPAOs highlighted the highest sensitivity to the lowering temperature. Thus, low temperature could significantly affect the selection of DPAOs and TPAOs and then also the efficiency of the process in terms of sludge reduction. However, down to 15 °C it could be actually possible to enhance the P-release and uptake of each phase by increasing the biomass in the ASSR to about 16 g/L. At temperatures lower than 15°C, too high solid concentrations should be needed, making the solution not easily applicable. A lower temperature effect on the SRBs activity have been measured.

Concerning the performances of the entire SBR-ASSR system, under each experimental phase the process was effective in the removal of sCOD, TN, $NH_4^+$-N and $PO_4^{3-}$-P. The best carbon and nutrient removal was obtained under Phase III reaching an efficiency of 86%, 83%, 63% and 88% for sCOD, $NH_4^+$-N, $PO_4^{3-}$-P and TN removal, respectively.

The q-PCR analyses encoding 16 rRNA gene revealed a wide diversity of phylogenetic groups in each phase. The qPCR results showed that the abundance of total Bacteria increased in each experimental phase. An increasing selection of fermenting bacteria able to release EPS, denitrifying phosphate accumulating bacteria (DPAOs) and heterotrophic denitrifying bacteria was observed from Phase I to Phase III. Further, specific qPCR analyses targeted *apsA* gene showed an increase of sulphate reducing bacteria (SRBs) in Phase III. The total number of Archaea was almost the same for each experimental Phase. However, a shift from hydrogenotrophic methanogens to methylotrophic and acetoclastic methanogens was detected.

Definitively, the present work allowed the definition of the importance of DPAO selection over the entire TPAO population in the sludge reduction process.

However, further study with a bigger pilot-scale system to confirm the results and investigate every single aspect would be most needed in order to apply the process to full scale.

Based on the results of this PhD Thesis, a patent was presented entitled *Plant and methods for sludge reduction in wastewater treatment – UTN (University of Trento) System*. The invention (UTN System) relates to systems and methods for wastewater treatment in general and particularly to sludge treatment systems and methods that enable minimized sludge generation and reduction of effluent nutrients. The UTN wastewater treatment plant/system comprises: a mainstream reactor configured to perform alternate aerobic and anoxic phases (and optionally anaerobic) able to perform nutrient and organic matter removal; an ASSR in the returned sludge line configured to treat a portion of the settled or thickened sludge and configured to provide an anaerobic environment; a controller that implements the logics to manage the whole WWTP, developing the mechanisms of biological sludge reduction.

# References

# Reference


An K., Chen G. 2008. Chemical Oxygen Demand and the Mechanism of Excess Sludge Reduction in an Oxic-Settling-Anaerobic Activated Sludge Process. J. Environ. Eng. 134:469–477.

APAT-CNR-IRSA. 2003. Metodi analitici per le acque Volume Primo. .

APHA, AWWA, WEF. 2005. Standard methods for the examination of water and wastewater. Am. Public Heal. Assoc.

Bai H., Kang Y., Quan H., Han Y., Sun J., Feng Y. 2013. Bioremediation of copper-containing wastewater by sulfate reducing bacteria coupled with iron. J. Environ. Manage. 129:350–356.

Barker D.J., Stuckey D.C. 1999. A review of soluble microbial products (SMP) in wastewater treatment systems. Water Res. 33:3063–3082.

Beer M., Stratton H.M., Griffiths P.C., Seviour R.J. 2006. Which are the polyphosphate accumulating organisms in full-scale activated sludge enhanced biological phosphate removal systems in Australia? J. Appl. Microbiol. 100:233–243.

Bernardet J.-F., Segers P., Vancanneyt M., Berthe F., Kersters K., Vandamme P. 1996. Cutting a Gordian Knot: Emended Classification and Description of the Genus Flavobacterium, Emended Description of the Family Flavobacteriaceae, and Proposal of Flavobacterium hydatis nom. nov. (Basonym, Cytophaga aquatilis Strohl and Tait 1978). Int. J. Syst. Bacteriol. 46:128–148.

Bertolini V., Gandolfi I., Ambrosini R., Bestetti G., Innocente E., Rampazzo G., Franzetti A. 2013. Temporal variability and effect of environmental variables on airborne bacterial communities in an urban area of Northern Italy. Appl. Microbiol. Biotechnol. 97:6561–6570.

van den Bosch P.L.F., van Beusekom O.C., Buisman C.J.N., Janssen A.J.H. 2007. Sulfide oxidation at halo-alkaline conditions in a fed-batch bioreactor. Biotechnol. Bioeng. 97:1053 – 1063.

Brdjanovic D., Logemann S., Van Loosdrecht M.C.M., Hooijmans C.M., Alaerts G.J., Heijnen J.J. 1998. Influence of temperature on biological phosphorus removal: Process and molecular ecological studies. Water Res. 32:1035–1048.

Brdjanovic D., van Loosdrecht M.C.M., Hooijmans C.M., Alaerts G.J., Heijnen J.J. 1997. Temperature effects on physiology of biological phosphorus removal. J. Environ. Eng. 123:144–153.

Brdjanovic D., Mithaiwala M., Moussa M.S., Amy G., van Loosdrecht M.C.M. 2007. Use of modelling for optimization and upgrade of a tropical wastewater treatment plant in a developing country. Water Sci. Technol. 56:21–31.

Carrère H., Dumas C., Battimelli A., Batstone D.J., Delgenès J.P., Steyer J.P., Ferrer I. 2010. Pretreatment methods to improve sludge anaerobic degradability: a review. J. Hazard. Mater.

183:1–15.

Chen G., Liu Y. 1999. Modeling of energy spilling in subtrate - suffient cultures. J. Environ. Eng. 125:508–513.

Chen G., Yip W., Mo H., Liu Y. 2001. Effect of sludge fasting/feasting on growth of activated sludge cultures. Water Res. 35:1029–1037.

Chen G.-H., An K.-J., Saby S., Brois E., Djafer M. 2003. Possible cause of excess sludge reduction in an oxic-settling-anaerobic activated sludge process (OSA process). Water Res. 37:3855–66.

Chon D.-H., Park C. 2012. Method to reduce sludge generation in wastewater treatment systems. .

Chon D.H., Rome M., Kim H.-S., Park C. 2011a. Investigating the mechanism of sludge reduction in activated sludge with an anaerobic side-stream reactor. Water Sci. Technol. 63:93 – 99.

Chon D.H., Rome M., Kim Y.M., Park K.Y., Park C. 2011b. Investigation of the sludge reduction mechanism in the anaerobic side-stream reactor process using several control biological wastewater treatment processes. Water Res. 45:6021–9.

Christophersen C.T., Morrison M., Conlon M.A. 2011. Overestimation of the abundance of sulfate-reducing bacteria in human feces by quantitative PCR targeting the Desulfovibrio 16S rRNA gene. Appl. Environ. Microbiol. 77:3544–3546.

Chu L., Wang J., Wang B., Xing X.H., Yan S., Sun X., Jurcik B. 2009a. Changes in biomass activity and characteristics of activated sludge exposed to low ozone dose. Chemosphere. 77:269–272.

Chu L., Yan S., Xing X.-H., Sun X., Jurcik B. 2009b. Progress and perspectives of sludge ozonation as a powerful pretreatment method for minimization of excess sludge production. Water Res. 43:1811–22.

Chudoba P., Morel A., Capdeville B. 1992. The case of both energetic uncoupling and metabolic selection of microorganisms in the OSA activated sludge system. Environ. Technol. 13:761 – 770.

Coma M., Rovira S., Canals J., Colprim J. 2013. Minimization of sludge production by a side-stream reactor under anoxic conditions in a pilot plant. Bioresour. Technol. 129C:229–235.

Converti A., Rovatti M., Del Borghi M. 1995. Biological removal of phosphorus from wastewaters by alternating aerobic and anaerobic conditions. Water Res. 29:263–269.

Curtis B.-A., Doyle M., Marc Roehl P.J.P. 2007. Screening of inert solids from a low-yield wastewater treatment process. .

Curtis B.-A., Kutcher T., Marc E.R. 2011. Conditioning system for activated sludge wastewater treatment processes. .

Cyganecka A., Podedworna J., Żubrowska-sudoł M. 2011. Analysis of Methods for Determining

DPAO Fraction in Phosphorus Accumulating Organisms. 20:303–309.

Datta T., Liu Y., Goel R. 2009. Evaluation of simultaneous nutrient removal and sludge reduction using laboratory scale sequencing batch reactors. Chemosphere. 76:697–705.

DuBois M., Gilles K.A., Hamilton J.K., Rebers P.A., Smith F. 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28:350–356.

Edgar R.C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods. 10:996–8.

Fahrbach M., Kuever J., Remesch M., Huber B.E., Kampfer P., Dott W., Hollender J. 2008. Steroidobacter denitrificans gen. nov., sp. nov., a steroidal hormone-degrading gammaproteobacterium. Int. J. Syst. Evol. Microbiol. 58:2215–2223.

Fahrbach M. 2006. Denitratisoma oestradiolicum gen. nov., sp. nov., a 17beta-oestradiol-degrading, denitrifying betaproteobacterium. Int. J. Syst. Evol. Microbiol. 56:1547–1552.

Feng X.-C., Guo W.-Q., Yang S.-S., Zheng H.-S., Du J.-S., Wu Q.-L., Ren N.-Q. 2014. Possible causes of excess sludge reduction adding metabolic uncoupler, 3,3′,4′,5-tetrachlorosalicylanilide (TCS), in sequence batch reactors. Bioresour. Technol. 173:96–103.

Ferrentino R., Langone M., Gandolfi I., Bertolini V., Franzetti A., Andreottola G. 2016a. Shift in microbial community structure of anaerobic side-stream reactor in response to changes to solid retention time and interchange ratio. PhD Thesis. Chapter 4.

Ferrentino R., Langone M., Merzari F., Tramonte L., Andreottola G. 2016b. A review of Anaerobic Side-Stream Reactor for excess sludge reduction: configurations, mechanisms and efficiency. Crit. Rev. Environ. Sci. Technol. 46:382–405.

Ferrentino R., Langone M., Villa R., Andreottola G. 2016c. Simultaenous biological processes for excess sludge reduction in anaerobic side-stream reactor. PhD Thesis. Chapter 3.

Figueroa L. a, Silverstein J. 1992. The effect of particulate organic matter on biofilm nitrification. Water. Environ. Res. 64:728–733.

Florentz M., Caille D., Bourdon F., Sibony J. 1987. Biological phosphorus removal in France. Water Sci. Technol. 19:1171 – 1173.

Flowers J.J., He S., Yilmaz S., Noguera D.R., McMahon K.D. 2009. Denitrification capabilities of two biological phosphorus removal sludges dominated by different “Candidatus Accumulibacter” clades. Environ. Microbiol. Rep. 1:583–588.

Foladori P., Andreottola G., Ziglio G. 2010. Sludge Reduction Technologies in Wastewater Treatment Plants. IWA Publishing.

Frolund B., Griebe T., Nielsen P.H. 1995. Enzymatic activity in the activated-sludge floc matrix. Appl. Microbiol. Biotechnol. 43:755–761.

Gagliano a. L., Tagliavia M., D'Alessandro W., Franzetti a., Parello F., Quatrini P. 2015. So close, so different: geothermal flux shapes divergent soil microbial communities at neighbouring sites. Geobiology.:n/a–n/a.

Gao D.W., Zhang T., Tang C.Y.Y., Wu W.M., Wong C.Y., Lee Y.H., Yeh D.H., Criddle C.S. 2010. Membrane fouling in an anaerobic membrane bioreactor: Differences in relative abundance of bacterial species in the membrane foulant layer and in suspension. J. Memb. Sci. 364:331–338.

Ghasimi D.S.M., Tao Y., de Kreuk M., Zandvoort M.H., van Lier J.B. 2015. Microbial population dynamics during long-term sludge adaptation of thermophilic and mesophilic sequencing batch digesters treating sewage fine sieved fraction at varying organic loading rates. Biotechnol. Biofuels. 8:171.

Goel R.K., Noguera D.R. 2006. Evaluation of Sludge Yield and Phosphorus Removal in a Cannibal Solids Reduction Process. :1331–1337.

Grady C.P.L., Daigger G.T., Lim H.C. 1999. Biological Wastewater Treatment. New York: Marcel Dekker Inc.

Grady C.P.L., Daigger G.T., Love N.G., Filipe C.D.M. 2011. Biological wastewater treatment. New York: CRC press.

Gujer W., Henze M., Mino T., Matsuo T., Wentzel M., Marais G. 1995. The activated sludge model no. 2: Biological phosphorus removal. Water Sci. Technol. 31:1–11.

Han J.I., Choi H.K., Lee S.W., Orwin P.M., Kim J., LaRoe S.L., Kim T.G., O'Neil J., Leadbetter J.R., Lee S.Y., Hur C.G., Spain J.C., Ovchinnikova G., Goodwin L., Han C. 2011. Complete genome sequence of the metabolically versatile plant growth-promoting endophyte Variovorax paradoxus S110. J. Bacteriol. 193:1183–1190.

Han X., Wang Z., Ma J., Zhu C., Li Y., Wu Z. 2015. Membrane bioreactors fed with different COD/N ratio wastewater: impacts on microbial community, microbial products, and membrane fouling. Environ. Sci. Pollut. Res.:11436–11445.

Hao O.J., Chen J.M., Huang L., Buglass R.L. 1996. Sulfate reducing bacteria - Critical reviews. Environ. Sci. Technol. 26:155 – 187.

Henze M., Gujer W., Mino T., van Loosdrecht M.C.M. 2000. Activated Sludge Models ASM1, ASM2, ASM2d and ASM3. IWA Publ.:121.

Henze M., van Loosdrecht M.C.M., Ekama G.A., Brdjanovic D. 2008. Biological wastewater treatment: principles modelling and design. London: IWA Publishing.

Hii K., Baroutian S., Parthasarathy R., Gapes D.J., Eshtiaghi N. 2013. A review of wet air oxidation and Thermal Hydrolysis technologies in sludge treatment. Bioresour. Technol. 155C:289–299.

Hu J.Y., Ong S.L., Ng W.J., Lu F., Fan X.J. 2003. A new method for characterizing denitrifying

phosphorus removal bacteria by using three different types of electron acceptors. Water Res. 37:3463–3471.

Hu Z.R., Wentzel M.C., Ekama G. a. 2002. Anoxic growth of phosphate-accumulating organisms (PAOs) in biological nutrient removal activated sludge systems. Water Res. 36:4927–4937.

Huber J. a, Mark Welch D.B., Morrison H.G., Huse S.M., Neal P.R., Butterfield D. a, Sogin M.L. 2007. Microbial population structures in the deep marine biosphere. Science. 318:97–100.

Jang H.M., Kim J.H., Ha J.H., Park J.M. 2014. Bacterial and methanogenic archaeal communities during the single-stage anaerobic digestion of high-strength food wastewater. Bioresour. Technol. 165:174–182.

Jin W.B., Wang J.F., Zhao Q.L., Lin J.K. 2008. Performance and mechanism of excess sludge reduction in an OSA (oxic-settling-anaerobic) process. Huanjing Kexue/Environmental Sci. 29:726–732.

Johnson B.R., Daigger G.T., Novak J.T. 2008. The Use of ASM based Models for the Simulation of Biological Sludge Reduction Processes. 3.

Jørgensen K.S., Pauli A.S. 1995. Polyphosphate accumulation among denitrifying bacteria in activated sludge. Anaerobe. 1:161–8.

Khanal S.K., Huang J.C. 2003. ORP-based oxygenation for sulfide control in anaerobic treatment of high-sulfate wastewater. Water Res. 37:2053–2062.

Kim J.M., Lee H.J., Kim S.Y., Song J.J., Park W., Jeon C.O. 2010. Analysis of the fine-scale population structure of “candidatus accumulibacter phosphatis” in enhanced biological phosphorus removal sludge, using fluorescence in situ hybridization and flow cytometric sorting. Appl. Environ. Microbiol. 76:3825–3835.

Kim Y.M., Chon D.-H., Kim H.-S., Park C. 2012. Investigation of bacterial community in activated sludge with an anaerobic side-stream reactor (ASSR) to decrease the generation of excess sludge. Water Res. 46:4292–300.

Kleerebezem R., Mendez R. Autotrophic denitrification for combined hydrogen sulfide removal from biogas and post-denitrification. :349–356.

Kong Y., Nielsen J.L.J.J.L., Nielsen P.H.P.P.H. 2005. Identity and Ecophysiology of Uncultured Actinobacterial Polyphosphate-Accumulating Organisms in Full-Scale Enhanced Biological Phosphorus Removal Plants. Appl. Environ. Microbiol. 71:4076–4085.

Kortstee G.J.J., Appeldoorn K.J., Bonting C.F.C., van Niel E.W.J., van Veen H.W. 1994. Biology of polyphosphate-accumulating bacteria involved in enhanced biological phosphorus removal. FEMS Microbiol. Rev. 15:137–153.

Krishna C., Van Loosdrecht M.C.M. 1999. Effect of temperature on storage polymers and

settleability of activated sludge. Water Res. 33:2374–2382.

Kristiansen R., Thi H., Nguyen T., Saunders A.M., Lund Nielsen J., Wimmer R., Le V.Q., Mcilroy S.J., Petrovski S., Seviour R.J., Calteau A., Lehmann Nielsen K., Nielsen P.H. 2012. A metabolic model for members of the genus Tetrasphaera involved in enhanced biological phosphorus removal. ISME J. 7:543–554.

Laspidou C.S., Rittman B.E. 2002. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert bi omass. Water Res. 36:2711–2720.

Lee H., Yun Z. 2014. Comparison of biochemical characteristics between PAO and DPAO sludges. J. Environ. Sci. 26:1340–1347.

Lee H.W., Lee S.Y., Lee J.O., Kim H.G., Park J.B., Choi E., Park Y.K. 2003. The microbial community analysis of a 5-stage BNR process with step feed system. Water Sci. Technol. 48.

Lens P.N.L., Vissera A., Janssena A.J.H., Hulshoff Pola, L.W. Lettingaa G. 1998. Biotechnological treatment of sulfate-rich wastewaters. Crit. Rev. Environ. Sci. Technol. 28:41 – 88.

Liu J., Tanner R.S., Schumann P., Weiss N., Mckenzie C. a, Janssen P.H., Seviour E.M., Lawson P. a, Allen T.D., Seviour R.J. 2002. Emended description of the genus Trichococcus, description of T. collinsii sp. nov., and reclassification of Lactosphaera pasteurii as T. pasteurii comb. nov. and of Ruminococcus palustris as T. palustris comb. nov. in the low-GMC Gram-positive bacteria. Int. J. Syst. Evol. Microbiol. 52:1113–1126.

Liu Y., Fang H.H.P. 2003. Influences of Extracellular Polymeric Substances (EPS) on Flocculation, Settling, and Dewatering of Activated Sludge. Crit. Rev. Environ. Sci. Technol. 33.

Liu Y., Tay J.H. 2001. Strategy for minimization of excess sludge production from the activated sludge process. Biotechnol. Adv. 19:97–107.

Liu Y., Whitman W.B. 2008. Metabolic, Phylogenetic, and Ecological Diversity of the Methanogenic Archaea. Ann. N. Y. Acad. Sci. 1125:171–189.

Liu Y. 1996. Bioenergetic interpretation on the S0/X0 ratio in substrate-sufficient batch culture. Water Res. 30:2766–2770.

Liu Y. 2000. The So/Xo-dependent dissolved organic carbon distribution in substrate-sufficient batch culture of activated sludge. Water Res. 34:1645–1651.

Liu Y. 2003. Chemically reduced excess sludge production in the activated sludge process. Chemosphere. 50:1–7.

Van Loosdrecht M.C.M., Henze M. 1999. Maintenance, endogeneous respiration, lysis, decay and predation. Water Sci. Technol. 39:107–117.

Lopez-Vazquez C.M., Hooijmans C.M., Brdjanovic D., Gijzen H.J., van Loosdrecht M.C.M. 2009a. Temperature effects on glycogen accumulating organisms. Water Res. 43:2852–2864.

Lopez-Vazquez C.M., Oehmen A., Hooijmans C.M., Brdjanovic D., Gijzen H.J., Yuan Z., van Loosdrecht M.C.M. 2009b. Modeling the PAO–GAO competition: Effects of carbon source, pH and temperature. Water Res. 43:450–462.

Low E.W., Chase H.A. 1998. The use of chemical uncouplers for reducing biomass production during biodegradation. Water Sci. Technol. 37:399 – 402.

Lu H., Wang J., Li S., Chen G.-H., van Loosdrecht M.C.M., Ekama G. a. 2009. Steady-state model-based evaluation of sulfate reduction, autotrophic denitrification and nitrification integrated (SANI) process. Water Res. 43:3613–21.

Lu S.P., Ryu S.H., Chung B.S., Chung Y.R., Park W., Jeon C.O. 2007. Simplicispira limi sp nov., isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 57:31–34.

Lv X.-M., Shao M.-F., Li C.-L., Li J., Gao X., Sun F.-Y. 2014. A Comparative Study of the Bacterial Community in Denitrifying and Traditional Enhanced Biological Phosphorus Removal Processes. Microbes Environ. 00:261–268.

Ma J., Wang Z., Yang Y., Mei X., Wu Z. 2013. Correlating microbial community structure and composition with aeration intensity in submerged membrane bioreactors by 454 high-throughput pyrosequencing. Water Res. 47:859–869.

Mason C.A., Hamer G., Bryers J.D. 1986. The death and lysis of microorganisms in environmental processes. FEMS Microbiol. Lett. 39:373 – 401.

McClintock S.., Randall C.W., Pattarkine V.M. 1993. Effects of Temperature and Mean Cell Residence Time on Biological Nutrient Removal Processes. Water Environ. Res. 65:110–118.

Meijer S. 2004. Theoretical and practical aspects of modelling activated sludge processes. .

Mino T., Van Loosdrecht M.C.M., Heijnen J.J. 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. Water Res. 32:3193–3207.

Morita R.Y. 1975. Psychrophilic Bacteria. Microbiology. 39:144–167.

Mulkerrins D., Dobson A.D.W., Colleran E. 2004. Parameters affecting biological phosphate removal from wastewaters. Environ. Int. 30:249–259.

Muyzer G., Stams A.J.M. 2008a. The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. 6:441–54.

Muyzer G., Stams A.J.M. 2008b. The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. 6:441–54.

Nadkarni M., Martin F.E., Jacques N.A., Hunter N. 2002. Determination of bacterial load by real-time PCR using a broad range (universal) probe and primer set. Microbiology. 148:257–266.

Narihiro T., Terada T., Ohashi A., Kamagata Y., Nakamura K., Sekiguchi Y. 2012. Quantitative detection of previously characterized syntrophic bacteria in anaerobic wastewater treatment

systems by sequence-specific rRNA cleavage method. Water Res. 46:2167–2175.

Nedwell D.D.. 1999. Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature. FEMS Microbiol. Ecol. 30:101–111.

Nelson M.C., Morrison M., Yu Z. 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102:3730–3739.

Neyens E., Baeyens J. 2003. A review of thermal sludge pre-treatment processes to improve dewaterability. J. Hazard. Mater. 98:51–67.

Nielsen P.H., Keiding K. 1998. Disintegration of activated sludge flocs in presence of sulfide. Water Res. 32:313–320.

Nielsen P.R., Jahn A. 1999. Extraction of EPS. In: Wingender J., Neu T.R., Flemming H.-C., editors. Microbial Extracellular Polymeric Substances. Springer Berlin Heidelberg. p. 49–72.

Norberg A.B., Enfors S.O. 1982. Production of extracellular polysaccharide by Zoogloea ramigera. Appl. Environ. Microbiol. 44:1231–1237.

Novak J.T., Chon D.H., Curtis B.-A., Doyle M. 2007. Biological Solids Reduction Using the Cannibal Process. Water Environ. Res. 79:2380–2386.

Novak J.T., Sadler M.E., Murthy S.N. 2003. Mechanisms of floc destruction during anaerobic and aerobic digestion and the effect on conditioning and dewatering of biosolids. Water Res. 37:3136 – 3144.

Park C., Abu-Orf M.M., Novak J.T. 2006. The digestibility of waste activated sludges. Water Environ. Res. 78:59–68.

Park C., Novak J.T. 2007. Characterization of activated sludge exocellular polymers using several cation-associated extraction methods. Water Res. 41:1679–1688.

Pilli S., Bhunia P., Yan S., LeBlanc R.J., Tyagi R.D., Surampalli R.Y. 2011. Ultrasonic pretreatment of sludge: a review. Ultrason. Sonochem. 18:1–18.

Pinzon A., Brdjanovic D., Moussa M., Lopez-Vazquez C.M., Meijer S., Van Straaten H., Janssen A., van Loosdrecht M.C.M., Amy G. 2007. Modeling of an Oil Rafinery Wastewater Treatment Plant. Environ. Technol. 29.

Ragazzi M., Rada E.C., Ferrentino R. 2015. Analysis of a real scale experiences of novel sewage sludge treatments in an Italian pilot region. Desalin. Water Treat.

Rocher M., Goma G., Pilas Begue a., Louvel L., Rols J.L. 1999. Towards a reduction in excess sludge production in activated sludge processes: Biomass physicochemical treatment and biodegradation. Appl. Microbiol. Biotechnol. 51:883–890.

Saby S., Djafer M., Chen G.H. 2003. Effect of low ORP in anoxic sludge zone on excess sludge production in oxic-settling-anoxic activated sludge process. Water Res. 37:11–20.

Satoh H., Oshima K., Suda W., Ranasinghe P., Li N., Gunawardana E.G.W., Hattori M., Mino T. 2012. Bacterial Population Dynamics in a Laboratory Activated Sludge Reactor Monitored by Pyrosequencing of 16S rRNA. Microbes Environ. 28:65–70.

Semblante G.U., Hai F.I., Bustamante H., Guevara N., Price W.E., Nghiem L.D. 2016. Biosolids reduction by the oxic-settling-anoxic process: Impact of sludge interchange rate. Bioresour. Technol.:1–7.

Semblante G.U., Hai F.I., Ngo H.H., Guo W., You S.-J., Price W.E., Nghiem L.D. 2014. Sludge cycling between aerobic, anoxic and anaerobic regimes to reduce sludge production during wastewater treatment: performance, mechanisms, and implications. Bioresour. Technol.

Seviour R.J., Blackall L.. 1999. The Microbiology of activated sludge. .

Seviour R.J., Mino T., Onuki M. 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol. Rev. 27:99–127.

Sheng G.-P., Yu H.-Q., Li X.-Y. 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnol. Adv. 28:882–894.

Smolders G.J.F., van der Meij J., van Loosdrecht M.C.M., Heijnen J.J. 1994. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol. Bioeng. 44:837–848.

Song K., Suenaga T., Hamamoto A., Satou K., Riya S., Hosomi M., Terada A. 2014. Abundance, transcription levels and phylogeny of bacteria capable of nitrous oxide reduction in a municipal wastewater treatment plant. J. Biosci. Bioeng. 118:289–297.

Sun L., Randall C.W., Novak J.T. 2010. The Influence of Sludge Interchange Times on the Oxic-Settling-Anoxic Process. Water Environ. Res. 82:519–523.

Takai K., Horikoshi K. 2000. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Appl. Environ. Microbiol. 66:5066–5072.

Tchobanoglus G., Burton F., Stensel H. 2003. Wastewater engineering: Treatment and Reuse. New York: American Works Association.

Toerien D.F., Gerber A., Lotter L.H., Cloete T.E. 1990. Enhanced biological phosphorus removal in activated sludge systems. .

Torregrossa M., Di Bella G., Di Trapani D. 2012. Comparison between ozonation and the OSA process: analysis of excess sludge reduction and biomass activity in two different pilot plants. Water Sci. Technol. 66:185 – 192.

Troiani C., Eusebi A.L., Battistoni P. 2011. Excess sludge reduction by biological way: from

experimental experience to a real full scale application. Bioresour. Technol. 102:10352–8.

Wagner M., Loy A., Klein M., Lee N., Ramsing N.B., Stahl D.A., Friedrich W.F. 2005. Functional Marker Genes for Identification of Sulfate-Reducing Prokaryotes. Methods Enzymol. 397:469 – 489.

Wagner M., Loy A. 2002. Bacterial community composition and function in sewage treatment systems. Curr. Opin. Biotechnol. 13:218–227.

Wang J., Zhao Q., Jin W., Lin J. 2008. Mechanism on minimization of excess sludge in oxic-settling-anaerobic (OSA) process. Front. Environ. Sci. Eng. China. 2:36–43.

Wang J.F., Zhao Q.L. 2011. Microbial Community Analysis on Oxic-Settling-Anaerobic Process by Using PCR-DGGE Assay. Adv. Mater. Res. 255-260:2934–2939.

Wang Q., Garrity G.M., Tiedje J.M., Cole J.R. 2007. Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl. Environ. Microbiol. 73:5261–5267.

Wang S.H., Liang P., Wu Z.Q., Su F.F., Yuan L.L., Sun Y.M., Wu Q., Huang X. 2015. Mixed sulfur-iron particles packed reactor for simultaneous advanced removal of nitrogen and phosphorus from secondary effluent. Environ. Sci. Pollut. Res. 22:415–424.

Wang Y., Qian P.-Y. 2009. Conservative Fragments in Bacterial 16S rRNA Genes and Primer Design for 16S Ribosomal DNA Amplicons in Metagenomic Studies. PLoS One. 4:e7401.

Weemaes M.P.J., Verstraete W.H. 1998. Evaluation of current wet sludge disintegration techniques. J. Chem. Technol. Biotechnol. 73:83–92.

Wei Y., Van Houten R.T., Borger A.R., Eikelboom D.H., Fan Y. 2003. Minimization of excess sludge production for biological wastewater treatment. Water Res. 37:4453–67.

Welles L., Tian W.D., Saad S., Abbas B., Lopez-Vazquez C.M., Hooijmans C.M., van Loosdrecht M.C.M., Brdjanovic D. 2015. Accumulibacter clades Type I and II performing kinetically different glycogen-accumulating organisms metabolisms for anaerobic substrate uptake. Water Res. 83:354–366.

Wentzel M.C., Lötter L.H., Ekama G.A., Loewenthal R.E., Marais G. v. R. 1991. Evaluation of Biochemical Models for Biological Excess Phosphorus Removal. Water Sci. Technol. 23:567–576.

Westgarth W., Sulzer F., Okum D. 1964. Anaerobiosis in the activated sludge process. 2nd IAWPRC Conf.:43 – 55.

Wong M.T., Mino T., Seviour R.J., Onuki M., Liu W.T. 2005. In situ identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. Water Res. 39:2901–2914.

Yang S.-S., Guo W.-Q., Zhou X.-J., Meng Z.-H., Liu B., Ren N.-Q. 2011. Optimization of operating parameters for sludge process reduction under alternating aerobic/oxygen-limited conditions by response surface methodology. Bioresour. Technol. 102:9843–51.

Yang X.F., Xie M.L., Liu Y. 2003. Metabolic uncouplers reduce excess sludge production in an activated sludge process. Process Biochem. 38:1373–1377.

Ye F., Li Y. 2010. Oxic-settling-anoxic (OSA) process combined with 3,3′,4′,5-tetrachlorosalicylanilide (TCS) to reduce excess sludge production in the activated sludge system. Biochem. Eng. J. 49:229–234.

Ye F., Zhu R., Li Y. 2008. Effect of sludge retention time in sludge holding tank on excess sludge production in the oxic-settling-anoxic ( OSA ) activated sludge. 114:109–114.

Ye F.X., Li Y. 2005. Uncoupled metabolism stimulated by chemical uncoupler and oxic-settling-anaerobic combined process to reduce excess sludge production. Appl. Biochem. Biotechnol. 127:187–199.

Yeoman S., Hunter M., Stephenson T., Lester J.N., Perry R. 1988. An assessment of excess biological phosphorus removal during activated sludge treatment. Environ. Technol. Lett. 9:637–646.

Young R., Cheng S., Fedorak P. 2006. Aerobic biodegradation of 2, 2′-dithiodibenzoic acid produced from dibenzothiophene metabolites. Appl. Environ. …. 72:491–496.

Zhang J., Zhou J., Han Y., Zhang X. 2014. Start-up and bacterial communities of single-stage nitrogen removal using anammox and partial nitritation (SNAP) for treatment of high strength ammonia wastewater. Bioresour. Technol. 169C:652–657.

Zhang T., Shao M.-F., Ye L. 2012. 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. ISME J. 6:1137–1147.

Zhang X., Wang Z., Wu Z., Wei T., Lu F., Tong J., Mai S. 2011. Membrane fouling in an anaerobic dynamic membrane bioreactor (AnDMBR) for municipal wastewater treatment: Characteristics of membrane foulants and bulk sludge. Process Biochem. 46:1538–1544.

Zhou S., Zhang X., Feng L. 2010. Effect of different types of electron acceptors on the anoxic phosphorus uptake activity of denitrifying phosphorus removing bacteria. Bioresour. Technol. 101:1603–1610.

Zhou Z., Hu D., Jiang L., Xing C., Zhu Y., Jiang M. 2015a. Nitrification kinetics of a full-scale anaerobic / anoxic / aerobic wastewater treatment plant. 56:2054.

Zhou Z., Qiao W., Xing C., An Y., Shen X., Ren W., Jiang L., Wang L. 2015b. Microbial community structure of anoxic–oxic-settling-anaerobic sludge reduction process revealed by 454-pyrosequencing. Chem. Eng. J. 266:249–257.

Zhou Z., Qiao W., Xing C., Wang C., Jiang L.-M., Gu Y., Wang L. 2015c. Characterization of dissolved organic matter in the anoxic–oxic-settling-anaerobic sludge reduction process. Chem. Eng. J. 259:357–363.

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