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Microbial ecology of novel bioprocesses for biogas recovery and short-cut nutrients removal from wastewater

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ABSTRACT:

The science of microbial ecology is focused on how microbial populations assemble to form communities and how these communities interact with each other and their environment. The performance of biological processes in operation in the wastewater treatment plants strongly depends on the activities and interaction of the microbial community. Thus, information on the identity of microorganisms responsible for specific activities are important for optimizing these processes.

The characteristics of problematic biofilms (i.e., fouling and clogging layers) were studied with regards to the removal and fate of trace metals during the long-term operation of a pilot-scale membrane bioreactor for the treatment of real wastewaters from a large industrial area. Results showed that clogging layer was more effective than suspended activated sludge in the biosorption of As > Zn > Ni > Cd > Sb > Fe > Se due to the synergic effects of extracellular polymeric compounds and metal-resistant bacteria. In fact the selective microbial speciation of the phylum of *Bacteroidetes*, which is highly resistant to heavy metals, was observed in the clogging sludge in spite of the very low concentration of dissolved metals in the bioreactor. Compared to the suspended activated sludge, the clogging layer enhanced the biosorption of very toxic substances such as As, Cd and Ni. Then, the potential desorption of metals during the membrane acid cleanings was estimated as relevant as 10–15% of the metals associated to the clogging sludge. The combined effects of pH and the selected microbial community, and the minor effect of the redox potential, let us conclude on the major importance of bio-sorption/desorption mechanisms with respect to bio-precipitation/dissolution.

A thermophilic two-phase anaerobic digestion process for the concurrent production of H2 and CH4 through the treatment of source sorted organic fraction of municipal solid waste was carried out over a long-term pilot scale experience. The results showed that stable production of bio-hythane without inoculum treatment could be obtained. The pH of the dark fermentation reactor was maintained in the optimal range for hydrogen producing bacteria activity through sludge recirculation from a methanogenic reactor. An average specific bio-hythane production of 0.65 m3/kg of volatile solids fed was achieved when the recirculation flow was controlled through an evaporation unit in order to avoid inhibition problems for both microbial communities. Microbial analysis indicated that dominant bacterial species in the dark fermentation reactor are related to the *Lactobacillus* family, while the population of the methanogenic reactor was mainly composed of *Defluviitoga tunisiensis*. Archaeal community of the methanogenic reactor shifted, moving from

Methanothermobacter-like to *Methanobacteriales* and *Methanosarcinales*, the last one found also in the dark fermentation reactor, with considerable methane production.

Pig manure (PM) can be an excellent base substrate for anaerobic digestion due to its inherent buffering capacity and high content of a wide range of nutrients required for the development of anaerobic microorganisms. However, PM has a low biogas yield and high ammonium concentrations. Consequently, PM is preferably co-digested with high carbon content wastes, to improve the C/N ratio and increase the biogas production, essential for the plant's economy. Lipid wastes are ideal substrates for methane production, since theoretically their degradation produces more biogas with higher methane content, when compared with proteins or carbohydrates. Studies investigating the anaerobic digestion of high-strength lipid wastes have reported a wide assortment of operational challenges. The aims of this project were to understand the degradation pathways of real wastes containing high percentages of lipids, to determine the optimal conditions to treat lipid wastes in anaerobic co-digestion with pig manure and to characterize the microbial community responsible for specific Long Chain Fatty Acid degradation pathways using PCR-DGGE and FISH analyses. In this work we demonstrated that anaerobic biomass can be adapted to high lipid concentration using a continuous feeding strategy in co-digestion with pig manure as a source of humidity and alkalinity and the adaptation is not related to the type of LCFA composing the lipid substrate. In our case the maximum reached OLR was 4 g COD/Ld, where the lipid OLR was 3 g COD/Ld. The Archaeal community did not change between adapted and non-adapted biomasses, while the Bacterial community of the lipid degrading bacterial community was dominated by the phyla *Firmicutes*, where members of the *Pseudomonas* genus are probably responsible of the LCFA degradation.

Biological nutrients removal via-nitrite has gained interest recently due to several advantages over the conventional processes. In this processes the role of certain short chain fatty acids as carbon source is crucial for the nutrients removal. but a gap of knowledge about their influence on the speciation of heterotrophic denitrifying microbial populations. including denitrifying phosphorus accumulating organisms. The purpose of this study is to investigate the microbiological composition of a via-nitrite process when different carbon sources for nutrients removal are used. A demonstration sequencing batch reactor treating supernatant from anaerobic co-digestion of sewage sludge and OFMSW was stably operated via-nitrite pathway. In long term operation different external carbon sources were tested. In particular. microbial community of the sludge during acetic

acid and fermentation liquid of the organic fraction of municipal solid waste (OFMSW) dosage was analyzed through PCR-DGGE technique using primer set for the universal eubacterial V3 region within 16S rRNA gene. Results showed that the microbial community was mainly composed of *Bacteroidetes*. including *Sphingobacteriaceae*. and *Proteobacteria*. In particular a strong speciation to a few species of denitrifying bacteria commonly present in reactors with high F/M was observed. This result is in accordance with the operating conditions and observed denitrifying activity of the biomass (NUR 0.65÷1.14 $kgNO_2\text{-}N\ kgVSS^{-1}day^{-1}$). Using the OFMSW fermentation liquid as external carbon source. the highest biological phosphorous removal was observed. with a Maximum Phosphorus Uptake Rate of 0.37±0.09 $kgPO_4\text{-}P\ kgVSS^{-1}day^{-1}$. In this specific case. a high NLR (1.14 $gN\text{-}NH_3/d$) and F/M. coupled with OFMSW fermentation liquid were the driving forces to the speciation of a biomass composed of members of the CFB group related to the genus *Cytophaga* and *γ-Proteobacteria* (genus *Thermomonas*). which are related to the enhanced phosphorus biological removal via-nitrite.

RIASSUNTO:

L'ecologia microbica è focalizzata su come le popolazioni microbiche si associno per formare comunità e come queste comunità interagiscano tra loro e con l'ambiente. Le performances dei processi biologici dipende strettamente dall'attività e dalle interazioni tra le comunità microbiche. Perciò avere informazioni sulle identità dei microrganismi responsabili di specifiche attività è importante ai fini di ottimizzare questi processi.

Le caratteristiche di biofilm problematici (come fouling e clogging) sono stati studiati ponendo l'attenzione sulla rimozione e il destino dei metalli in tracce durante l'attività di un impianto a membrane in scala pilota per il trattamento di acque reflue reali di una zona industriale. I risultati mostrano che il biofilm era più efficace della biomassa sospesa nell'assorbimento di Az>Zn>Ni>Cd>Sb>Fe>Se a causa dell'effetto sinergico dei composti polimerici extracellulari e la presenza di batteri resistenti ai metalli. Infatti è stata osservata la speciazione selettiva verso il phylum *Bacteriodetes*, altamente resistente ai metalli, nel fango di clogging di membrana, nonostante la bassa concentrazione di metalli disciolti nel bioreattore. In confronto alla biomassa sospesa, il biofilm incrementa il bioassorbimento di sostanze molto tossiche come As, Cd e Ni. L'effetto combinato del pH e della comunità microbica selezionata, e il minor effetto del potenziale redox, ci permette di concludere che sono maggiormente importanti i meccanismi di bio-assorbimento/rilascio piuttosto che la bio-precipitazione/dissoluzione.

È stato effettuato un processo di digestione anaerobica in 2 fasi per la produzione contestuale di idrogeno e metano attraverso il trattamento della frazione organica di rifiuti solidi urbani in scala pilota. I risultati mostrano che anche senza un inoculo è possibile la produzione stabile di bio-hytane. Analisi sulla popolazione microbica mostrano che le specie dominanti il fermentatore sono appartenenti alla famiglia Lactobaccillus, mentre la popolazione del digestore anaerobico era composta prevalentemente da *Defluviitoga tunisiensis*. La popolazione *Archaea* cambiò da *Methanothermobacter* a *Methanobacteriales* e *Methanosarcinales*, l'ultimo trovato anche nel fermentatore, dove veniva prodotta una considerevole quantità di metano.

Il liquame suino può essere un eccellente substrato per la digestione anaerobica, ma ha una bassa resa in biogas e produce alte concentrazioni di ammoniaca. Rifiuti contenenti grassi possono essere co-substrati ideali per la produzione di metano. Studi hanno evidenziato una serie di problematiche dovute all'utilizzo di questo substrato. Nel nostro caso abbiamo raggiunto il carico organico di 3 g COD/Ld di lipidi. La comunità *Archaea* non cambiò tra biomassa adattata e non adattata ai lipidi, mentre la comunità batterica della biomassa capace di degradare i lipidi era dominata dal phylum

Firmicutes , dove membri del genere *Pseudomonas* erano probabilmente responsabili della degradazione degli acidi grassi a catena lunga.

1. STATE OF THE ART

1.1. BIOGAS PRODUCTION

The global energy demand is growing rapidly, and about 88% of this demand is met at present time by fossil fuels. Scenarios have shown that the energy demand will increase during this century by a factor of two or three (IEA 2006). At the same time, concentrations of greenhouse gases (GHGs) in the atmosphere are rising rapidly, with fossil fuel-derived CO2 emissions being the most important contributor. In order to minimize related global warming and climate change impacts, GHG emissions must be reduced to less than half of global emission levels of 1990 (IPCC 2000). Another important global challenge is the security of energy supply, because most of the known conventional oil and gas reserves are concentrated in politically unstable regions.

In this context, biogas from wastes, residues, and energy crops will play a vital role in future. Biogas is a versatile renewable energy source, which can be used for replacement of fossil fuels in power and heat production, and it can be used also as gaseous vehicle fuel. Methane-rich biogas (**biomethane**) can replace also natural gas as a feedstock for producing chemicals and materials.

The production of biogas through anaerobic digestion offers significant advantages over other forms of bioenergy production. It has been evaluated as one of the most energy-efficient and environmentally beneficial technology for bioenergy production (Fehrenbach et al. 2008).

It can drastically reduce GHG emissions compared to fossil fuels by utilization of locally available resources. The digestate is an improved fertilizer in term of its availability to crops which can substitute mineral fertilizer. The European energy production from biogas reached 6 million tons of oil equivalents (Mtoe) in 2007 with a yearly increase of more than 20% (EurObserv'er 2008).

1.1.1. ANAEROBIC DIGESTION

Methane fermentation is a complex process, which can be divided up into four phases: hydrolysis, acidogenesis, acetogenesis/dehydrogenation, and methanation (Figure 1).

The individual degradation steps are carried out by different consortia of microorganisms, which partly stand in syntrophic interrelation and place different requirements on the environment (Angelidaki et al. 1993). Hydrolyzing and fermenting microorganisms are responsible for the initial attack on polymers and monomers and produce mainly acetate and hydrogen and varying amounts of volatile fatty acids such as propionate and butyrate. Hydrolytic microorganisms excrete hydrolytic enzymes, e.g., cellulase, cellobiase, xylanase, amylase, lipase, and protease.

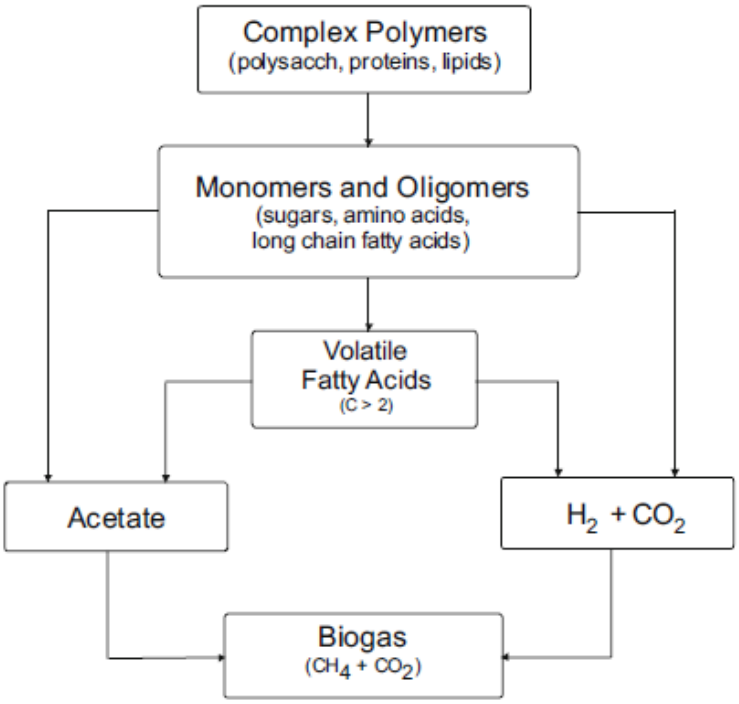

FIGURE 1 THE STAGES OF THE METHANE FERMENTATION PROCESS (TAKEN FROM WEILAND, 2010)

A complex consortium of microorganisms participates in the hydrolysis and fermentation of organic material. Most of the bacteria are strict anaerobes such as *Bacteriocides*, *Clostridia*, and *Bifidobacteria*. Furthermore, some facultative anaerobes such as *Streptococci* and *Enterobacteriaceae* take part. The higher volatile fatty acids are converted into acetate and hydrogen by obligate hydrogen-producing acetogenic bacteria. The hydrogen-producing acetogenic bacteria are not well characterized. Typical homoacetogenic bacteria are *Acetobacterium woodii* and *Clostridium aceticum*. The accumulation of hydrogen can inhibit the metabolism of the acetogenic bacteria. The maintenance of an extremely low partial pressure of hydrogen is, therefore, essential for the acetogenic and H2-producing bacteria. Although many microbial details of metabolic networks in a methanogenic consortium are not clear, present knowledge suggests that hydrogen may be a limiting substrate for methanogens (Bagi et al. 2007). This assumption is based on the fact that addition of H2-producing bacteria to the natural biogas-producing consortium increases the daily biogas production. At the end of the degradation chain, two groups of methanogenic bacteria produce methane from acetate or hydrogen and carbon dioxide. These bacteria are strict anaerobes and require a lower redox potential for growth than most other anaerobic bacteria. Only few species are able to degrade acetate into CH4 and CO2, e.g., *Methanosarcina barkeri*, *Metanonococcus mazei*, and *Methanotrix soehngenii*, whereas all methanogenic bacteria are able to use hydrogen to form methane. The first and second groups of

microbes as well as the third and fourth groups are linked closely with each other (Schink 1997). Therefore, the process can be accomplished in two stages.

A balanced anaerobic digestion process demands that in both stages the rates of degradation must be equal in size. If the first degradation step runs too fast, the acid concentration rises, and the pH drops below 7.0 which inhibits the methanogenic bacteria. If the second phase runs too fast, methane production is limited by the hydrolytic stage. Thus, the rate-limiting step depends on the compounds of the substrate which is used for biogas production. Undissolved compounds like cellulose, proteins, or fats are cracked slowly into monomers within several days whereas the hydrolysis of soluble carbohydrates takes place within few hours. Therefore, the process design must be well adapted to the substrate properties for achieving a complete degradation without process failure (Weiland et al., 2010).

1.1.2. DARK FERMENTATION

Hydrogen produced from biomass is a renewable energy carrier. Among the various hydrogen production methods, dark fermentation of organic wastes seems to be the most promising and environmentally friendly method. The feasibility of such method has been demonstrated in several studies (Cai et al., 2004; Liu et al., 2006). However, the main obstacles in such process are the lower hydrogen yield (<4 mol H2/mol Glucose) and higher residual organic concentration in the effluent (Xie et al., 2008). The effluents of the dark fermentation process contain mainly acetate, propionate, butyrate, etc., which should be further utilized to increase the total energy recovery efficiency. Combined hydrogen and methane production in a two-stage process is a concept which has been developed in recent years (Kyazze et al., 2007; Liu et al., 2006; Ueno et al., 2007). It is similar to the traditional two-phase process that separates hydrolysis/acidogenesis and methanogenesis, and optimizes each process separately, leading to a larger overall reaction rate and biogas yield (Fox and Pohland, 1994). The main difference is that hydrogen is retrieved in the first stage of the two-stage process for hydrogen and methane production. The co-production of hydrogen and methane is more promising from an energy perspective. Liu et al. (2006) have demonstrated that more methane could be obtained by two-stage hydrogen and methane process. Also, the mixture of hydrogen and methane has many advantages than methane alone, which could improve the efficiency of the methane combustion motors and decrease the emissions of CO2 and CO (Akansu et al., 2004). Several studies have been conducted to investigate the hydrogen and methane production in the two-stage process. However, they mainly focused on the optimization of hydrogen and methane reactors individually (Antonopoulou et al., 2008; Venetsaneas et al., 2009).

It is necessary to optimize the whole system for higher total energy production. In addition, the mechanisms involved in the two-stage process and the microbial community structures have not been investigated and clarified, which is crucial for better understanding of the process. Concerns about instability of fossil fuels supply, limits on fossil fuel reserves and not the least environmental pollutions and climate changes have brought new insights into the utilization of biomass in biorefinery concepts, where biomass is used as feedstock instead of fossil fuels for production of bio-based fuels, chemicals, solvents, etc. by biological conversion processes (Luo et al., 2011).

1.2. NITROGEN POLLUTION

In the last few decades are increasing concerns about the health of the environment and for consequence it is increased the propensity to reduce the human pollution. One of the major environmental themes is the quality of water, in particular regarding the presence of toxic compounds like nitrates and nutrient enrichment (N and P) that causes eutrophication phenomena (ERSAF, 2008). The decrease in water quality has been attributed to an increased contribution of agriculture and livestock farming, which are considered the main pollution source of nutrients to water (Infascelli et a., 2009).

Groundwater characterized by high concentration of nitrate is unsuitable for human consumption (Thayalakumaran et al., 2008). Nitrate itself is a compound of low toxicity, but nitrate can be reduced to nitrite, the most toxic form with regard to human health, which can cause infantile methemoglobinemia. In addition, carcinogenic Nitrosamines can be formed from nitrite (Fine et al., 1977), which is suspected of causing gastric cancer and other malignancies.

The European Union (EU) regulation Directive 96/61 EC, Integrated Pollution Prevention and Control (IPPC), requires that an integrated approach to pollution deriving from livestock activities should be implemented. This takes into consideration the possible impacts on the three environmental compartments water, air and soil and aims to decrease pollutants at the origin of the production chain.

The Italian Ministry for Agricultural and Forestry Policies has ruled, by means of the Ministerial Decree issued on 7 April 2006, that the regions are required to practice an integrated management of effluents by promoting ways of livestock farming and feed selection aimed at reducing nitrogen content in manure. Some authors (Burton and Turner, 2003; Westerman and Bicudo, 2005) maintain that, in this context, the goal should be to make the ‘‘waste’’ a resource that can be utilized and not just discarded. The large quantities of slurry and manure that are produced every year in areas in which animals are raised, could be an important source of organic matter and nutrients.

Recycling this waste via land application could also lead to soil improvement with regard to porosity, structure and waterholding capacity (Ramos et al., 2006). In order to decide the right balance between reuse and disposal, "best management practices" need to be identified. Although manure is a valuable source of plant nutrients, it can also be a source of pollution and a threat to the environment unless managed carefully.

1.2.1. CONVENTIONAL AND INNOVATIVE PROCESSES

Ammonium can be removed from wastewaters by a variety of physicochemical and biological processes. Because biological nitrogen removal is effective and inexpensive, it has been adopted widely in favor of the physico-chemical processes (EPA, 1993).

Biological nitrogen removal proceeds slowly because the microorganisms responsible for the elimination reactions grow slowly. In addition, the operational control of aerobic and anaerobic conditions needed for nitrification and denitrification, respectively, can be difficult. To cope with these problems, various kinds of bioreactors have been studied for enhancing the efficiency of nitrogen removal. Examples of enhanced processes include the combined nitrification and denitrification (Kuenen and Robertson, 1994.); immobilization of bacteria on polymeric gel beads in a moving bed biofilm reactor (Hem et al., 1994); and the formation of bacterial film on the surface of rotating disks or other packing in a moving bed biofilm reactor or aeration tank (Klees and Silverstein, 1992; Rusten et al., 1992).

Unfortunately, these enhanced processes have generally not performed well when faced with wastewaters containing a high concentration of nitrogen. Variously, poor performance has been ascribed to the low nitrification and denitrification rates, low stability of immobilized bacteria and insufficient/unavailable carbon source for denitrification. To overcome existing limitations, several novel nitrogen removal processes have been developed, including the SHARON process, the ANAMMOX process, the combined SHARON and ANAMMOX process and CANON process.

1.2.1.1. Conventional Nitrification and Denitrification

Conventional microbial nitrogen removal is based on autotrophic nitrification and heterotrophic denitrification. The removal involves aerobic nitrification (i.e., the conversion of NH_4^+ to NO_2^- and further to NO_3^-) with molecular oxygen as the electron acceptor. The relevant reactions are as follows:

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + 2H_2O$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^-$$

The anoxic denitrification (i.e., the conversion of NO_3^- and NO_2^- to gaseous nitrogen) is accomplished with a variety of electron donors, including methanol, acetate, ethanol, lactate and glucose (Grabinska-Loniewska, 1991; Tam et al., 1992; Akunna et al., 1993). The anoxic denitrification involves the following reactions:

$$2NO_3^- + 10H^+ + 10e^- \rightarrow N_2 + 2OH^- + 4H_2O$$

$$2NO_2^- + 6H^+ + 6e^- \rightarrow N_2 + 2OH^- + 2H_2O$$

As nitrification and denitrification are carried out under different conditions and by different microorganisms, experience shows that these processes have to be separated in time or space to function effectively. The conventional nitrification/denitrification reactions have been known for a long time (Winogradsky, 1890; Beijerinck and Minkman, 1910; Kluyver and Donker, 1926). The nitrification reaction consumes a large amount of oxygen, requiring 4.2 g of oxygen for each gram of ammonium nitrogen nitrified (Gujer and Jenkins, 1974; EPA, 1975). During denitrification, the requirement of organic carbon is significant. For example, 2.47 g of methanol is required per gram of nitrate nitrogen for complete denitrification (McCarty et al., 1969). The requirement of added electron donors such as methanol makes full-scale denitrification quite expensive.

Because the organic carbon present naturally in the wastewater is quite limited, the complete removal of nitrogen from wastewaters that contain a high nitrogen concentration requires a large amount of an added carbon source for denitrification (van Dongen et al., 2001). Furthermore, most existing wastewater treatment facilities were not designed for nitrogen removal, and meeting the demands of the nitrification/denitrification steps in these facilities can be difficult. Thus, many wastewater treatment plants do not meet the current discharge standard of 10 mg N/l (Jetten et al., 2002). This was what drove the development of the new low-cost biotreatments for nitrogen-rich wastewaters.

1.2.1.2. Short-Cut Nitrification

Various novel biological nitrogen removal processes such as short-cut nitrification and denitrification, anaerobic ammonium oxidation (AnAmmOx), completely autotrophic nitrogen

removal over nitrite (CANON) process and oxygen-limited autotrophic nitrification-denitrification (Oland) process, have been developed (Verstraete and Philips 1998). However, partial nitrification is a critical procedure for implementing these novel processes owing to nitrite is required as substrate or intermediary media (Philips et al. 2002). To date, nitrification and denitrification via nitrite technology have attracted more and more interests after successful application of SHARON (Single reactor system for High Ammonia Removal Over Nitrite process) in practice. Partial nitrification process is based on the fact that nitrite is an intermediary compound in both nitrification and denitrification steps: a partial nitrification up to nitrite is performed followed by nitrite denitrification (Ferhan 1996; Fdz-Polanco et al. 1996), as shown in the next figure (Figure 2).

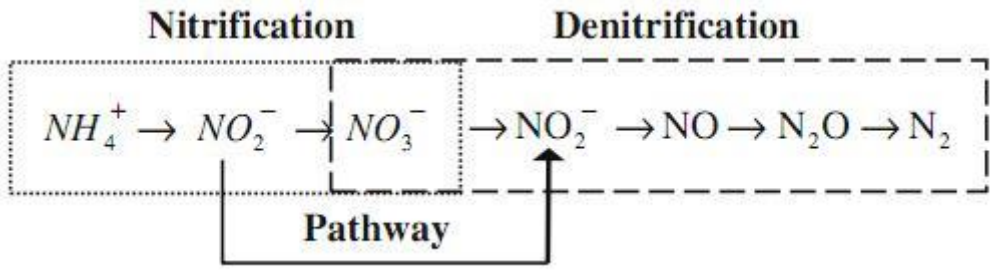

FIGURE 2 SCHEME OF THE SHORT-CUT NITROGEN REMOVAL PROCESS

Compared to the traditional nitrification and denitrification via nitrate, the main advantages of partial nitrification with respect to complete nitrification (Beccari et al. 1983; Turk and Mavinic 1987; van Kempen et al. 2001) were reported as followed:

- 25% lower oxygen consumption in the aerobic stage implies 60% energy savings;
- in the anoxic stage the electron donor requirement is lower (up to 40%);
- nitrite denitrification rates are 1.5 to 2 times higher than with nitrate;
- reduces CO2 emission by 20%;
- 33~35% lower sludge production in nitrification process and 55% in denitrification process.

1.2.1.3. References

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1.3. PROGRESS IN REAL-TIME CONTROL APPLIED TO BIOLOGICAL NITROGEN REMOVAL FROM WASTEWATER. A SHORT-REVIEW

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Abstract

Real-time control of biological nitrogen removal is a major issue to advance the performances and the efficiencies of the wastewater treatment, especially when coupled to the processes via-nitrite pathway. Over the last three decades many researchers focused on indirect parameters (namely pH, DO, ORP, conductivity) to control the conventional nitrification and denitrification, up to several full scale applications. On the other hand, the real-time control to achieve and operate the processes via-nitrite is under investigation over the last years. This short review analyzes and discusses the main real-time control strategies and their most common and successful applications in sequencing batch reactors. In conclusion, the knowledge concerning the conventional processes will be outlined together with the next developments for short-cut nitrogen removal.

1.3.1. INTRODUCTION

Even though nitrogen recovery from wastewater should be more and more pursued, nitrogen removal by means of biological process is still the most economically viable treatment for a number of urban and industrial wastewaters. Within this framework, the realtime control of the bioprocesses is definitely a key-issue to optimize the process performances, and the sequencing batch reactors (SBR) have shown great success in implementing real-time control systems for nitrogen removal. In addition, SBRs allow to completely achieve nitrogen removal via-nitrate or via-nitrite within the same reactor, which is also used as final settler and offer kinetic advantages compared to Completely Stirred Tank Reactors (CSTRs). The common practice used in SBR is based on the execution of a predefined cycle over time. This rigid scheme is far from the optimal one since every cycle has different requirements, depending on the loading influent. However, there is the possibility to operate by information gained from on-line parameters which are directly (i.e. $N\text{-}NH_4$ and N-NOx) or indirectly (i.e. pH, DO, ORP, conductivity) related to nitrogen removal. In addition, short-cut nitrogen removal recently provided an innovative improvement to the conventional process, allowing significant costs reduction and performances betterment. In this review, dealing

with three types of wastewaters widely studied, we first describe the main widely applied control strategies for conventional processes via-nitrate, then we deal with the promising strategies to implement the innovative short-cut nitrogen removal.

1.3.2. THE WASTEWATERS: MAIN CHARACTERISTICS

Most of scientific literature related to the SBRs for nitrogen biological removal are related to: (1) municipal wastewater; (2) livestock effluents, (3) anaerobic digestate or, generally, liquors with low BOD:N. In order to outline the framework of the wastewater discussed in this review, we hereby summarize the main characteristics of the real substrates fed to these SBRs. SBRs for municipal wastewater treatment were well developed for small and decentralized applications (Lens et al., 2001) up to 20000–25000 population equivalent (PE) while recently these were even applied to large treatment plants up to 1.2 million PE (Demoulin, 2006).

TABLE 1 TYPICAL CHARACTERISTICS OF RAW DOMESTIC WASTEWATER. VALUES ARE EXPRESSED IN MG/L (ADAPTED FROM WANG ET AL.,2008)

COD	BOD5	TKN	NH4-N	pH
mg/L	mg/L	mg/L	mg/L	
160–500	80–300	60–110	50–100	7.0–8.0

Table 1 reports the typical composition of a municipal waste water, which can represent the influent to the SBRs hereby reviewed. Mechanical separation and biological treatment of pig slurry is included as Best Available Techniques (BAT) in the European BRef (BAT, 2003) and, generally, SBR technology is of wide interest even to face the environmental impact of intensive farming, which may involve the discharge of strong nitrogenous waste streams. Tables 2 and 3 report examples of livestock effluents and let us know the differences between papers from different continents and different farming practices. Another common source of strong nitrogenous wastewater is the anaerobic digestion of bio-waste, which is increasingly used for biogas production. Anaerobic digestion brings undoubtedly several benefits both from environmental (such as greenhouse related gasses reduction) and economical points of view (incomes from the energy recovered). However, nitrogen contained in organic matter, specifically in proteins, gets released when organic compounds are degraded. As a result, liquors effluent from anaerobic digesters represents a high-strength ammonia stream, which should be adequately treated before disposal or recirculation to the liquid treatment train, when integrated in a wastewater treatment plants

(WWTP) (Cervantes, 2009) (Table 4). Table 3 reports examples of anaerobic digestates which will represent those treated in SBRs for nitrogen removal.

TABLE 2 AVERAGE SWINE SLURRY AND WASTEWATER CHARACTERISTICS (VALUES ARE EXPRESSED IN MG/L, ADAPTED FROM KISHIDA ET AL., 2003)

	Swine wastewater	Swine slurry
BOD5	30000	10000
TOC	1200	NA
TSS	300	115000
TN	1130	3600
NH4-N	1120	2700
NO3-N	<0.01	NA
NO2-N	<0.01	NA
TP	21	3000
pH	8.87	NA

TABLE 3 CHARACTERISTICS OF SWINE WASTEWATER FROM AN ITALIAN CASE-STUDY (ADAPTED FROM TICHE ET AL., 2001)

	Swine wastewater	Range
pH	7.8	–
TS g/Kg	8.2	±3.7
TSS g/L	4.43	±2.4
CODtot mg/L	10580	±4969
NH4-N	844	±310
TKN	1258	±262
TP	236	±124

TABLE 4 COD, BOD AND N AVERAGE CONCENTRATION IN WASTE STREAMS WITH HIGH NITROGEN CONTENT. (VALUE ARE EXPRESSED IN MG/L, ADAPTED FROM YAMAMOTO ET AL., 2008; DOSTA ET AL., 2007, UYSAL ET AL., 2010, FATONE ET AL., 2011)

Feeding	Reject water	Sewage sludge	WAS + OFMSW
pH	8.3–8.8	7.6	7.6
TS (mg/L)	8000–33900	25 100–25 600	–
VTS (mg/L)	3900–20700		–
SS (mg/L)	1900–10000	22 610–23 200	3–47
VSS (mg/L)	1400–2200	11 450–11 590	–
Total COD (mg/L)	3900–17000	23 970–27 790	49–170
Soluble COD (mg/L)	2700–5000	367–430	51–136
N-NH4 + (mg/L)	2000–4000	938–960	355–535
T-N (mg/L)	3000–5000	1024–1060	355–535
N-NO2-(mg/L)	na	–	na
N-NO3-(mg/L)	na	–	na
T-P (mg/L)	–	388–395	33–120
COD/TKN	~1	<1	<1

1.3.3. REAL-TIME CONTROL IN CONVENTIONAL NITRIFICATION–DENITRIFICATION VIA-NITRATE

According to the conventional nitrification–denitrification via-nitrate, during oxic phase ammonia is converted to nitrate which, subsequently in the anoxic phase, is further denitrified to molecular nitrogen. In the last decades full-scale SBRs for the treatment of the raw and/or pre-digested livestock effluents were widely applied all over the world using a fixed-time control strategy, even because of the late development of instrumentation, control and automation (ICA). In order to describe the conventional design and operation parameters, we reviewed a recent work (Bortone, 2009) as an example for the successful integration of SBR technology in an Italian case study. Each day-long cycle was divided into (i) 6 h first anoxic phase, (ii) 7 h first oxic phase, (iii) 4 and (iv) 6 h second anoxic and oxic phases respectively. Figure 3 shows NH4, NO3, and NO2 trends within a representative SBR cycle.

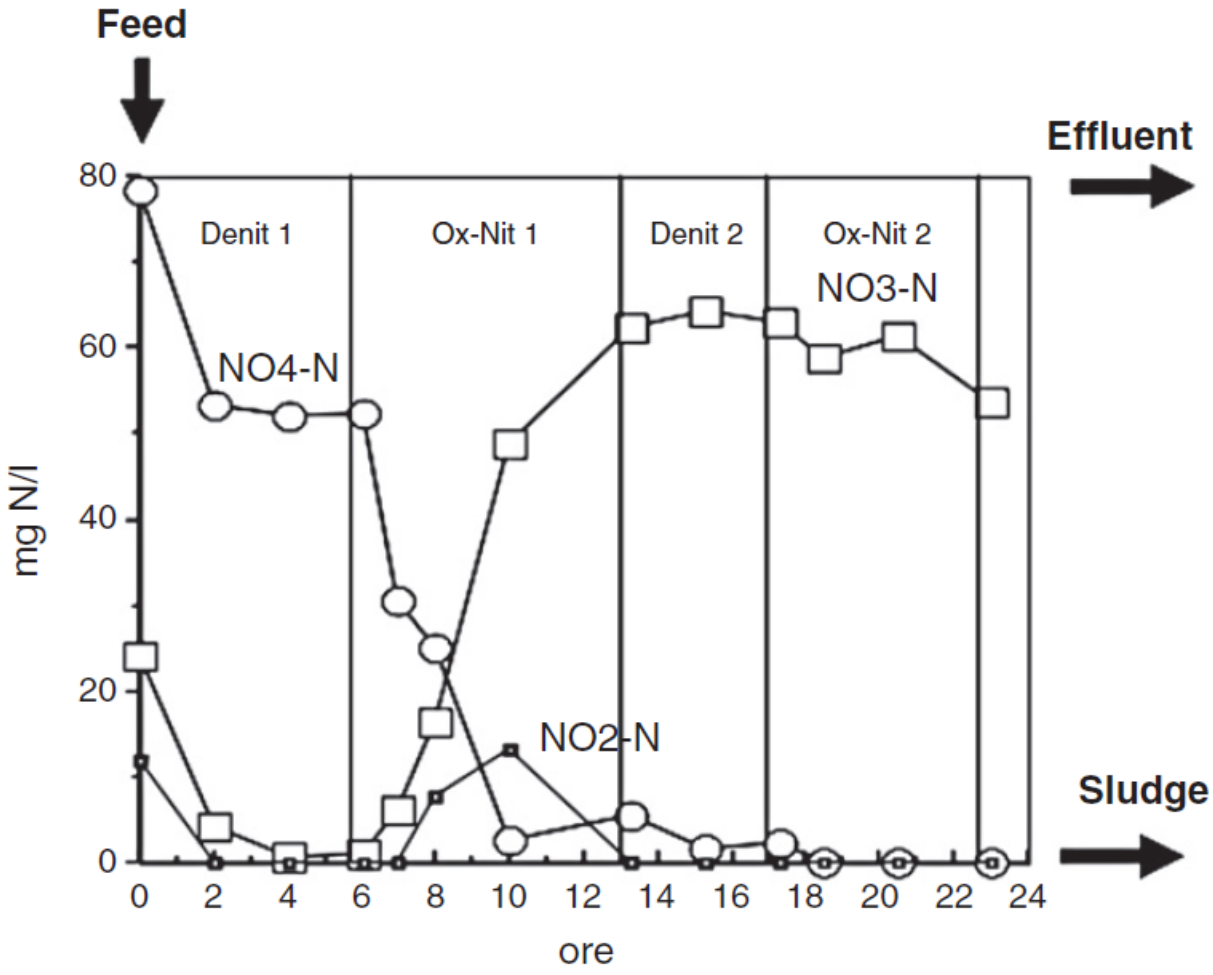

FIGURE 3 TYPICAL SBR OPERATIONAL CYCLE. BORTONE ET AL., 2009

As it can be seen from Figure 3, actual depletion of NH_4-N takes place evidently much earlier than the end of the fixed-time controlled oxic phase. Therefore, even if high ammonia removal is successfully achieved, the fixed-time strategy leads undoubtedly to a consistent waste of energy and thus to high and superfluous operation costs. The real-time control of SBR is therefore the major driver for the best performing application and success of SBR technology (Yang et al., 2010). Real time control proved to be an efficient way to increase process performances since it allows to (i) improve effluent quality (ii) decrease energy consumption and (iii) increase the specific amount of waste-water treated. Real time may take advantages of two types of parameters: directs and indirects. Direct on line control focuses on parameters such as NH4, NO3 and NO2 content. The general problems associated with establishing control strategy based on direct pollutants sensors (i.e. nutrients and COD sensors) include high sensor costs, complex maintenance and lag time (Casellas et al., 2006). However, the recent progresses in ICA are strengthening the affordability and reliability of nutrients on-line meters and increasing the feasibility of the realtime control strategies (Olsson et al., 2005). Considering the low-cost operations within SBRs processes are ever

increasingly adopted, cheap and reliable indirect parameters are necessarily of a key importance in the success of this technology. Over the last three decades many researchers have been focusing on the typical patterns of these parameters and a lot of advances have been done in both conventional and short-cut nitrogen removal. Indirect parameters studied and directly connected to biological reaction taking place inside the SBR are Dissolved Oxygen (OD), Oxidation Reduction Potential (ORP), pH, Oxygen Uptake Rate (OUR) and conductivity (Puig et al., 2005). In fact, the evolution of the process variables (pH, ORP, DO and conductivity) shows some characteristic patterns that indicate the end of nitrification and denitrification (Chang et al., 1996). When focusing on nitrogen removal, different end-points for the anoxic and aerobic phases could be observed during a cycle (Ra et al., 1998). In particular, the end points for nitrification can be: the ammonia valley (Chang and Hao, 1996); the residual carbon manipulation point (Battistoni et al., 2007); flex-point on the OD (Battistoni et al., 2003) and average variability of the OUR (Vanrolleghem and Coen, 1995). The end points for denitrification can be: nitrate knee and (Paul et al., 1998) nitrate apex (Hao, Huang, 1996) (Table 5). In particular it has been widely reported how pH and ORP show specific bending points. Ammonia valley, which takes place during aerobic phase, it is a minimum point in the pH corresponding to the complete ammonia depletion. Moreover, a sudden increase in DO is also contextually observable. A sudden fall in the ORP signal, on the other hand, during anoxic phase shows the characteristic nitrate knee and corresponds to the termination of the nitrate in the reactor. In the past decades several researches faced the problem of how to automatically detect these particular bending points and a lot of solutions have been proposed; nevertheless the most applied methods are: (i) detection of derivatives (Kishida et al., 2008) (ii) fuzzy neural networks (Marsili-Libelli, 2006; Peng et al., 2002) (iii) artificial neural networks (Yu et al., 1998; Cho et al., 2001) and (iv) artificial intelligence using episode representations (Rubio et al., 2004).

TABLE 5 PHASE PROCESS INDICATORS IN A SBR PROCESS (ADAPTED FROM MARSILI-LIBELLI ET AL., 2008)

Phase	Process	Indicators
Anaerobic/Anossic	Denitrification	Nitrate Knee point (NKP) Nitrate apex
Aerobic	Nitrification	DO breakout, Ammonia Valley ORP discontinuity (NBP or RCMP) OUR average variability

1.3.3.1. Substrate: municipal wastewater

Suitability for both start-up and long term operations in municipal waste water is extensively demonstrated in literature; notably Marsili-Libelli (Marsili-Libelli et al., 2008) incorporated real-time control in a stand-alone monitoring system for long-term unattended operation on a 20 Liter SBR. The monitoring system was composed of successive operations on the data starting from acquisition, validation and de-noising using a wavelet filter. Numerical derivation was performed and a fuzzy inference algorithm was used to detect end of the different phases. The key to successful monitoring was the Preliminary Data Validation (PDV) which is the ability to decide whether the acquired data is meaningful and possibly correct potential sensor error. Marsili-Libelli (2008) continuously operated the system for 6 months under the supervision of the fuzzy monitoring system. Results proved the robustness of the method, showing a correct phase detection in excess of 95%. Furthermore the fuzzy monitoring system was found to be capable of steering the process through the seasonal temperature variations and several feed changes. Another example of application of real-time control to municipal wastewater treatment might be found in the research work of Puig et al. (2006). In particular the authors presented a newly developed start-up control system which allowed each aerobic and anoxic reaction to be automatically adjusted to their optimal duration in order to achieve complete nitrification and denitrification. The control strategy was based on online monitoring of both OUR and ORP, to detect the end points of the aerobic and anoxic reactions phases, respectively. The research was carried out on a pilot-scale reactor fed with municipal wastewater. DO was kept at 2.0 mg/L and pH, ORP, and DO signals were collected continuously. The control system acted on the aerobic and anoxic phases of the SBR cycle. The aerobic phase length was controlled by the OUR measurements, while the ORP profile was used during the anoxic phase. The control system module was responsible for detecting the aerobic and anoxic phase end points using minimum OUR and ORP values (OURmin and ORPmin respectively which were found to be 35 mg $O_2L{-}1$ $h{-}1$ and -120 mV respectively). The control system was employed in the pilot-plant SBR for 3 months. In comparison with the fixed-cycles, the cycle length applying the real-time control system was shortened of about 56%. Effluent quality was found in agreement with requirements of the European Directive. On-line ORP and pH measurement have been proposed as useful process control parameters for biological nutrient removal in continuous-flow reactors (Hao and Huang, 1996), while Fatone et al. (Fatone et al., 2008) found the best control for nitrogen removal from wastewater in membrane bioreactors adopting ORP and DO and Paul et al. (Paul et al., 1998) evaluated pH, DO and ORP. Notably, the real-time control of intermittent aeration was found suitable for the best integration of the wastewater and foodwaste, when foodwaste disposers are applied in small and decentralized towns (Battistoni et al., 2007).

TABLE 6 COMPARISON OF VARIOUS NITROGEN REMOVAL PROCESS ADAPTED FROM AHN, 2006

	gO_2 needed/gN	gCOD needed/gN
Nitrification–denitrification	4.57	4
Nitritation–denitritation	3.43	2.4
Partial nitritation–Anammox	1.72	NA

1.3.3.2. Substrate: strong nitrogenous wastewater

Biological heterotrophic denitrification is known to occur by the action of heterotrophic bacteria using available carbon sources (Lee et al., 1997). Because swine wastewater can be particularly low in BOD/N ratio, the overall biological denitrification process may be limited and organic source must be supplied. As any external carbon source necessary results in an increase in operational costs, which can count up to 40–50%, the real-time control of the dosage is a major issue. Kim et al. (Kim et al., 2004) evaluated a possible integrated real-time system which could optimize the addition of the external carbon source to enhance nitrogen removal. The experimentation was carried out on a 9 Liter bench-scale SBR fed with swine wastewater. For the purpose of the research, swine waste as external carbon source for denitrification was examined and a pulsed pattern of addition was determined. Practically, after the feeding, external carbon source was continuously added at intervals of 10 min each. If the quantity of swine waste was deficient for complete denitrification, the next addition cycle was started until nitrite knee point appeared. Results showed that by the mean of the integrated real-time control for carbon addition a relatively constant final effluent was obtained with a nitrogen removal efficiency up to 96%. The importance of BOD/N ratio was also pointed out from other authors. Kishida et al. (2003) investigated the effectiveness of ORP, pH and DO as on-line control parameters for indicating denitrification followed by nitrification in a bench-scale SBR at fixed-HRT treating swine wastewater with 8 hour cycles. During start-up of the reactor (run1) incomplete denitrification occurred because of the low BOD/N ratio of the influent and raw pig slurry was therefore added as carbon source. Nevertheless slower nitrate and ammonium accumulation still occurred. Real-time control parameters were evaluated for their ability to regulate the SBR within this transient period. Interestingly neither ORP nor pH was found to be a helpful indicator of both nitrification and denitrification, and only false real-time control points appeared. It is therefore highlighted the importance of BOD/N ratio on effective real-time control within this experimentation since it is found that a low BOD/N ratio not only limited nitrogen removal from swine waste-water but also it makes real-time control impossible. As for the long term operation, Kishida et al. (2003) pointed out as once stability of the

reactor was achieved, the possibility for the process to be successfully controlled by real-time parameters was also investigated. Results indicated a Real-time Control Point (RCP) appearance in more than 200 cycles in a continuous way. In particular, in the ORP profile, RCPs appeared in both oxic and anoxic phases. pH profile, on the other hand, did not show to be as reliable as ORP since during anoxic phase the correspondent RCP-anoxic was not always clearly detected. Ga et al. (2009) evaluated the feasibility of a newly developed realtime control strategy of the aerobic treatment duration using pH (mV)–time profile. Experimentation was carried out on a 6 m^3 SBR fed with swine wastewater and operated in anoxic/aerobic sequence. The duration of the oxic phase was determined by real-time control system. After feeding the swine wastewater into the SBR, the remote on-line controller took registering ORP and pH every second and calculated an average of 60 data points every minute. The Moving Slope Change (MSC) of the pH value was also calculated every minute with a data sample size of five samples. The real-time control point was detected by tracking the change pattern of the MSC. With respect to ORP profile results indicated a poor reliability of this parameter since Nitrate Breaking Point (NBP) detection rarely happened. On the other hand even when NBP did not occur on the ORP-profile, the pH (mV)–time profiles consistently revealed the NBP and, successful real-time control was consequently achieved. Furthermore the authors investigated MSC applicability for anoxic reaction. Both ORP and pH profiles allowed identification of Nitrate Knee Point resulting in enhanced nitrogen removal up to 100% but it is also pointed out how, in some cases, pH (mV)–time was not able to detect the point (Table 7).

TABLE 7 SUMMARY OF THE MAIN RESULTS OBTAINED FROM REAL-TIME CONTROL OF CONVENTIONAL BIOLOGICAL NITROGEN REMOVAL.

Type of wastewater	Scale	RCP start-up	RCP long-term	Strategy	Performances
Municipal	Bench-scale	Not included	ORP,pH	Preliminary data validation, fuzzy control	95% correct phase detection
Municipal	Pilot-scale	ORP,OUR	ORP,OUR	ORP<120 mV, OUR<35mgO2/l/h	–
Swine wastewater	Bench-scale	Neither ORP nor pH	ORP,pH	Detection of NKP, detection of NBP	
Swine wastewater	Bench-scale	Not-included	ORP	Continuous carbon supply until NKP detection	96% nitrogen removal
Swine wastewater	Pilot-scale	pH MSC	pH MSC	RCPs detection on pH(mV)–time	100% nitrogen removal

1.3.4. REAL-TIME CONTROL OF SHORT-CUT NITROGEN REMOVAL

In conventional nitrification–denitrification process, ammonia is oxidized to nitrate by two different groups of bacteria. The first bacterial group, named ammonia-oxidizing bacteria (AOB) converts ammonia to nitrite, later in the reaction the second group named nitrite oxidizing bacteria (NOB) further transforms nitrite to nitrate. Short-cut Biological Nitrogen Removal (scBNR) is an innovative technology that oxidizes ammonia to nitrite and reduces this latter directly to nitrogen

gas (Chung et al., 2007). Over the last few years, interesting progresses have been reached in the use of the on-line process control to achieve and maintain both conventional and short-cut nitrification. For the latter all of these progresses aim at creating the most favorable growing condition for AOB biomass promoting contemporaneous NOB washout over the start-up phase. Nevertheless few major drawbacks still limit the widespread application of this technology since specific critical conditions are required to suppress nitrite oxidation without retarding ammonia oxidation. The nitrite route can be achieved according to two alternatives: (i) combining the partial nitrification of ammonium to nitrite and the subsequent heterotrophic denitrification or (ii) combining the partial nitrification of half of the ammonia contained the waste-water followed by the Anammox process (ammonia oxidation using nitrite as elector acceptor in anoxic environment). Factors affecting shortcut nitrogen removal have been extensively studied over the past few decades. It has widely reported (Jenicek et al., 2004) that selection of AOB over NOB, and thus highly efficient nitritation process, is particularly influenced by: temperature, Free Ammonia (FA), Free Nitrous Acid (FNA), Dissolved Oxygen (DO) and pH. In addition, it is widely accepted that DO plays a major role in nitritation. Activation energy of the ammonia oxidation step is higher than that of the nitrite oxidation step (Hellinga et al., 1998). Oxygen, and carbon requirements for different types of processes are compared in Table 6. Furthermore, scBNR demonstrated to be up to twice faster when compared to conventional nitrification denitrification. For these reasons, partial nitrification to nitrite was recently widely investigated especially with relation to a large variety of wastewater seriously lacking in biodegradable carbon source. It comes that the maximum specific growth rate of the AOB will be higher than that of the NOB at the temperatures above 25 °C. Free ammonia over 10 mg N-NH3/L is reported in literature to inhibit Nitrosomonas, while Nitrobacter is inhibited just only with 0.1–1.0 mg N-NH3/L (Anthonisen et al., 1976). Oxygen saturation constants of Monod Kinetics for nitritation and nitratation are known to be 0.3 and 1.1 mg/L respectively (Wiesmann, 1994). Therefore it is reported that a limited oxygen concentration between 0.5 and 1.5 mg/L would act as an effective selective pressure factor leading to the complete washout of NOB over AOB biomass. Nevertheless some authors found limited oxygen concentration to be an obstacle for nitritation. The possibility of applying realtime control to optimize such important parameters would be an enticing opportunity to reduce start-up time to a minimum and consequently to achieve stable partial nitrification in a shortened period. Therefore, below we discuss first the start-up to short-cut the nitrogen cycle, then the long-term operation (Table 8).

TABLE 8 SUMMARY OF THE MAIN RESULTS OBTAINED FROM REAL-TIME CONTROL OF SCBNR (GUO ET AL., 2009, VAN LOOSDRECHT AND HEIJNEN, 1993, VÀZQUEZ-PADÌN ET AL., 2010, GU ET AL., 2011, ZENG ET AL., 2010, GAO ET AL., 2009)

Type of wastewater	Scale	RCP start-up	RCP long-term	Strategy	Performances
Municipal	Bench-scale	DO	DO	Evaluation of O2 concentration	98% nitrite accumulation
Municipal	Bench-scale	DO	pH, ORP	Detection of ammonia valley, determination of NKP	–
Municipal	Bench-scale	DO,FA	–	Determination of FA inhibition	–
Municipal	Bench-scale	pH	pH	Aeration duration based on NH4 valley	95% partial nitrification
Strong nitrogenous	Pilot-demonstration scale	Blower frequency	Blower frequency	Detection of NBP in blower frequency	Achieving of Shortcut in 40 days
Swine wastewater	Pilot scale	ORP,DO,pH	–	Intermittent aeration DO<2.0 mg/L	83% nitrite accumulation

1.3.5. START-UP TO ACHIEVE THE SCBNR

1.3.5.1. Substrate: municipal wastewater

It is reported that partial nitrification is difficult to achieve when treating weak nitrogenous wastewater (Guo et al., 2009). Ma et al. (Ma et al., 2009) applied real time control to start up shortcut nitrification–denitrification in domestic waste-water treatment. Temperature, MLSS and DO in reactor were kept at 28 °C, 2400 mg/L and higher than 2.0 mg/L respectively during start-up period. After 2 months Nitrogen Accumulation Rate (NAR) reached 98% but after a few days it dropped to 80% because of a sharp increase in ammonia concentration in raw influent which, moreover, caused filamentous sludge bulking occurrence. In order to restore the original performances a pre-anaerobic operation mode was adopted. Since anaerobic condition might have stronger inhibition on anabolism of NOB than AOB, NOB were likely to be washed out through long-term pre-anaerobic operation mode. Vázquez-Padín et al. (2010) achieved start-up of short-cut nitrification with nitrite accumulation of 130 mg N-NO2/L in a batch nitrifying granular sludge reactor treating domestic wastewater with dissolved oxygen concentration around 8 mg/L and at room temperature. The mean diameter of the granules was 3.0±0.4 mm which was particularly large possibly causing important oxygen limitation in the deep layers of the granule. Experiments were carried out at different DO concentrations to assess effects of oxygen on the granular SBR. By working with biofilms, the importance of the volume of bulk liquid ratio was indicated as one of the key points in the oxygen transport from the gas phase to the granule surface. Van Loosdrecht et al. (1993) reported that the oxygen penetration in the biofilm varies typically in a range from 75 to 200 μm and therefore it is important to maximize the surface area of the granule to maximize reactor performances. During a single cycle DO was varied and monitored obtaining total oxidation of the ammonium by working at DO concentrations from 4 to 30 mg/L; however AOB activity fall of 75%

when DO was 2 mg/L. NOB activity increased considerably when DO was raised up to 8 mg/L. Maximal NOB activity was reached only at DO of 22 mg/L. Experimentation showed the role of mass transfer limitations involved when working with granules with large diameter. Effects of free ammonia on granular sludge were also evaluated. As already reported in several research papers the authors found FA to affect NOB but they also point out how it was not expected to be the main factor affecting the decrease in nitrite oxidation of the reactor at long term operations. Guo et al. (2009) applied real-time control to start-up partial nitrification to nitrite in a 10 Liter SBR treating domestic wastewater. Differently from other research works, DO was not controlled since it was meant to decelerate nitrification rate. Instead aeration duration was real-time controlled. DO was kept at 2.5 mg/L, temperature lower than 26 °C, free ammonia was below 1 mg/L while SRT was kept above 30 days. Aeration duration was determined by detecting the ammonia valley on the pH profile. Results indicated the achieving of 20% in partial nitrification during the first day of operation, 92% on day 10 and a further improvement up to 95% at day 30. The initial nitrite accumulation was important to start an effective aerobic duration control scheme. Real-time aeration control was not only favorable for stable partial nitrification to nitrite, but also beneficial for complete ammonia oxidation. In particular it is shown how such real time control could reliably indicate the exact consumption of ammonium oxidation, leading to a much greater ammonium utilization by AOB than nitrite by NOB. Therefore, nitrite accumulation could be maintained and sludge population optimization could be achieved. Blackburne et al. (2008) investigated the aerobic duration control to achieve nitrogen removal via nitrite for a laboratory-scale representation of a domestic wastewater SBR treatment process. After initially inducing 40% nitrite accumulation with formic acid addition, the process proved effective in achieving a steady state whereby over 80% nitritation was sustained. Investigation of the cause of nitritation by a calibrated ammonium and nitrite oxidation model showed aerobic duration control as the key factor leading to nitritation. The process robustness could be enhanced with a better measure than OUR for the aerobic duration control. OUR is clearly affected by processes other than ammonium oxidation (e.g. heterotrophic oxidation of COD). Volcke et al. (2006) proposed (based on simulation studies) a method to control the extent of nitritation in a SHARON reactor using a cascade feedback control loop with an oxygen set point and pH control. This type of system may also be applicable to this application. Alternatively, the direct measurement of the ammonium concentration to determine the end of ammonium oxidation may be effective but will rely on a robust ammonium probe, which, in the past, has not been available.

1.3.5.2. Substrate: strong nitrogenous wastewater

Jenicek et al. (2004) during their experimentation with high nitrogen load waste water noticed the inhibitory effects of low DO (0.3–0.8 mg/L) not only in nitrate formation but also in nitrite generation. The efficiency of nitrite production was limited by decreasing pH and oxygen concentration. When oxygen concentration was raised up to 4 mg/L, 20% improvement of the nitrite production efficiency was reached. The Authors proposed the reason for this finding to be due to the decreasing pH during NH4 oxidation with high nitrite concentration leading to HNO2 formation, which concentration, if too high, would result both in nitrite and ammonia oxidizers inhibition. Other researchers also found a tight correlation between DO and pH in promoting nitrite formation. Authors also found Free Ammonia (FA) and Free Nitrous Acid concentration a considerable inhibiting factor during start-up. Particularly, it was found that low pH (<6.5) and high concentration of nitrite (700–1200 mg/L) cause severe inhibition of further nitritation. At such condition HNO2 was found to be around 1–1.5 mg/L which is supposed to repress both AOB and NOB activity. Free ammonia and free nitrous acid activity on partial nitrification achieving were also evaluated by Vadivelu et al. (2007). For the purpose of the research an 11 Liter reactor was fed with synthetic waste water at high nitrogen-load (1000 mg/L N-NH3). The authors studied FA and FNA effects on nitrite accumulation while the reactor was kept at DO=3 mg/L and pH 7.3.They found that the biosynthesis of *Nitrobacter* totally stopped at above 6 mg N-NH3/L and 0.02 mg NO2/L. *Nitrosomonas* biomass was not inhibited by FA at a concentration up to 16 mg N-NH3/L while 0.40 mg N-HNO2 was enough to completely stop any metabolic process. Recently, Gu et al. (Gu et al., 2011) introduced a novel real-time control strategy based on pH and blower frequency to achieve short-cut nitrification at low temperature in an 8800 Liter SBR. Oxygen concentration within the reactor was kept at 2.0 mg/L by mean of a centrifugal blower equipped with a frequency converter. The blower frequency–time curve was the parameter utilized by the automatic system to identify the specific control points which were also compared to conventional derivatives such as dpH and dORP.

In particular the authors highlighted how, at the end of nitrification, ammonia valley not always appeared, while the specific NBP emerged in the frequency–time curve consistently. Therefore blower frequency is indicated as a more reliable parameter than pH for realtime control within this specific process. Furthermore, in the paper, it is also demonstrated the start-up of short-cut nitrification at low temperature (twater=11–16 °C) in 40 days with the application of realtime control strategy based on frequency and pH in pilot scale SBR process and proper solid retention time (SRT). The optimal SRT for improving the growth of AOB and NOB is different with the temperature. Particularly, maintaining the SRT of the system in a range lower than the optimal

AOB SRT but higher than the optimal NOB SRT, NOB biomass was washed-out and sludge was gradually AOB-enriched. Occurrence of short-cut nitrogen removal at normal conditions for high nitrogen load wastewaters with real time control was also investigated by Gao et al. (2009). Experimentation was carried out on a 38 Liter reactor fed with soy-bean raw wastewater. In order to assess the influence of excessive aeration under fixed time control DO was varied between 0.2 mg/L and 7.2 mg/L. With the increase of the number of running cycles under excess aeration, the concentration of NO_3 gradually increased. After 13 cycles the authors found nitrosation ratio to be decreased indicating that short-cut nitrification turned to conventional nitrification. It is concluded that within this experimentation excess aeration indeed adversed short-cut nitrification. Reactor start-up was simply achieved by temperature control. In fact, since it is reported that the growing rate of AOB to be much higher than that of NOB at temperatures above 30 °C (Hellinga et al.,1998) domesticating sludge at 31 °C allowed to get 96% of nitrosation rate. In order to improve organic carbon management and reduce the sludge production in the case of combined anaerobic digestion and biological nitrogen removal of piggery wastewater with low BOD/N ratio, partial nitrification is suggested. For this purpose, Rajagopal et al. (2011) applied ORP, pH and DO real-time monitoring in order to achieve short-cut nitrification.

1.3.5.3. Substrate: swine wastewater

The experiments were performed in a 125 Liter pilot scale continuously stirred reactor kept at ambient temperature. To obtain nitrite pathway, dissolved oxygen and aeration pattern were controlled in the anoxic/oxic phases. In particular as observed by Boursier (2003), application of an intermittent aeration pattern largely favors nitritation during biological nitrogen removal process. Based on this observation, DO concentration of 2 mg/L was considered as a threshold value such that whenever DO surpassed this value aeration was switched off. With this strategy, the authors report a nitrite accumulation up to 83% of the initial nitrogen. As result of partial nitrification, an average reduction on the N-NH4 was observed close to 98–99% resulting in a Total Kjeldahl Nitrogen (TKN) removal up to 75%. Further research is planned to assess the strategy reliability on longterm operations.

1.3.6. LONG-TERM OPERATION OF THE SCBNR

Ma et al. (2009) also investigated the effect of different DO levels on stabilization of short-cut nitrogen removal (0.5 mg/L, 1.0 mg/L and 1.5 mg/L), with real time control strategy as good

judgment of whether nitrification–denitrification was accomplishing. Unexpectedly long-term high NAR of more than 95% happened not only at low DO level (0.5 mg/L) but also at the comparative abundant circumstances (1.0 and 1.5 mg/L). Long term real-time control strategy, which could have led to bacterial colony optimization, is proposed by the authors to be responsible for this observation. Gao et al. (2009) in order to evaluate the efficiency of real-time control for short-cut nitrification in long term operation gradually dropped the temperature from 31 °C to 25 °C. Aeration length was real-time controlled by pH and ORP values. In particular aeration period was automatically stopped when detection of ammonia valley occurred. Stability of short-cut nitrification under real-time ORP/pH supervision was tested for 2 months. The study demonstrated the high efficiency of using ORP and pH to achieve a stable short-cut nitrification at normal operational condition. Jianlong et al. (2004) studied partial nitrification in an 8.21 Liter reactor under limited dissolved oxygen conditions and compared Ka (maximum ammonia oxidation rate) varying DO concentrations at different pH. When pH was kept at 7.5, DO concentration was raised up to 1.5 mg/L and it demonstrated to effectively enhance nitrite accumulation when compared to that at 0.5 mg/L. Experimentation carried out at pH of 6.5, 7.5, and 8.5 showed respectively 80%, 50% and 15% improvements in the performance when DO was raised from 0.5 up to 1.5 mg/L. Obviously the increase in Ka depended on the pH value. A series of ammonia oxidation experiments was also performed under various FA concentrations keeping DO constantly at 1.5 mg/L and varying pH from 6.5 to 9.5. Results showed a positive influence of FA on nitrite accumulation for concentration between 6.5 and 7.5 mg/L but when FA reached 9.5 mg/L a dramatic AOB inhibition occurred. The authors concluded comparing the effect of operational parameters such as DO, pH and temperature on ammonia oxidation rate Ka and nitrite accumulation rate Kn. What is shown is that the best results were obtained at pH=7.5 DO=1.5 mg/L and temperature set at 30 °C. Furthermore nitritation under progressive changes in DO concentration was evaluated by Ruiz et al. (2003). Results showed that nitrite accumulation was not influenced by DO concentration between 2.7 and 5.7 mg/L but they found a linear correlation with ammonia disappearances and nitrite accumulation at DO=0.5 mg/L. Lower DO inhibited both ammonia oxidizer and nitrite oxidizer biomass.

1.3.7. CONCLUSIONS

This short review analyzes the potential of the indirect parameters for the real-time control of the conventional and the short-cut nitrogen biological removal. To date, the conventional via-nitrate processes are controllable by robust and reliable systems mostly based on the ORP for the

denitrification, and DO and pH for the aerobic nitrification. On the other hand, the DO and pH (indirectly to control free-ammonia and free nitrous acid) are recognized as key parameters to start up towards the short-cut nitrogen removal, while ORP may be useful for heterotrophic denitritation.

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1.3.8. REFERENCES

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1.4. INTEGRATING PROCESS ENGINEERING AND MICROBIOLOGY TOOLS TO ADVANCE ACTIVATED SLUDGE WASTEWATER TREATMENT RESEARCH AND DEVELOPMENT

Wastewater treatment is arguably the largest biological process industry worldwide. Both the size of the treatment plants and the total number of full-scale installations worldwide are unmatched by any other industry utilizing biological processes. Yet, despite this major importance, the level of research and development (R&D) investment in this industry is generally a very small fraction of the total capital and operating costs. Considering that the most common wastewater process technology, the activated sludge system, was invented early in the last century, today's systems are still surprisingly similar to these early ones. One of the main reasons for this apparent lack of progress has been the slow development of public awareness of environmental values and the corresponding slow increase in demands for the treated effluent quality. For a long time, the "basic" activated sludge technology was sufficient for most situations. The main demands in this technology were related to the hydraulic flow through the system and the hydraulic (civil) engineers were therefore the main experts in this technology. With the arrival of nutrient (nitrogen and phosphorus) removal demands grew the level of complexity of the systems. Increasingly, process or chemical engineers became involved in the development, design and operation of such treatment processes. Today, a significant fraction of professionals in this industry have such a background, particularly those involved in concept design and optimization.

However, the biological fundamentals behind most treatment technologies are often treated as "black boxes" with presumably known inputs and outputs (Keller et al., 2002). The science of microbial ecology is focused on how microbial populations assemble to form communities and how these communities interact with each other and their environment.

The performance of biological processes in operation in the wastewater treatment plants strongly depends on the activities and interaction of the microbial community. Thus, information on the identity of microorganisms responsible for specific activities, on interactions between cells of the same or different populations and on the influence of changing environmental conditions are important for optimizing these processes. Microbial diversity can be seen in many ways besides phylogeny, including cell size and morphology, physiology, motility, mechanisms of cell division, pathogenicity, developmental biology, adaptation to environmental extremes and so on. In this context, traditional microbiological and conventional microscopic techniques are not able to answer the many questions about species composition, structure and bacterial distribution as well as the spatial activity, because the vast majority of microorganisms, over 99% of all the species, have

never been grown in laboratory cultures. The disparity between culturable and *in situ* diversity has increased the importance of culture-independent molecular approaches. Molecular techniques based on the comparative analysis of specific gene sequences are now being widely used in microbial ecology and have allowed the discovery of many novel and yet unculturable microbes. This constitutes a significant progress for microbial ecology studies even though the enrichment and isolation of microorganisms is necessary since is the only way to fully characterize their properties and predict their impact on an environment.

The rRNA approach has become the gold standard for the cultivation-independent assessment of bacterial diversity in natural and engineered systems. The importance of these 16Sand 23S rRNA genes is that they code for the ribosomes, needed for protein synthesis; they are about 1500 and 3000 nucleotides in length, respectively, and contain both highly variable and conserved regions.

Commonly used approaches in molecular microbial ecology are based on: the extraction of nucleic acids, their amplification by Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE) of the 16S rRNA genes, followed by cloning, sequencing and finally identification and affiliation of the isolated clone together with adequate reference sequences with the aid of phylogenetic software. Subsequently specific oligonucleotide probes for these organisms can be designed and used for in situ hybridisation (FISH) of the original sample.

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2. ROLE AND CHARACTERISTICS OF PROBLEMATIC BIOFILMS WITHIN THE REMOVAL AND MOBILITY OF TRACE METALS IN A PILOT-SCALE MEMBRANE BIOREACTOR

This chapter of the thesis is published: Di Fabio S., Lampis S., Zanetti L., Cecchi F., Fatone F., 2013. Role and characteristics of problematic biofilms within the removaland mobility of trace metals in a pilot-scale membrane bioreactor. Process Biochemistry 48, 1757–1766.

Abstract

The characteristics of problematic biofilms (i.e., fouling and clogging layers) were studied with regards to the removal and fate of trace metals (contents well under 100 g/L) during the long-term operation of a pilot-scale membrane bioreactor for the treatment of real wastewaters from a large industrial area. Results showed that clogging layer was more effective than suspended activated sludge in the biosorption of As > Zn > Ni > Cd > Sb > Fe > Se due to the synergic effects of extracellular polymeric compounds and metal-resistant bacteria. In fact the selective microbial speciation of the phylum of *Bacteroidetes*, which is highly resistant to heavy metals, was observed in the clogging sludge in spite of the very low concentration of dissolved metals in the bioreactor. Compared to the suspended activated sludge, the clogging layer enhanced the biosorption of very toxic substances such as As, Cd and Ni. In fact, the metal contents were respectively: 7.9–7.4 vs.690–840 µgAs/kgTS; 1.5–2.2 vs. 149–219 µgCd/kgTS; 58.8–71.7 vs. 227–298 µgNi/kgTS. Then, the potential desorption of metals during the membrane acid cleanings was estimated as relevant as 10–15% of the metals associated to the clogging sludge. The combined effects of pH and the selected microbial community, and the minor effect of the redox potential, let us conclude on the major importance of bio-sorption/desorption mechanisms with respect to bio-precipitation/dissolution.

2.1. INTRODUCTION

The application of membrane bioreactors (MBRs) is widely recognised as an effective option for enhanced wastewater treatment in the industrial and urban sectors (Judd, 2008; Judd, 2006; Lin et al., 2012). However, early misconceptions about the technology are persistent, and false statements are commonly encountered in articles and conferences. In fact, previous statements that MBRs are better for removing organic micropollutants cannot be generalised because under similar biological operating conditions (i.e., temperature, load and process), regular MBRs show very similar

performances to conventional activated sludge processes (CASP) (Lesjean et al., 2011; Fatone et al., 2011; Bolzonella et al., 2010). However, membrane bioreactors were reported to potentially enhance metals removal primarily by reducing effluent suspended solids (Lesjean et al., 2011). Also, the role of the biological operating parameters was investigated by a number of authors (Di Fabio et al., 2013; Santos and Judd, 2010; Katsou et al., 2011; Fatone et al., 2008). It was found that the sludge age was a key-parameter in influencing biosorption and/or bioaccumulation (Santos and Judd, 2010). However, a gap in knowledge currently exists with respect to the relationship between the fouling and clogging layers and the removal and mobility of trace metals during waste water treatment. In fact, due to their negative influence on membrane permeability, these problematic layers were extensively studied (Drews, 2010), but rarely the biosorption of heavy metals (Flemming, 2011) and the possible desorption during the membrane acid cleanings were adequately considered. According to these remarks and considering the scale of our experimental research, the fouling and clogging sludge form a layer compartment acting like a biofilm (referred as "biofilm layer") that can play an important role in biosorption/transformation of trace metals in membrane bioreactors. To date, a number of engineered biofilms for metal ion removal have been developed and applied to treat highly contaminated industrial wastewater, wherein metal concentrations are in the range 1–10^3 mg/L (i.e., discharges from mining, metal processing, electroplating, electronics) (Le Cloirec et al., 2003). However, information on trace metals and slightly contaminated wastewater is necessary as this scenario is very widespread in wastewater treatment plants. In addition, a lot of research studied the metals biosorption in pure microbial cultures and/or lab-scale researches. On the other hand, this paper presents what Plant was observed in a real membrane bioreactor, which is subject to sudden and drastic changes in the quality and quantity of the influent wastewater coming from a large industrial area. Therefore, this paper addresses the behaviour and the role of clogging sludges (CSs) in the operation of a MBR used for wastewater treatment, specifically on the removal and fate of the heavy trace metals. The removal of metals by the CS was assessed in a pilot plant MBR treating real industrial wastewater that contained heavy metals in the range of nanograms to micrograms per litre. Aside from the characterisation of solids, organic contents, nutrients and extracellular polymeric substances (EPSs), gene-based identification was used to study the microbial communities of both the suspended (SAS) and clogging sludges (CS). In addition, the effect of membrane maintenance cleaning on metal desorption was studied. Therefore, the results of this paper will also contribute to support the decisions about the best environmental strategies for MBRs operations and maintenance.

2.2. MATERIALS AND METHODS

2.2.1. THE PILOT PLANT AND THE OPERATING PARAMETERS

In parallel with a large centralised full scale MBR, the pilot-scale MBR (Di Fabio et al., 2013) (Figure 4) was operated for two years, treating real petrochemical wastewater. Before biological treatment, the raw wastewater collected from a large industrial area underwent conventional clariflocculation which abated the heavy metals content below 100 g/L. The pilot-scale MBR had a reaction volume of 4.2 m^3 and consisted of multizone preanoxic–oxic scheme (V_{anoxic}= 1.46 m^3; $V_{aerobic}$= 2.2 m^3). Finally, the aerobic filtration chamber had volume of 0.58 m^3. The submerged hollow-fibre membrane module (ZeeWeed 230; GE Water and Process Technologies) had membrane surface area 21.7 m^2 and nominal pore size of 0.04 μm and were made of polyvinylidene fluoride (PVDF). Over the experimental period, the pilot MBR was operating with an hydraulic retention time (HRT) of 13–14 h and specific loading rates of 0.15–0.25 kgBOD_5/kgVSS day and 0.018–0.023 mgN/gVSS day. SRT was approximately 90 days to enhance the biodegradation of priority hazardous compounds (i.e., PAHs and PCBs) (Fatone et al., 2011). The temperature of the mixed liquor varied in the range of 18–27 ˚C. Several online sensors were installed to monitor the most important parameters in the biological and filtration processes, including dissolved oxygen (DO-Hach-Lange), oxidation reduction potential (ORP-Yokogaw), mixed liquor temperature (Hach-Lange), permeate turbidity (Hach-Lange), transmembrane pressure (TMP-Endress Hauser). The data were recorded using a multi-channel recorder model Ecograph (Endress Hauser). Mechanical cleaning of the membrane module was conducted through backwash and coarse bubble aeration (0.2 Nm^3/m^2h^{-1}). As with the operation of the parallel full scale MBRs, maintenance cleanings in place were performed once a week by 4-h soakings in cleaning solutions of hypochlorite (0.05 wt% NaOCl) or citric acid (0.1 wt% $C_6H_8O_7$), occasionally with chloridric acid up to a pH of 2–3. According to the technical recommendations of the membrane manufacturer, the whole filtration section was designed and operated to conform to the fluiddynamic and operating conditions of the industrial-scale MBRs. Thus, it operated with a net flux of 10 LMH and a filtration cycle of 650 s and 40 s for permeation and backwashing, respectively. MLSS concentration in the ultrafiltration tank was maintained constant by applying sludge recirculation (R = 2.5) to the first anoxic compartment of the pilot plant. The specific air demand (SAD) was in the range of 0.23–0.30 Nm^3/m^2h (SADp 25 $m^3_{air}/m^3_{permeate}$) while the aeration cycle was set for10 s on and 10 s off. Thus the typical operation of MBRs was followed in terms of air supply (0.25–0.54 Nm^3/m^2h for SADm and 12−28 $m^3_{air}/m^3_{permeate}$for SADp - Judd, 2006).

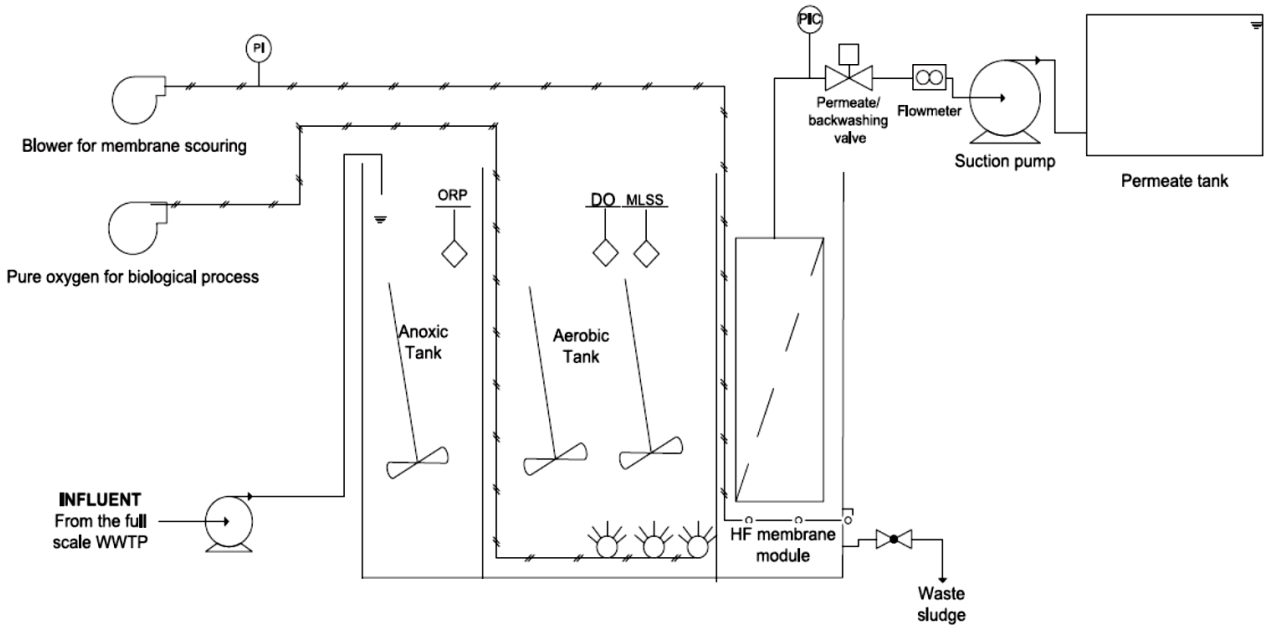

FIGURE 4 P&ID OF THE MBR PILOT PLANT

2.2.2. SUSPENDED AND CLOGGING SLUDGE: SAMPLING AND CHARACTERISATION

After one year of continuous and pseudo steady-state operation of the MBR, the CS and the SAS were sampled over six months. The membrane module was periodically lifted from the ultrafiltration tank and the clogging sludge was sampled from three different zones over the length of the membrane fibres. A composite and homogeneous sample was then considered for further analytical measurements. We analysed the dry solids and the liquids for conventional parameters (i.e.,COD, nitrogen (N) and phosphorus (P)) and metal content according to the Standard Methods (APHA, 1998) and U.S. Environmental Protection Agency (EPA) methods, respectively. In particular, Be, B, Al, V, Cr, Mn, Co, Ni, Cu, Zn, Mo, Ag, Cd, Sn, Te, Ba, Tl, Pb, As, Se and Sb were determined using the EPA method, EPA 200.8/94; Fe was determined according to APAT IRSA-CNR 29/2003 3020 (APAT, 2003); Al and Mg were measured according to IRSA Q 100/94-3110; and Ca was determined using IRSA Q 100/94-3070. To evaluate the role of extracellular polymeric substances (EPSs) in the removal and/or release of metals, both soluble and bound EPS were measured. The soluble from the bound EPS were separated through centrifugation (4000 × g for 20 min at 4 ˚C) followed by additional thermal treatment at 80 ˚C (Judd, 2006). In addition, proteins and carbohydrates were measured using the photometric methods of Lowry et al. (Lowry et al., 1951) and Dubois et al. (Dubois et al., 1956), respectively. Finally, gene-based identification by

PCR-DGGE analysis was used to study the composition of the microbial communities in both SAS and CS.

2.2.3. PCR-DGGE ANALYSIS

Total DNA extraction was performed using the FAST DNA®Spin Kit for Soil (MO BIO, Carlsbad, CA), following the manufacturer's instructions. Approximately 0.5 g of material was used per extraction and the total DNA obtained was polymerase chain reaction (PCR) amplified and then analysed by denaturing gradient gel electrophoresis (DGGE). Samples from both SAS and CS were analysed. The whole 16S rDNA was first amplified using primers F8 and R11 (Weisburg et al., 1991). Then, a nested PCR was performed starting from the 16S rRNA gene amplicons obtained from the first amplification. In this second PCR reaction, the ipervariable V3 region of the 16S rRNA gene was amplified using primers p3 (with a GC clamp) and p2 (Muyzer et al.,1993). The PCR reactions' master mix composition and the temperature conditions for the amplification were as described in Lampis et al. (Lampis et al., 2009). The PCR products were quantified using the Low DNA MassTM Ladder (Celbio, Italy) in a 2.0% agarose gel. DGGE analysis was performed on the V3 region amplicons. The gel (8% acrylamide/bisacrylamide 19:1, BioRad) was cast using a denaturing gradient of 30–60%, with 100% denaturant defined as 7 M urea and 20% (v/v) formamide. Electrophoresis was performed at 50 V for 16 h at 65 ˚C with the DcodeTM Universal Detection System (Biorad), and the gels were stained with SYBR Green I (Sigma, Italy). The PCR products corresponding to the different samples were loaded in the same DGGE gel. Equal masses of the PCR products were loaded in each lane to allow for a semi-quantitative comparison among lanes. The interpretation of the DGGE gels with respect to the Similarity Index was performed with the software SPSS 8.0 for the calculation of the Pearson coefficient, while the NTSYS software was used for the dendrogram formation, according to the UPGMA method (Kropf et al., 2004).

2.2.4. CLONING, SEQUENCING AND PHYLOGENETIC ANALYSIS

The major bands in the DGGE profiles were excised, eluted and re-amplified using p1 and p2 primers (Muyzer et al., 1993). The different amplicons were then cloned in a pGEM vector through the pGEM-T Easy vector system (Promega, Italy), according to the manufacturer's instructions. Clones were then checked against their corresponding original bands by DGGE analysis, which compared the bands excised in the profile with those cloned. Afterwards, the cloned bands were sequenced (PRIMM Biomedical, San Raffaele, Milan, Italy) on both strands, checked for the presence of artefacts through the Decipher's Find Chimeras tool (Wright et al., 2012) and finally, a search for similarities was performed using the BLASTN database (Altschul et al., 1997). Twelve

nucleotide sequences related to the major bands in the DGGE profiles determined in this study were deposited in NCBI database under the following accession numbers: KC797676–KC797680 for SAS sequences and KC797681–KC797687 for CS sequences. The sequences were initially aligned using the multiple alignment program CLUSTAL X 1.83 (Thompson et al., 1997). A phylogenetic tree was then constructed using the neighbour-joining method with the MEGA version 5.0 software package (Tamura et al., 2011). A bootstrap analysis was performed on the basis of 1000 bootstrap replications.

2.2.5. BATCH TEST TO ASSESS METAL DESORPTION DURING CHEMICAL MAINTENANCE CLEANING

Whenever the membrane module was lifted from the filtration chamber, the clogged part of the fibres were measured by means of a common metric tape. Over three measurements, we observed that CS stably clogged the membrane fibres for around 10%. In order to estimate the quantity of clogging sludge in the filtration chamber, we considered that the membrane hollow fibres occupied 60% of the working volume of the filtration chamber (Judd, 2006). Therefore, the jar test was used to gently mix 2.1 g of the clogging sludge per litre of citric acid solution (0.1 and 0.2 wt% C6H8O7). HCl was used to evaluate the impact of the boundary acid conditions on the desorption of metals. Temperature was in the range 25–27 ˚C, and the test conditions were as reported in Table 9. Four different batch tests were performed and repeated twice. As the actual membrane cleaning lasted for 3–4 h, the metal desorption from CS in a 4-h soaking in the cleaning solution was investigated.

TABLE 9 DESORPTION FROM THE CS: CONDITIONS OF THE BATCHE TEST (TEMPERATURE STABLE AT 26°C)

	Clogging sludge (g/L)	$C_6H_8O_7$ concentration (mg/L)	HCl dosage	pH
Blank	2.1	0	No	7.6
Test 1	2.1	1000	No	3.2
Test 2	2.1	1000	Yes	2
Test 3	2.1	2000	No	2.9
Test 4	2.1	2000	Yes	2

TABLE 10 LONG-TERM METAL OCCURRENCE AND REMOVAL IN THE MBR PILOT PLANT (NUMBER OF SAMPLES = 95 COLLECTED OVER ONE YEAR, 8 PER MONTH)

	Frequency of occurrence (%)	Influent concentration (average ± variation coefficient) (μg/L ± %)	Removal (average ± variation coefficient) (% ± %)
Al	100	74.90 ± 77.3	27.0 ± 32.6
Ag	5	0.27 ± 27.0	54.5 ± 6.5
As	91	2.38 ± 36.2	18.8 ± 25.6
Ba	100	20.12 ± 55.5	20.2 ± 26.7
Be	0	–	–
B	100	302.07 ± 68.9	17.2 ± 26.7
Cd	0	–	–
Co	21	0.33 ± 51.6	40.1 ± 32.3
Cr	98	11.81 ± 71.3	62.3 ± 32.4
Cr6+	0	–	–
Cu	94	4.25 ± 87.8	41.4 ± 31.8
Fe	100	889.44 ± 61.6	85.0 ± 14.3
Hg	79	0.23 ± 58.2	52.1 ± 29.1
Mn	100	22.50 ± 55.8	66.3 ± 26.0
Mo	100	8.70 ± 34.8	13.6 ± 22.8
Ni	97	4.37 ± 72.1	25.0 ± 30.2
Pb	97	2.25 ± 110	45.4 ± 37.1
Sb	21	0.62 ± 38.9	38.1 ± 32.9
Se	56	4.28 ± 51.1	9.2 ± 23.1
Sn	72	1.54 ± 75.1	42.3 ± 31.2
Tl	0	–	–
Te	0	–	–
V	98	4.08 ± 82.5	31.3 ± 28.8
Zn	99	33.53 ± 77.1	28.2 ± 32.6

2.3. RESULTS AND DISCUSSION

2.3.1. METAL CONTENT AND ITS FATE IN MEMBRANE BIOREACTORS:LONG-TERM VARIABILITY AND BEHAVIOUR

To account for the variability of industrial discharges collected in the centralised MBR, a year-long monitoring was performed before focusing on the role of CS and SAS. The suspended solids at the MBR's inlet varied greatly depending on the full scale operation of the primary clariflocculation treatment, while the metal contents are shown in Table 10. Concentrations of metals after

clariflocculation were in the range of 0.1–100 µg/L, as high as the levels that commonly occur in urban wastewater (Carletti et al., 2008; Chipasa, 2003). Having average concentration of 33 µg/L, Zn was the most prevalent trace element with toxicity. On the other hand, Cr, Pb, Ni, Cu, Se and As were present at levels below that of typical urban wastewater (Fatone et al., 2011), on average lower than 12 µg/L over the year. In addition, Co (0.25 µg/L), Ag (0.25 µg/L) andSb (0.5 µg/L) had frequencies of occurrence below 50%; Be, Cr^{VI}, Cd, Tl and Te were always under the limit of quantification. As observed by Santos et al. (Santos and Judd, 2010), the removal was not clearly correlated to the influent concentration. Basically, the average removals of metals by MBR (Table 10) were as follows:

- <30% for B, Ba, Al, Ni, Se and Zn.
- 40–70% for Pb, Hg, Cu, Ag, Cr and Co.
- >70% Fe.

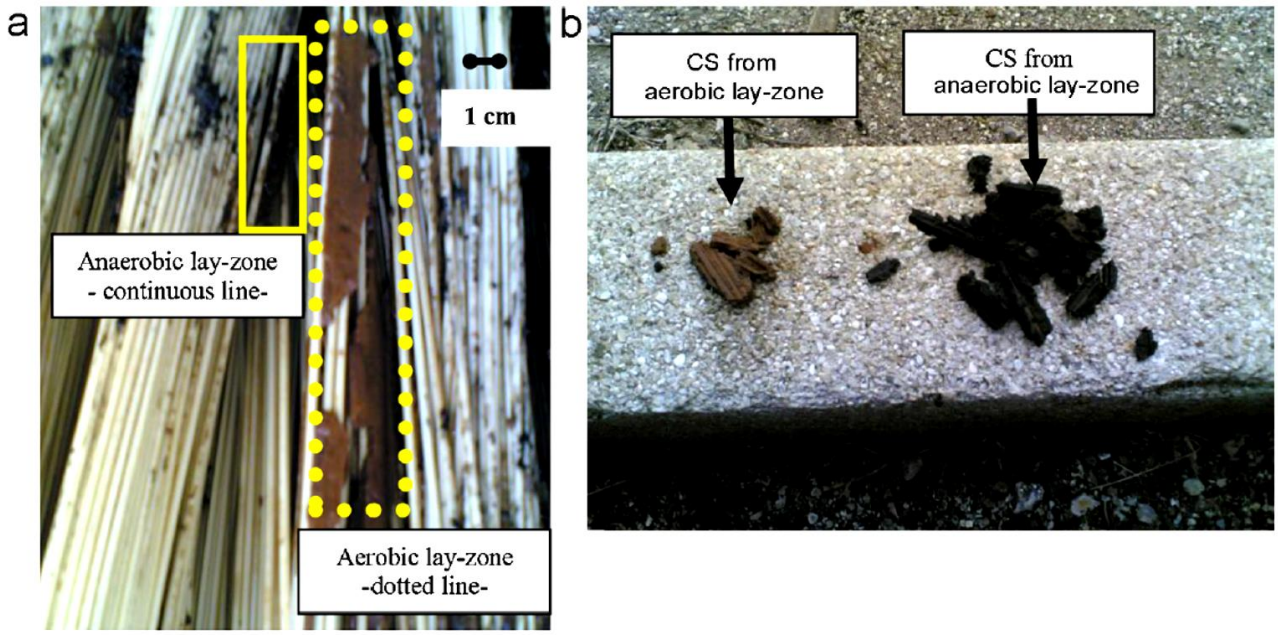

FIGURE 5 A) CLOGGING SLUDGE WITHIN THE HOLLOW FIBRES AND B) CS FROM THE ANAEROBIC LAY-ZONES

Despite the variability of the influent metal contents, the variation coefficients of the removal rates were lower than 40% (Table 2), showing stable removal capability as high as the ranges mentioned above. Therefore, pseudo-steady state conditions were assumed for the biosorption phenomena in the MBR. Assuming biosorption as the main removal mechanism for metals in the MBR, metal contents in the sludge was calculated as:

Calculated metal content in the sludge =

$$\frac{C_{influent} - C_{effluent}) \times Q_{in}}{L_{waste}} \rightarrow [\mu gMe/gTS] \qquad (3)$$

where $C_{influent}$ is the concentration of metals influent to the MBR [µgMe/L]; $C_{effluent}$ is the concentration of metals in the permeate[µgMe/L]; Q_{in} is an influent flowrate [m^3/g]; Q_{in} is the mass of waste activated sludge [gTS/day].

TABLE 11 COMPARISON OF METAL CONTENT IN THE SLUDGE: ANALYTICAL VS. CALCULATED (6 SAMPLES PER YEAR)

	Measured		Calculated		Measured vs. calculated
	Average (µg/g TS)	St. dev. (µg/g TS)	Average (µg/g TS)	St. dev. (µg/g TS)	(An-Mass. Bal)/newline Max (An; Mass. Bal)
Al	2501.00	59.40	1258.00	427.00	−50
Ag	2.85	0.21	0.00	0.00	−100
As	0.75	0.35	10.08	5.00	93
B	127.50	3.54	110.00		−14
Ba	32.00	1.41	48.75	33.00	34
Be	<0.01	–	0.01	0.03	0
Cd	<0.01	–	0.01	0.03	0
Co	2.50	0.00	2.46	2.00	−2
Cr	291.00	14.14	231.00	84.00	−21
Cu	69.00	0.00	68.95	40.00	0
Fe	17,857.00	188.09	23,300	9384	23
Hg	n.a.	n.a.	5.57	3.00	
Mn	350.50	16.26	493.00	268.00	29
Mo	3.35	0.64	1.23	3.00	−63
Ni	21.50	4.95	4.33	5.00	−80
Pb	20.50	0.71	39.90	28.00	49
Sb	0.60	0.00	6.84	2.00	91
Se	2.60	0.28	3.56	8.00	27
Sn	84.50	9.19	22.94	17.00	−73
Te	<0.01	–	0.03	0.05	0
Tl	<0.01	–	0.00	0.00	0
V	35.00	1.41	53.89	31.00	35
Zn	124.50	4.95	63.42	36.00	−49

To cope with the low concentrations and high variability of the metals in the influent, the waste activated sludge was also analysed to validate efficiency and biosorption mechanisms of removal. Thus these analytical data were used to assess the long-term reliability of our results, so as to validate our sampling and analytical methods (Karvelas et al., 2003). In general, a good agreement between the measured and the calculated metal contents in the waste activated sludge was observed. Despite the low influent concentration, even the errors for B, Ba, Be, Cd, Co, Cr, Cu and Fe met the literature values (Goldstone and Lester, 1991). Conversely, discrepancies were observed for Zn, Sn, Ni, As, Al and Ag due to influent variability. With the exception of Ag, the variation coefficients for these metals were greater than 70%. Generally our results showed the average removal efficiencies were generally lower than urban MBRs (Lin et al., 2012; Fatone et al., 2011; Chipasa, 2003). In fact, Santos and Judd (Santos and Judd, 2010) found >90% removals for Cd, Cr, Cu, Pb and Hg; removals in the ranges of 50–95% for Zn and 30–95% for Ni. However, we should

consider the minor influent occurrence and the higher dissolved portion of metals, as the MBR was preceded by a clariflocculator. In fact, when our wastewater contained metals similar to urban wastewater, the removal rates (Fatone et al., 2008) were similar as well (see B, As, Se in Table 10).

2.3.2. CHARACTERISTICS AND ROLES OF THE FOULING AND CLOGGING LAYERS (CS)

Despite the coarse-bubble membrane scouring and high turbulence in the filtration chamber, CS often accumulated within the membrane fibres (Figure 5A). Depending on the specific lay-zone in the outer or inner part of the membrane module, the CS was found under aerobic or anoxic/anaerobic conditions (Figure 5B) and was characterised by different redox conditions and, consequently, different influences on the bio-precipitation-dissolution of the trace metals (Grybos et al., 2007). The characteristics of the two sludges in the bioreactor were initially studied for solids (TS), COD, N and P contents (Table 12).

TABLE 12 COD, TKN AND TOTAL P CONTENTS IN THE SUSPENDED AND CLOGGING SLUDGES

	Suspended activated sludge (mg/gTS)	Clogging sludge – aerobic lay-zone (mg/gTS)	Clogging sludge – anaerobic lay-zone (mg/gTS)
COD	525	723	719
TKN	46.2	75.6	78.5
Total *P*	4.2	7.2	7.7

Concentrations of COD, N and P was higher in CS. This likely confirms the role of CS in the removal of colloidal and soluble compounds. In fact, Chang et al. (Chang et al., 2006) observed that COD removal during the ultrafiltration of activated sludge was mainly attributed to sieving/adsorption onto the cakes deposited over the membrane surface and partly to the adsorption on the membrane pores/surfaces. With regard to the effect of anaerobic and aerobic conditions on CS, major influences on the COD, TKN or P contents were not observed. As EPS is recognised to have a key role in the biosorption of metals (Comte et al., 2006), SAS and CS were analysed for their EPS contents. Apart from similar contents of soluble carbohydrates in CS and SAS, much higher content of EPS was generally observed (Table 13) in CS.

TABLE 13 EPS IN THE CLOGGING AND SUSPENDED ACTIVATED SLUDGES

		Clogging sludge	Suspended activated sludge
Proteins			
Soluble	mgProt/gTS	8.0 ± 1.0	3.9 ± 0.4
Bound	mgProt/gTS	61.1 ± 2.1	50.5 ± 3.8
Carbohydrates			
Soluble	mgCOH/gTS	2.9 ± 0.5	3.0 ± 0.6
Bound	mgCOH/gTS	46.9 ± 2.4	21.3 ± 2.1

As carbohydrates and proteins have different functional groups for biosorption (carboxylic and amine groups in the case of proteins and mainly carboxylic functional groups in the case of carbohydrates) (Comte et al., 2006), EPS likely enhanced the removal of metals in CS. Thus, the metal contents in CS (Table 14) was also analysed and compared with metals in SAS (Table 11). Heavy metals in the CS were found in the following descending order: Fe > Al > Zn > Cr > Cu > Ni > Pb > As > Se > Cd. In addition, the CS was more effective than SAS in the removal of certain metals in the following order: As > Zn > Ni > Cd > Sb > Fe > Se. Additionally, oxidative (aerobic) conditions had a minor positive influence on metal bioprecipitation. A number of authors have linked the high content and crucial role of EPS to the enhanced metal-binding potential of the biofilm (Srivastava and Majumder, 2008; Chang et al., 2001). Depending on pKa of the main functional groups of the EPS (i.e., carboxylic, phosphoric and amino groups) (Comte et al., 2006), the deprotonated form of the reactive sites could be responsible for the binding of metal ions to the EPS. Chang et al. (Chang et al., 2006) found an activated sludge–biofilm sludge to be more effective than a SAS in the removal of Cd; bound EPS in AS-biofilm sludge showed a greater affinity for Cd compared to the bound EPS of a SAS. Environmental conditions, namely pH, influenced the ionisation state of these functional groups, while low concentrations enhanced the interaction with the available sorption sites. In addition, concentration polarisation at the membrane surface increased solute concentration and may have enhanced precipitation and ion exchange in or on the CS (Yuncu et al., 2006; Hammaini et al., 2007). Interestingly, Zn and Cd are both in group 12 of the periodic table, demonstrating the importance of the chemical properties of the material. Our results showed that the CS enhanced the biosorption of amphoteric substances (such as As and Se) (Carletti et al., 2008). Therefore, convection mechanisms can also have a role for bringing metals in the biofilm, where the microbial speciation may favour the metals biosorption. While

suspended sludge is only exposed to diffusion gradients, clogging sludge is also exposed to convection mechanisms.

TABLE 14 METAL CONCENTRATION IN THE CS AND SAS

	CS anaerobic lay-zone		CS aerobic lay-zone		SAS	SAS in urban Italian WWTPs [26]
	Concentration (mg/kgTS)	Hyper-accumulation (%)	Concentration (mg/kgTS)	Hyper-accumulation (%)	Concentration (mg/kgTS)	Concentration (mg/kgTS)
Fe	**38,258**	**113**	**54,932**	**205**	**17,990**	**4399–11,833**
Al	1391	−45	933	−63	2543	11,065–26,447
Zn	***715.2***	***491***	***627.5***	***419***	***121.0***	***433–2341***
Cr	265.3	−6	271.5	−3	281.0	17–560
Mn	184.1	−48	421.2	20	350.5	n.a.
B	119	−5	213.4	71	125.0	n.a.
Cu	83.4	21	72.7	5	69.0	165–348
Ni	***58.8***	***227***	***71.7***	***298***	***18.0***	***34–107***
Ba	34.7	5	46.3	40	33.0	n.a.
Pb	24.6	17	21.8	4	21.0	46–78
V	16	−53	24.1	−29	34.0	n.a.
Mo	10.2	252	19	555	2.9	n.a.
Sn	8.8	−89	11.8	−85	78.0	n.a.
As	***7.9***	***690***	***9.4***	***840***	***1.0***	***<2.5–15***
Se	***6.8***	***143***	***5.7***	***104***	***2.8***	n.a.
Co	3.5	40	4.1	64	2.5	n.a.
Ag	2.1	−30	3.5	17	3.0	n.a.
Cd	***2.2***	***>219***	***1.5***	***>149***	***<0.01***	***<0.05–3***
Sb	**1.4**	**133**	**1.7**	**183**	**0.60**	n.a.

Bold: hyper-accumulation of metals. Italics and bold: hyper-accumulation of heavy metals.

2.3.3. GENE-BASED IDENTIFICATION OF THE MICROBIAL COMMUNITIES IN SAS AND CS

Even though EPS matrixes can provide major sorption and/or sieving capacities for heavy metals, organic substances and particles (Drews, 2010; Hammaini et al., 2007), Spath et al. (Spath et al., 1998) demonstrated that EPS does not play a major role in the sorption of certain heavy metals, such as cadmium, zinc and cobalt, which accumulated directly on the cell walls of activated sludge organisms. Generally, interactions among bacteria present in active biofilms and pollutants can occur through different mechanisms such as accumulation, precipitation and transformation of various metalloids and heavy metals (Gadd, 2001). Biosorption and bioaccumulation may involve interactions and the concentration of toxic metals or organic pollutants (e.g., dyes) in the biomass, either living (bioaccumulation) or non-living (biosorption) (Chojnacka, 2010). Several bacterial strains belonging to both *Proteobacteria* and *Bacteroidetes* groups and capable of accumulating heavy metals have been isolated from activated sludges highly polluted with metals such as arsenic, cadmium, chromium, copper, nickel and silver (Leung et al., 2001; Yoon et al., 2009). With regards to microbial speciation, the CS was considered representative of both the fouling and clogging sludge. PCR-DGGE analysis was performed on the V3 hyper variable region of 16S rRNA gene starting from both SAS and CS. Data obtained are reported in Figure 6.

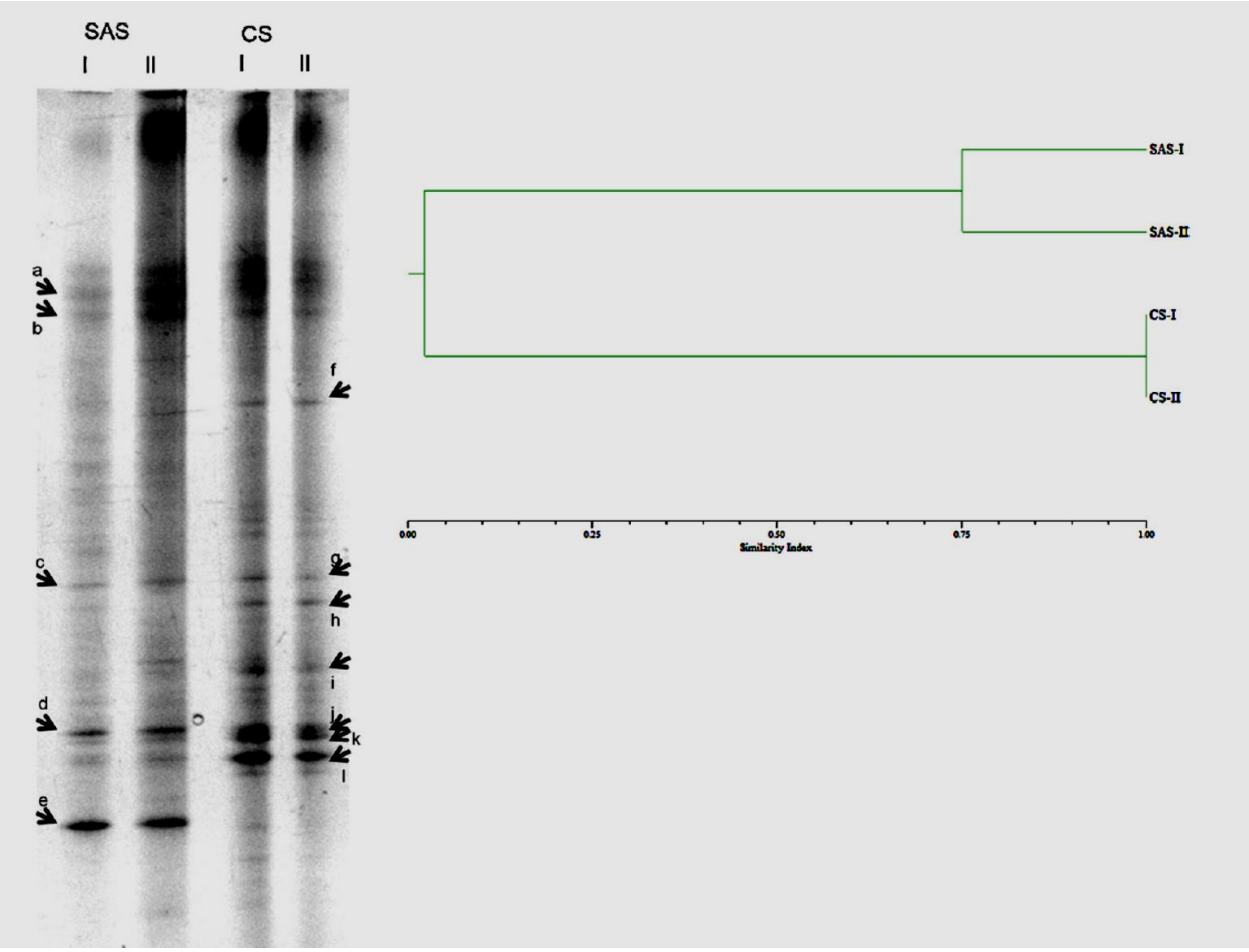

FIGURE 6 DGGE PROFILES OF EUBACTERIA IN THE SAMPLES COLLECTED FROM THE SUSPENDED ACTIVATED SLUDGE (SAS) AND CLOGGING SLUDGE (CS). ROMAN NUMERALS (I, II) CORRESPOND TO DIFFERENT REPLICATES. LETTERS IN THE GEL INDICATE BANDS THAT HAVE BEEN EXCISED, CLONED AND SEQUENCED. THE DENDROGRAM INDICATES THE SIMILARITY RELATIONSHIPS AMONG THE DIFFERENT DGGE PROFILES

Results indicate that a different speciation occurred between bacterial communities selected in SAS and CS samples. This observation is in agreement with the UPGMA analysis performed on the different electrophoretic patterns, which showed a low similarity (Similarity Index lower than 0.2) between the SAS and CS profiles. Several bands were cut off from the gel. However, only the major bands were successfully re-amplified, cloned and sequenced (marked in the figure as letters). This was due to the fact that minor and close bands were difficult to excise from the gel and could not be reliably amplified or because they only produced multiple DNA sequences. Sequencing data obtained for the major bands were first checked for the presence of chimaera artefacts through the Decipher software and then searched for similarities within the BLASTN database (Tamura et al., 2011); the results are listed in Table 15. A phylogenetic tree was constructed using the neighbour-joining method with the MEGA version 5.0 software package (Figure 7).

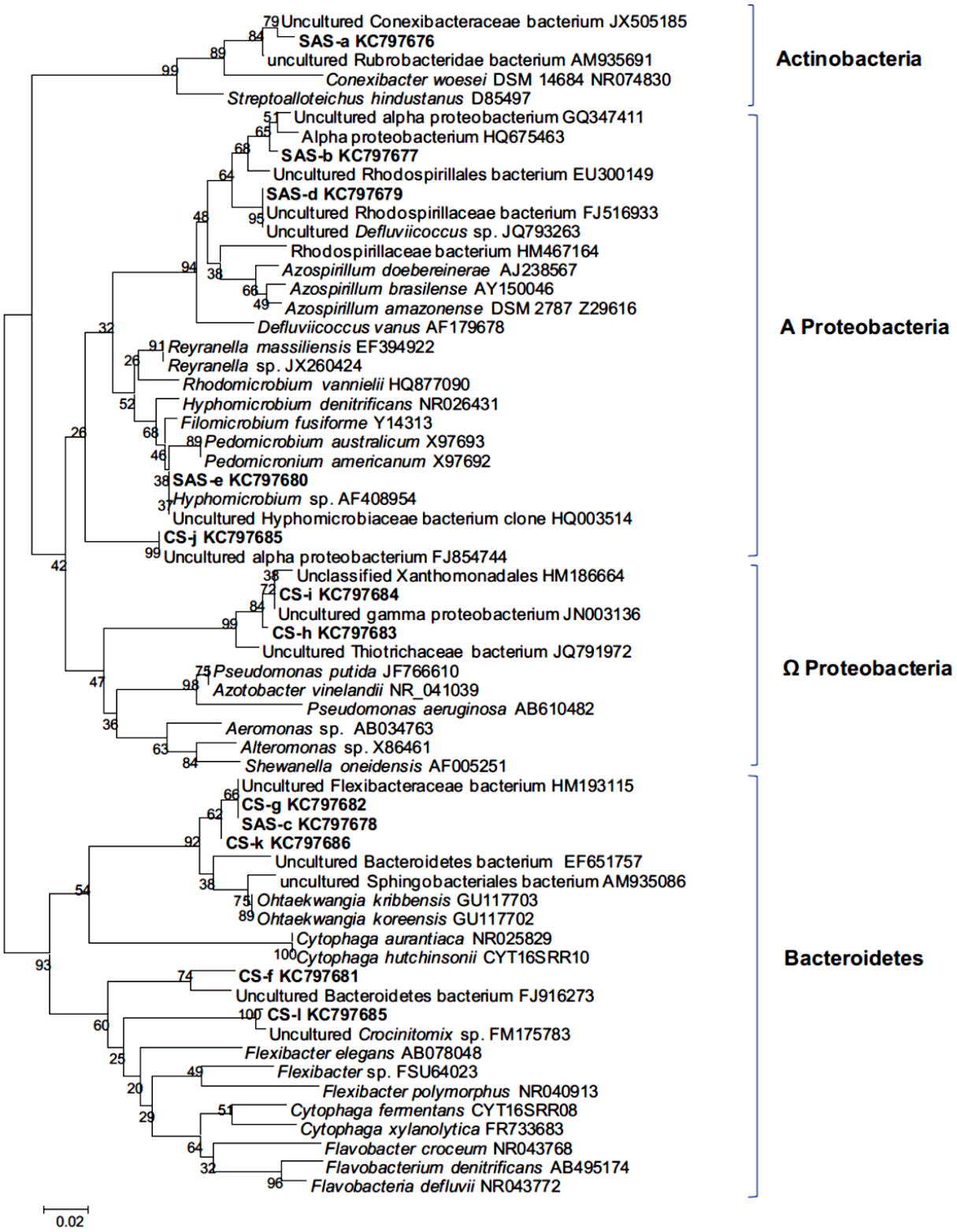

FIGURE 7 NEIGHBOUR-JOINING PHYLOGENETIC TREE, BASED ON THE SEQUENCE OF THE HYPERVARIABLE V3 REGION OF THE 16S RRNA GENE FOR EUBACTERIA, SHOWING THE RELATIONSHIPS OF DIFFERENT DGGE BANDS (INDICATED WITH LETTERS IN BOLD) AND RELATED SPECIES. BOOTSTRAP VALUES ARE GIVEN AT THE BRANCH NODES AND ARE BASED ON 1000 REPLICATES. BAR, 0.02 SUBSTITUTIONS PER NUCLEOTIDE POSITION

TABLE 15 TAXONOMIC CHARACHTERISATION OF THE MAJOR BANDS IN THE SAS ANS CS-DGGE PROFILES

Band	*Phylogenetic group*	TAXON	Similarity
SAS-a	*Actinobacteria*	Uncultured Conexibacteraceae bacterium	98%
SAS-b	*A-Proteobacteria*	Uncultured Rhodospirillaceae bacterium	98%
		Caedibacter caryophilus	96%
SAS-c	*Bacteroidetes*	Uncultured Flexibacteraceae bacterium	97%
SAS-d	*A-Proteobacteria*	Uncultured *Defluvicoccus* sp.	99%
		Defluvicoccus vanus	96%
SAS-e	*A-Proteobacteria*	*Filomicrobium fusiforme*	99.4%
CS-f	*Bacteroidetes*	Uncultured Bacteroidetes bacterium	96%
CS-g	*Bacteroidetes*	Uncultured Flexibacteraceae bacterium	97%
CS-h	*Γ-Proteobacteria*	Uncultured γ-Proteobacterium	99%
CS-i	*Γ-Proteobacteria*	Uncultured γ-Proteobacterium	100%
CS-j	*A-Proteobacteria*	Uncultured α-Proteobacterium	100%
CS-k	*Bacteroidetes*	Uncultured Cytophagaceae bacterium	97%
CS-l	*Bacteroidetes*	Uncultured Flavobacteriia bacterium	99%

Results indicated the predominance of alpha *Proteobacteria* in the SAS sample, whereas the *Bacteroidetes* and gamma *Proteobacteria* were the major bacterial phyla in the microbial community of the CS samples. In particular, it's worth nothing that band *e*, corresponding to a α-*Proteobacteria* strain, which was the major in SAS profile, disappeared in the CS pattern. At the same time, bands *k* and *l*, which were referred to *Bacteroidetes*, resulted the most abundant in the CS lane. This indicated a shift in the bacterial community composition from *Proteobacteria* to *Bacteroidetes* when SAS and CS are compared. On the other hand, gamma *Proteobacteria* are also present in the CS bacterial community as evidenced from sequencing results obtained for bands *h* and *i*. Bacterial strains belonging to either α- and γ-*Proteobacteria* or *Bacteroidetes* are currently found in activated sludges. Moreover, strains belonging to the phylum of *Bacteroidetes* also known as CFB group, which includes the *Cythophaga*, *Flavobacterium* and *Bacteroides* genera, are recognised to be highly resistant to heavy metals and to transform these toxic compounds through either reduction or oxidation reactions as well as to precipitate them (Yoon et al., 2009; Gillan and Pernet, 2007). Interestingly, many bacteria within CFB group, are capable of transforming heavy metals and metalloids such as Cadmium, Zinc, Arsenic and Selenium, that resulted to be hyperaccumulated in the clogging material (Reis et al., 2013; Gillan et al., 2005; De Souza et al., 2001). Actually, putative heavy metal binding protein (EMBL-CDS:BAD48778) and heavy metal efflux pump, czcA family precursor, for cadmium and zinc resistance have been isolated (EMBL-CDS:ADY35891) in strains belonging to *Bacteroides* genus. Cadmium and zinc-exporting ATPases (*cadA*) have been also identified in *Sphingobacterium* sp. (EMBL-CDS: EFK59886, EEI92281) and *Flavobacterium* sp. (EMBL-CDS: ABR09923) strains. Moreover, passive and active accumulation of zinc and cadmium has been demonstrated for a *Cytophaga johnsonae* strain (Donocik et al., 1995).

As far as Arsenic is concerned, arsenate reductase genes (*ArsC*) have been cloned in strains belonging to *Sphingobacterium* sp. (EMBL-CDS: EFK57102), *Cytophaga hutchinsonii* (EMBL-

CDS:ABG58858), *Flavobacterium johnsoniae* (EMBL-CDS: ABQ07783), *Bacteroides fragilis* (GCT8-3349 BF3083) and *Prevotella bergensis* (EMBL-CDS: EFA45563), all within the CFB group. Regarding Selenium, a SelT/selW/selH selenoprotein has been cloned in a *Sphingobacterium* sp. strain (EMBL-CDS: EEI92183) and bacterial strains belonging to the *Cytophagales* family have been isolated from a Selenium-Contaminated Hypersaline Evaporation Pond showing to be highly resistant to this metalloid (De Souza et al., 2001). Thus, it could be possible that the different biological conditions present in the CS have enhanced the selection of those heavy metal resistant strains that preferentially live in biofilm rather than in suspended sludge as planktonic cells.

2.3.4. EFFECT OF ACID MAINTENANCE CLEANING ON THE DESORPTION OF METALS FROM CS

During maintenance cleaning, the CS is exposed to acid conditions, and metals may desorb and dissolve in the cleaning solution, which can then either be recirculated to the headworks or discharged. The batch test run as long as necessary to reach the phase equilibrium. Figure 8 shows metal desorption from CS after 4, where metals in the permeate are the reference blank values. Desorption from CS was negligible for Cd and Sb. Major releases occurred for metals less toxic, but abundant in the CS, such as Fe, Al, Mn and Zn (Table 16).

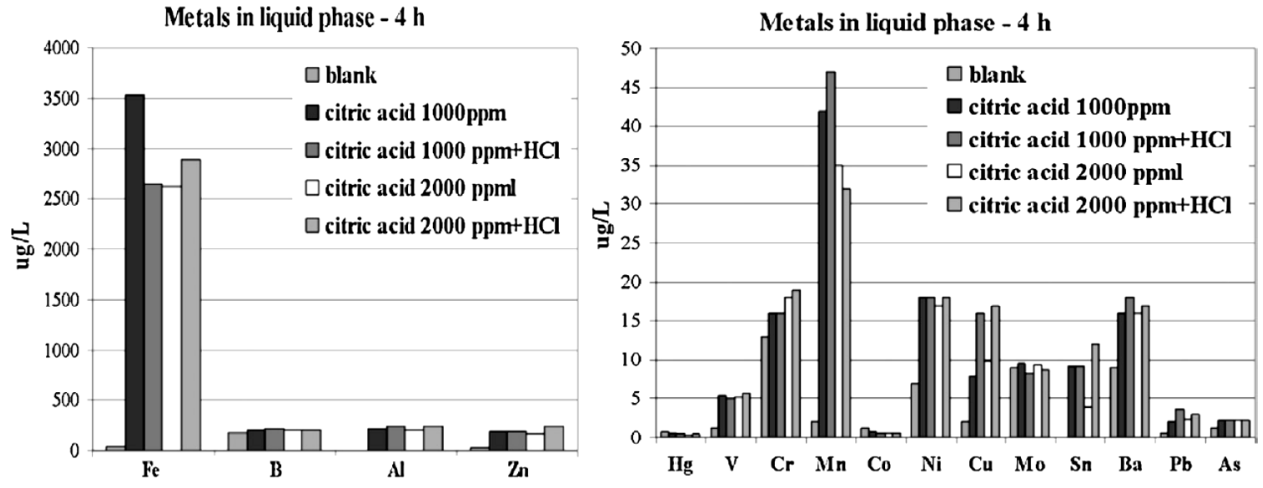

FIGURE 8 METAL CONCENTRATIONS IN THE CLEANING SOLUTIONS AFTER 4 H SOAKING OF THE CS, CD AND SB < LOD

TABLE 16 METAL CONCENTRARTIONS IN THE CLEANING SOLUTION (CITRIC ACID AT 1000PPM + HCL)

Metal	Metals dissolved in the cleaning solution (t = 4 h) µg Metal/L	Specific release µg Metal/gSS	Specific release from CS (%)
Fe	2600	1238	2.3
Hg	–0.3	–0.15	–
B	38.0	18.1	8.5
Al	237.6	113.1	12.1
V	3.8	1.8	7.5
Cr	3.0	1.4	0.5
Mn	45.0	21.4	5.1
Co	–0.5	–0.23	–
Ni	11.0	5.2	7.3
Cu	14.0	6.6	9.2
Zn	171.0	81.4	13.0
Mo	–0.8	–0.38	–
Sn	9.2	4.3	37.1
Ba	9.0	4.3	9.3
Pb	3.1	1.5	6.8
As	1.0	0.47	5.1

From Table 16 we observed descending order Sn > Zn > Al > Ba > Cu for the metals release rates, which is not proportional to the propensity to bio-sorption or to the content in the CS. It can therefore be concluded that the specific chemical bonds between the metals and the CS had a significant effect. Fe, Al, Zn, Mn, Ni, Cu, Sn, Ba and were the metals that were most released. pH played a positive or negative role in the metals' mobility of Cu, Mn, Ba, Pb, while this did not affect the release of Sn, Zn, Ni, Cr. Therefore, bio-sorption/desorption could be postulated as major mechanisms to explain metals mobility in CS. On the other hand, bio-precipitation/dissolution seemed to play a minor role as they are more influenced by redox conditions (Grybos et al., 2007).

2.4. CONCLUSIONS

The roles of the suspended activated and the clogging sludge were investigated with respect to the removal and fate of trace metals in a pilot scale membrane bioreactor used for real industrial wastewater treatment. The main conclusions are the following:

- Large and centralised industrial wastewater treatment plants may address trace metal contents that are even lower than urban wastewater.
- Being the influent metal contents under 100 µg/L, the removal efficiencies were under 30% for B, Ba, Al, Ni, Se and Zn; between 40% and 70% for Pb, Hg, Cu, Ag, Cr, and Co; and over 70% for Fe. Bio-sorption was likely the major removal mechanism, as after primary clariflocculation treatment the metals entered the membrane bioreactor as dissolved ions.
- Compared to suspended activated sludge, the clogging sludge enhanced the bio-sorption of heavy metals according to the descending order: As > Zn > Ni > Cd > Sb > Fe > Se. Thus, very remarkable enhanced biosorption was obtained with amphoteric and toxic substances such as the As.
- Both EPS content and microbial speciation enhanced the removal potential of the clogging sludge. In fact, in addition to the major content of EPS, strains belonging to the phylum of *Bacteroidetes* were more evident in the clogging sludge. These bacteria are known to be highly resistant to heavy metals and are able to adsorb the toxic metals to their outer membrane layers as well as precipitate them.
- Acid maintenance cleaning of the submerged membrane modules may result in 10–15% desorption of the metal in the clogging sludge. In particular, major releases were observed for Sn, Zn, Al, Ba, Cu and Mn, while bio-sorption/desorption was the most likely mobility mechanisms.

Acknowledgments

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3. ANAEROBIC DIGESTION PROCESSES

3.1. THERMOPHILIC TWO-PHASE ANAEROBIC DIGESTION OF SS-OFMSW FOR BIO-HYTHANE PRODUCTION: EFFECT OF RECIRCULATION SLUDGE ON PROCESS STABILITY AND MICROBIOLOGY OVER A LONG-TERM PILOT SCALE EXPERIENCE.

This chapter of the thesis is in publishing: A. Giuliano, L. Zanetti, F. Micolucci and C. Cavinato, 2014. Thermophilic two-phase anaerobic digestion of source sorted organic fraction of municipal solid waste for bio-hythane production: effect of recirculation sludge on process stability and microbiology over a long-term pilot scale experience. Water Science & Technology, in press.

Abstract:

A thermophilic two-phase anaerobic digestion process for the concurrent production of H2 and CH4 through the treatment of source sorted organic fraction of municipal solid waste was carried out over a long-term pilot scale experience. Two continuously stirred tank reactors were operated for about one year. The results showed that stable production of bio-hythane without inoculum treatment could be obtained. The pH of the dark fermentation reactor was maintained in the optimal range for hydrogen producing bacteria activity through sludge recirculation from a methanogenic reactor. An average specific bio-hythane production of 0.65 m3/kg of volatile solids fed was achieved when the recirculation flow was controlled through an evaporation unit in order to avoid inhibition problems for both microbial communities. Microbial analysis indicated that dominant bacterial species in the dark fermentation reactor are related to the Lactobacillus family, while the population of the methanogenic reactor was mainly composed of Defluviitoga tunisiensis. Archaeal community of the methanogenic reactor shifted, moving from Methanothermobacter-like to Methanobacteriales and Methanosarcinales, the latter found also in the dark fermentation reactor, with considerable methane production.

3.1.1. INTRODUCTION

The two-stage anaerobic digestion (AD) system is a promising technology since it allows for efficient stabilization of organic biodegradable waste, providing, at the same time, a valuable

energy carrier, the bio-hythane, characterized by an H2/CH4 ratio suitable for improving both combustion engine performance and environmental impact (Liu et al., 2006; Porpatham et al., 2007). In the two-stage AD system, H2 production occurs in a first reactor through the dark fermentation process, while methane production takes place in a second reactor fed with the acidic effluent that comes from the first reactor (Figure 9).

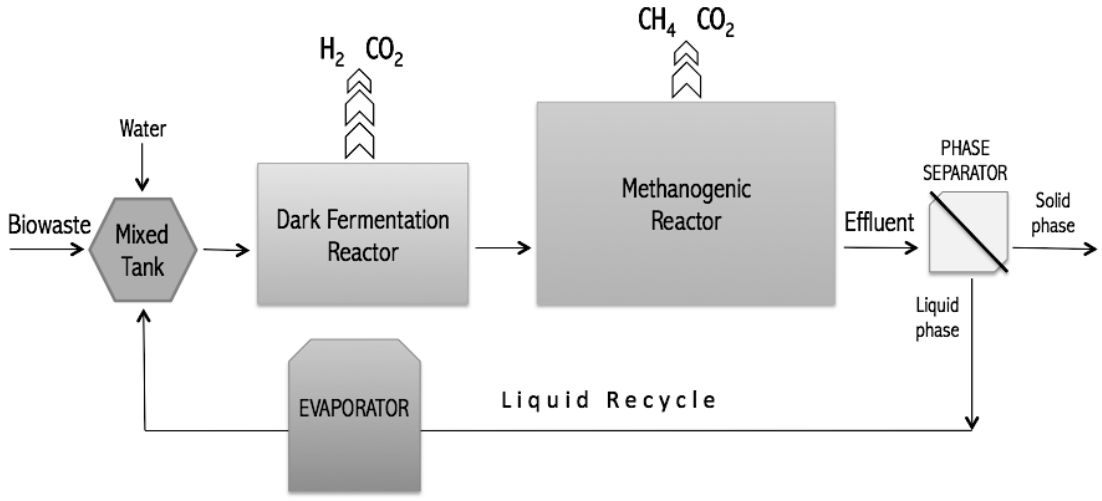


FIGURE 9 FLOW SCHEME OF TWO-STAGE TECHNOLOGY ADOPTED FOR BIO-HYTHANE PRODUCTION

Liu et al. (2012) reviewed the feasibility of bio-hythane production through the two-stage AD system and optimal design parameters such as pH, hydraulic retention time (HRT) and temperature, related to different biomass and process configuration. One of the main bottlenecks, which still makes the scale up of the two-stage process expensive is the alkali addition in the dark fermentation reactor in order to maintain the optimal pH range for efficient hydrogen production (Valdez Vazquez and Poggi-Varaldo, 2009). A cost-effective way to avoid external pH control in the dark fermentation reactor (H2-reactor) is to recirculate the effluent of the methanogenic reactor, since it is characterized by a high buffer capacity (Cavinato et al., 2011; Chinellato et al., 2013,). However, in long-term trials this choice may affect the reliability of the process because of the increase in ammonia concentration due to the high hydrolysis rate of food waste causing inhibition in both reactors (Cavinato et al., 2012). Among the different microorganisms of the anaerobic trophic chain, the methanogens are the least tolerant and free ammonia rather than total ammonia has been suggested to be the main cause of inhibition because of its ability to penetrate the cell membrane, causing proton imbalance and potassium deficiency (Chen et al., 2008; Sung and Liu, 2012). However, there is conflicting information in literature about the sensitivity of both acetoclastic and hydrogenotrophic methanogens, since the ammonia inhibition level depends on different factors

such as inocula, environmental conditions and acclimation level (Chen et al. 2008). Another important aspect that may affect the reliability of the process in long-term operation is the negative effect of H2-consuming microorganisms in the recirculation flow that can contaminate the dark fermentation reactor, negatively affecting the syntrophic association with H2-producing microorganisms for suitable hydrogen production (Wang and Wan, 2009). Several strategies have been adopted to avoid the involvement of H2-consuming microorganisms in the first stage reactor such as thermal treatment or chemical treatment of the recirculated digested sludge (Guo et al., 2010), but they significantly increase operation costs. In order to ensure the stability of the microbial communities and to design a strategy for enhanced bio-hythane production, it is important to understand microflora development during the experimental test, which depends mainly on the operating conditions adopted (Wang and Wan, 2009).

The aim of the present study is to provide information about the natural selection of the microbial communities structure involved in a two stage thermophilic AD approach for H2 and CH4 co-production, treating the source sorted organic fraction of municipal solid waste (SS-OFMSW) and applying the recirculation of the metahnogenic reactor liquid effluent as buffer supplier in the dark fermentation reactor. Ammonia accumulation in the system was controlled by an evaporation unit and the microbial community structure was investigated in a long term experimental test.

3.1.2. MATERIALS & METHODS

3.1.2.1. Substrates, inoculum and operating conditions

The pilot trial was carried out in two continuous stirred tank reactors (CSTR) fed with the food waste collected daily at the integrated Waste and Wastewater Treatment plant of Treviso municipality (North Italy). The incoming organic waste was mechanically pretreated in order to remove the inert fraction (plastics, metals, glass, etc.) (Bolzonella et al., 2006) and showed an average total solid (TS) content of about 270 g TS kg-1 with an inert fraction of less than 5% of total wet weight. The dark fermentation reactor (volume = 0.2 m3) was inoculated with a mixture of minced organic waste and tap water resulting in TS content of about 8%, while the methanogenic reactor (V= 0.76 m3) was inoculated with an active mesophilic biomass collected from a full scale anaerobic digester which co-treats both source sorted organic fraction of municipal solid waste (SS-OFMSW) and waste activated sludge (WAS).

The design parameters of the thermophilic (55°C) two-stage process are reported in Table 17.

TABLE 17 OPERATING CONDITIONS OF TWO-STAGE AD PROCESS

	RUN 1 (0-90 days)	RUN 2 (91-345)
Evaporator Unit	no	yes
Dark fermentation reactor		
HRT (days)	3.3	3.3
OLR (kg TVS m^3d^{-1})	16-18	16-18
T (°C)	55	55
V (m^3)	0.2	0.2
Methanogenic Reactor		
HRT (days)	12.6	12.6
OLR (kgTVS m^3d^{-1})	4-5	4-5
T (°C)	55	55
V (m^3)	0.76	0.76

maintenance period: (i)180-200 days; (ii) 220-240 days.

The authors' previous studies (Cavinato et al., 2011) have demonstrated that the best performance in terms of bio-hythane production was obtained with an organic loading rate (OLR) of about 16 kg TVS m-3d-1 and a retention time (HRT) of 3 days in the first fermentative reactor resulting in 4.2 kg TVS m-3d-1 applied in the second methanogenic reactor, with a retention time of 12 days. In accordance with these results, a long-term experience was designed in order to asses process stability (Cavinato et al., 2012). Recirculation of anaerobic digested sludge was applied from the methanogenic reactor to the dark fermentation reactor after a mild solids separation, in order to support the fermentative step with alkalinity and to keep the pH in the optimal range (5-6) for thermophilic hydrogen producing microorganisms (Guo et al., 2010). The process was continuously operated for 345 days with the exception of two maintenance periods (from 180 to 200 and from 220 to 240 days).

The long- term experience was divided into two RUNs based on different recirculation strategies: during RUN1, the recirculation flow was fixed as half of the total flow rate fed (data published, Cavinato et al. 2012) while during RUN2 the recirculation flow was treated by an evaporation unit (R150v3, Veolia Water S&T, Italy). The operation time unit was set daily in order to avoid ammonia accumulation. Ammonia removal was dynamically controlled depending on daily stability behavior observed through pH, VFA and alkalinity values.

3.1.2.2. Chemical analysis

The substrates and the effluents of both reactors were monitored weekly according to Standard Methods (APHA, 1998) in terms of TS and TVS, chemical oxygen demand (COD), total kjeldahl nitrogen (TKN) and total phosphorus (TP), whereas the process stability parameters, namely pH, volatile fatty acids (VFAs), alkalinity and TAN were checked at least three times per week. VFA concentration (acetate, propionate, i-butyrate, butyrate; i-valerate; valerate, i-caproate, caproate and heptanoate) was monitored using a gas chromatograph (Carlo Erba instruments) according to the procedure given in Cavinato et al. (2011).

3.1.2.3. Microbial Analysis

Molecular analysis of microbial communities was performed on the triplicate samples at two different times, when the process showed good performance in terms of bio-hythane production and composition: during RUN 1 at the end of start-up phase (day 35) and at the steady state conditions (SSC) of RUN 2 (day 300). Bacterial 16S rRNA genes were selectively amplified using F8/R11 primers (Weinsburg et al. 1991) with the following thermocycling program: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 30 s and extension at 72°C for 2.5 min; final extension at 72°C for 5 min. Afterwards a nested PCR was performed on the hyper-variable V3 region of the 16S rRNA gene using primers P3 (with a GC clamp) and P2 (Muyzer et al. 1993), conditions were as above, except for number of cycles, 35, the annealing temperature, 57°C, and extension time, 35 s. For Archaea, primers A109-f and A934b-r (Grosskopf et al. 1998) were used for nearly complete 16S rRNA gene amplification. Afterwards a nested PCR was performed on the hypervariable V2-V3 region using primers A109(T)-f and 515-GC-r (Roest et al. 2005), with a GC-clamp. The first archaeal PCR reaction was performed with the following thermocycle program: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 30 s, extension at 72°C for 1 min; and final extension at 72°C for 5 min. The nested PCR was as above but with 35 cycles. The PCR products were quantified using Low DNA Mass Ladder (Celbio, Italy) in a 2.0% agarose gel. DGGE analyses were performed in duplicates on amplicons obtained both for bacterial V3 and archaeal V2-V3 regions. Gels (8% acrylamide/bisacrylamide 19:1, BioRad) were cast using a denaturing gradient of 30–60%, with 100% denaturant defined as 7 mol L−1 urea and 20% (v/v) formamide. Electrophoresis was performed at 45 V for 18 h at 65◦C with the Dcode® Universal Detection System (Biorad) and gels were stained with EtBr (1 mgL−1). Representative DGGE bands were excised and incubated for 4 h

in 50 mL of sterile water. DGGE bands containing DNA to be sequenced were re-amplified. PCR amplification was carried out as described before, except for the use of non-GC-clamped primers. PCR products were transformed in *Escherichia coli* DH5α using the pGEM-T vector system according to the manufacturer's instructions (Promega, Italy), sequenced on both strands, and finally searched for homology using the BLASTN database (Altschul et al. 1997). Similarity of the sequences with Type Strains is checked using EzTaxon server 2.1 (Chun et al. 2007). The sequences were initially aligned using the multiple alignment program CLUSTAL_X 1.83 (Thompson et al. 1997). A phylogenetic tree was constructed using the neighbourjoining method with the MEGA version 5.1 software package (Tamura et al. 2011). Bootstrap analysis was performed from 1000 bootstrap replications. Chimeras were checked using DECIPHER database (Wright et al. 2012). Sequenced bands have been submitted in GeneBank database with accession numbers from KJ209714 to KJ209727.

FISH analysis was performed to investigate the presence of Archaea population in the dark fermentation reactor at 300 days, when significant methane production was detected. The probes used were EUB388mix for *Bacteria*, Arch915 for *Archaea*, MX825 for *Methanosaetaceae*, MS1414 for *Methanosarcinaceae*, MB1174 for *Methanobacteriaceae*, MG1200 for *Methanomicrobiaceae* and Methanospirillaceae and MC1109 for Methanococcales. The hybridization stringency was chosen based on Banks et al. (2012) and hybridized samples were observed using Leica DMRX epifluorescence microscopy. To quantify *Bacteria* and *Archaea*, Daime 1.3.1 software (Daims et al., 2006) was used to analyze the images.

3.1.3. RESULTS & DISCUSSION

3.1.3.1. Process stability

The stability of the process was evaluated considering the temporal profile of the pH, VFA, alkalinity, biogas production and composition. The data obtained during RUN 1 concerning process stability have already been exhaustively described in the authors' previous publication, where, after 30 days, the gas mixture produced by the reactors meet the typical composition of bio-hythane (Cavinato et al., 2012).Throughout the overall experimental trials, the behavior of the dark fermentation reactor was stable, showing an average specific gas production (SGP) of 0.175 m3 kg TVS-1 with H2, CH4 and CO2 content of 31%, 11% and 58 % (on average) respectively (Figure 10 A and B).On the other hand the SGP of the methanogenic reactor falls significantly at 85-109 days reaching values of less than 0.1 m3 kg TVS-1.

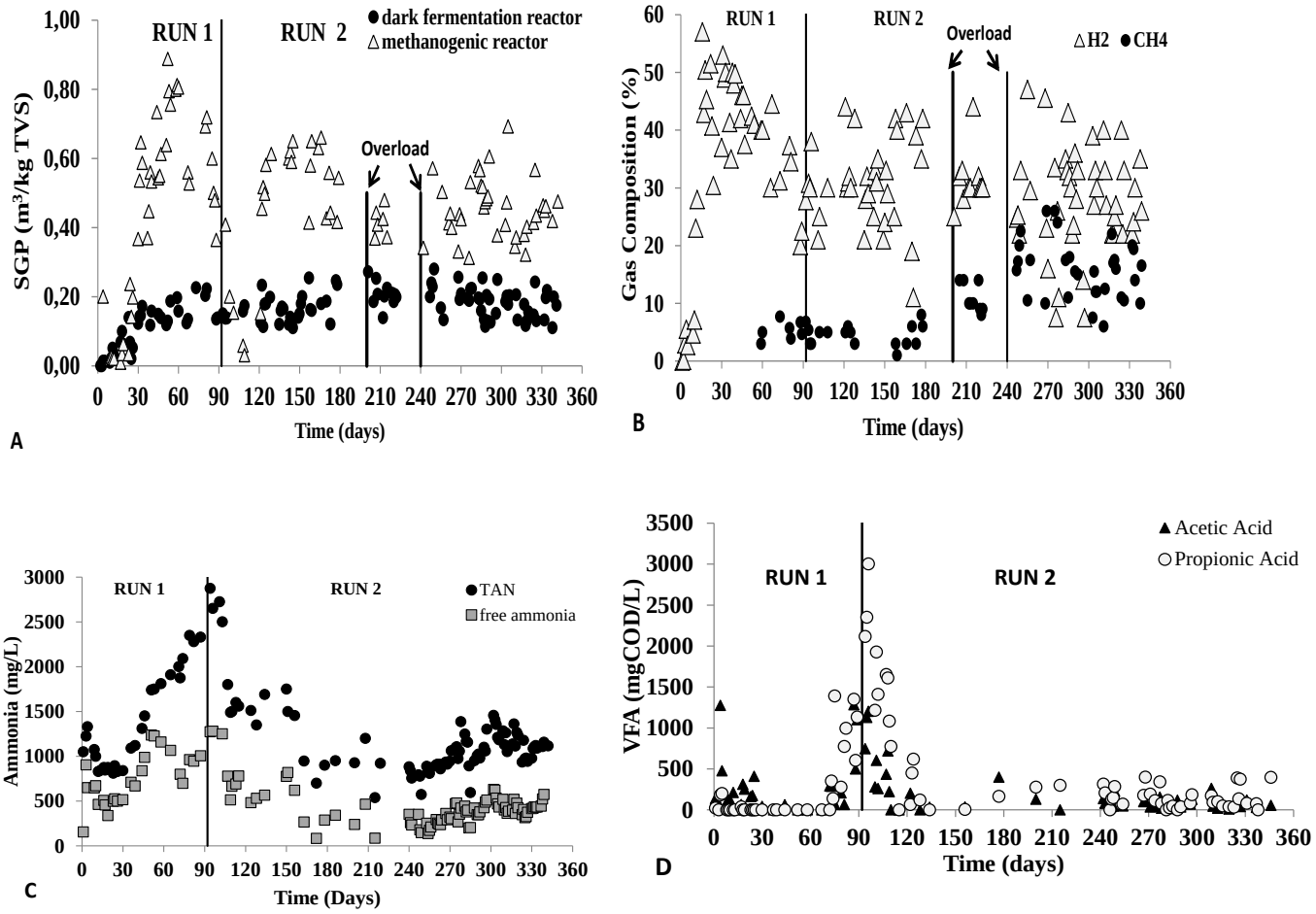


FIGURE 10 TIME PROFILE OF :[A] SPECIFIC GAS PRODUCTION (SGP) OF BOTH REACTORS; [B] GAS COMPOSITION OF THE DARK FERMENTATION REACTOR; [C] TOTAL AMMONIA NITROGEN AND FREE AMMONIA (FA) OF METHANOGENIC REACTOR; (D) ACETATE AND PROPIONATE OF DARK FERMENTATION REACTOR

The increase, over time, of TAN (Figure 10 C), which was formed during the degradation of proteineous organic materials, was observed. It is well known that the FA may restrain the growth rate of anaerobic microflora. Since the fraction of NH3 increase with both temperature and pH, its inhibition effect on thermophilic methanogens may become significant under specific conditions. Moreover, the authors have shown that under high ammonia concentrations, hydrogen-trophic methanogensis is the principle route to methane formation (Banks et al., 2012) and NH3 plays a role as a strong inhibitor of this metabolic pathway (Wigant et al., 1986). Gallaghert and Winter (1997) reported that NH3 of about 560 mg L-1 causes significant inhibition of methanogens at pH 7.6 In the present study, when biogas production falls (days 60-108), the NH3 content ranged from 698 to 1280 mg/L (Figure 10 C) at pH range 8.0 - 8.71. Moreover, propionate was the first VFA to increase (Figure 10 D) and it was the predominant methabolic by-product (31% of total VFA) when the total VFAs reached the maximum level (9346 mg COD L-1 at day 96), while the rest was represented by caproate (20%), butyrate (20%), acetate (13%) i-valerate (11%) and valerate (5%).

Angelidaki and Ahring, (1994) indicated that at thermophilic temperature NH3 above 700 mg L-1 registered poor treatment performance, affecting the propionate breakdown. Therefore, since propionate was the main by-product during the development of digestion instability, the poor process performance observed in the methanogenic reactor may be explained by the toxic effect caused by passive diffusion of the hydrophobic ammonia molecule in thermophilic methanogens.

In the light of these observations, an evaporation unit was adopted during RUN2 in order to control the ammonia content in the recirculation flow as well as to avoid inhibition problems in the reactors. As reported in Figure 10 C after twenty operation days of the evaporation unit the TAN concentration was reduced in the methanogenic reactor, reaching a value of around, 1500 mg L-1 (500 mg NH3 L-1). Consequently, a rapid increase of SGP was found after 10 days. The balance of the anaerobic trophic chain was confirmed by the low VFA concentration detected (less than 1 g COD L-1) until the end of experimental trials. On the other hand, since ammonium nitrogen is one of the most important parameters that affects the mechanism of buffering in the AD process, the partial removal of ammonia from the recirculation flow led to a significant decrease in total alkalinity (data not shown). Consequently, the decrease in alkalinity in the recirculation sludge also affected the pH of the dark fermentation reactor (Figure 11 A) with a negative impact on hydrogenase activity. In fact, the fraction of H2 in the gas decreased at days 166-173 reaching a value lower than 20% (v/v) (Figure 10 B).

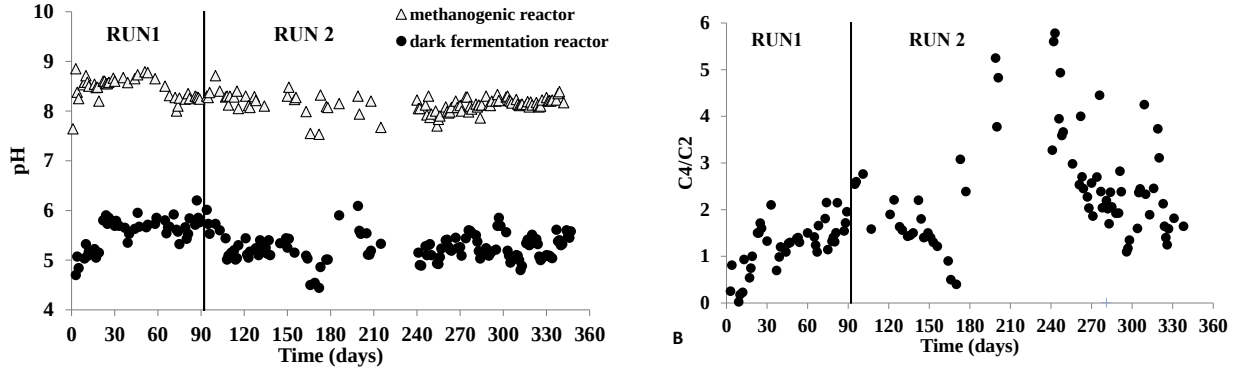


FIGURE 11 TIME PROFILE: [A] OF PH IN THE DARK FERMENTATION REACTOR; [B] BUTYRATE AND ACETATE RATIO (C4/C2) IN THE DARK FERMENTATION REACTOR

Moreover, a continuous decrease of C4/C2 ratio (butyrate/acetate) was observed (Figure 11 B), probably due to the shift from the H2/butyrate fermentation pathway to the solvent/lactate fermentation pathway (Valdez-Vazquez and Poggi-Varaldo, 2009). In fact, previous studies have shown that the hydrogen yield in thermophilic conditions is improved by maintaining the pH values within the range 5 - 6.5 (with an optimum value of 5.5 for food waste), since it optimises the microbial hydrogenase activity. Moreover, pH affects not only the hydrogenase activity in mixed

cultures, but can also modify the by-product spectrum as well as the structure of the microbial communities (Guo et al. 2010). Therefore, since pH reached values lower than 5 at day 166-178 (Figure 11 A), the recirculation flow treated through the evaporation unit was changed daily in order to maintain NH_3 concentration below the critical value observed (600 - 700 mg L^{-1}) in the methanogenic reactor, meanwhile ensuring a sufficient alkalinity supply to the fermentative reactor so as to buffer the high VFA content and to optimize H_2 production. This strategy allows all the main stability parameters such us pH, VFAs, biogas composition and alkalinity to be maintained within a suitable range, thus to avoiding the phenomena of imbalance of the anaerobic trophic chain. Steady-state operation (days 270-345) during RUN2 was reached when continuous bio-hythane production in terms of gas composition was detected. The overall performance of the reactors are summarized in Table 18.

TABLE 18 EFFLUENT CHARACTERISTICS, SPECIFIC GAS PRODUCTION (SGP), GAS PRODUCTION RATE (GPR) AND GAS COMPOSITION OBTAINED DURING STEADY STATE CONDITIONS (270-345 DAYS) OF RUN2

Effluent characteristics	*Units*	*average ± standard deviation*	
		H_2-reactor	*CH_4-reactor*
TS (n= 14)	mg L^{-1}	51 ± 11	31 ± 4
TVS (n= 14)	mg L^{-1}	42 ± 10	19 ± 8
COD (n= 14)	mg L^{-1}	33 ± 3	17 ± 1
TKN (n= 14)	mg L^{-1}	1.3± 0.3	1.2 ± 0.2
P (n= 14)	mg L^{-1}	0.33 ± 0.1	0.31 ± 0.1
pH (n= 47)	-	5.3 ± 0.2	8.2 ± 0.1
TAN (n= 47)	mg L^{-1}	745 ± 282	1115 ± 56
VFA (n= 25)	mg COD L^{-1}	13171± 5100	528 ± 393
acetate (n= 25)	mg COD L^{-1}	1958 ± 1090	71 ± 54
propionate (n= 25)	mg COD L^{-1}	1045 ± 766	137 ± 128
butyrate (n= 25)	mg COD L^{-1}	3994 ± 2312	116 ± 98
partial alkalinity(n= 47)	mg $CaCO_3$ L^{-1}	-	3602 ± 392
total alkalinity (n= 47)	mg $CaCO_3$ L^{-1}	4707±1070	5437 ± 471
Total gas composition and bio-hythane yields (n=36)			
H_2	%	8 ± 2	
CH_4	%	53 ± 3	
CO_2	%	38 ± 3	
SGP	m^3 kg TVS^{-1}	0.65 ± 0.10	
GPR	m^3 m^{-3} d^{-1}	2.5 ± 0.32	

n= number of samples

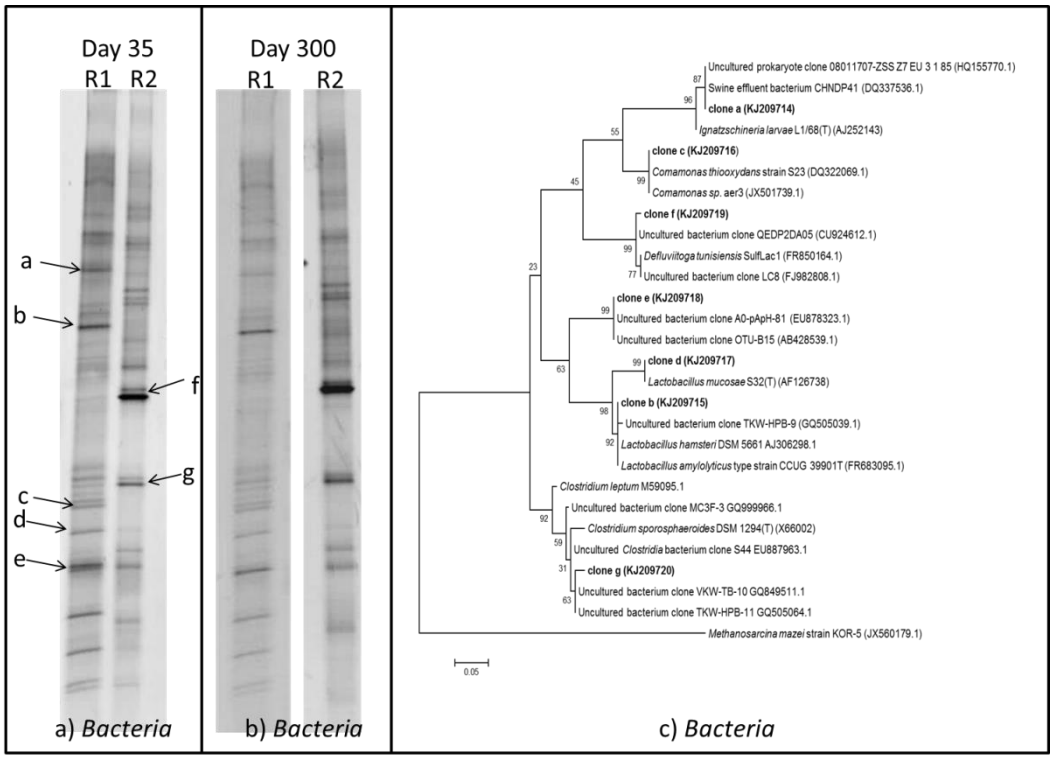
Despite the high VFA concentration measured (13171 mg COD L-1), the pH of the dark fermentation reactor was kept in the optimal range for hydrogenase activity with an average value of 5.3 ± 0.2. The fraction of CH4 detected in gas produced by the dark fermentation reactor experienced a marked increase during maintenance periods, probably due to the increase of HRT, which avoids washout of methanogens. Therefore, in order to inhibit the active methanogenic

biomass, we overloaded the fermentative reactor applying an OLR of more than 30 kg TVS m-3d-1 for a couple of days when the loading operations were reactivated (days 200 and 240). However, relevant production of CH4 (18%) was continuously detected (Figure 10 B), suggesting that acclimation of methanogen microorganisms could, in time, occur in the dark fermentation reactor (as confirmed by the FISH analysis: see next section) in spite of the low level of pH, high VFAs content and low HRT.

The methanogenic reactor reveleaded an average of 5437 and 3602 mg CaCO3 L-1 for total and partial alkalinity respectively, with a constant difference between these parameters along the CSS of RUN 2, indicating good balance of the anaerobic trophic chain. Indeed the methanogenic reactor showed low VFA concentration (528 mg COD L-1 on average) and constant pH (8.2 ± 0.1). The resulting TAN concentrations at CSS were 745 and 1115 mg L-1 for dark fermentation and methanogenic reactor respectively. From day 270 to the end of operations, the specific bio-hythane average was 0.65 m3 kg TVS-1 with an H2, CH4 and CO2 content of 8, 53, and 38% respectively. It can be remarked that TAN concentration was the major factor that influenced system performance during the different operation phases, since all other design parameters remained the same.

3.1.3.2. Bacterial Population Diversities

We investigated the microbial community using molecular analysis on two samples taken at day 35 and at day 300, when the process showed good performance in terms of bio-hythane production and composition. After about thirty days, a high H_2 production occurred in the first fermentative reactor, suggesting that the natural selection of H_2-producing bacteria was obtained starting from undefined mixed cultures by feeding the bio-waste directly. Indeed, the analysis of the bacterial community conducted on sample taken at day 35 from the dark fermentation reactor showed that the two major bands (band “b” and “e”) of the dark fermentation reactor (Figure 12 A, R1) included members affiliated with the family of the *Lactobacillacea* (band “b”) and phylum *Firmicutes* (band “e”): the first one (band “b”) showed a similarity of 99% to Type Strain *Lactobacillus hamsteri* DSM 5661 (Figure 12 C), found in thermophilic fermentors for hydrogen production fed with food waste (Wang et al., 2010); band “e”, showed a similarity of 100% to a member of the phylum *Firmicutes*, classified as uncultured cluster I (Tang et al., 2004), playing a role in the decomposition of complex and soluble organic matter during the initial step of hydrolysis (Sasaki et al., 2011).



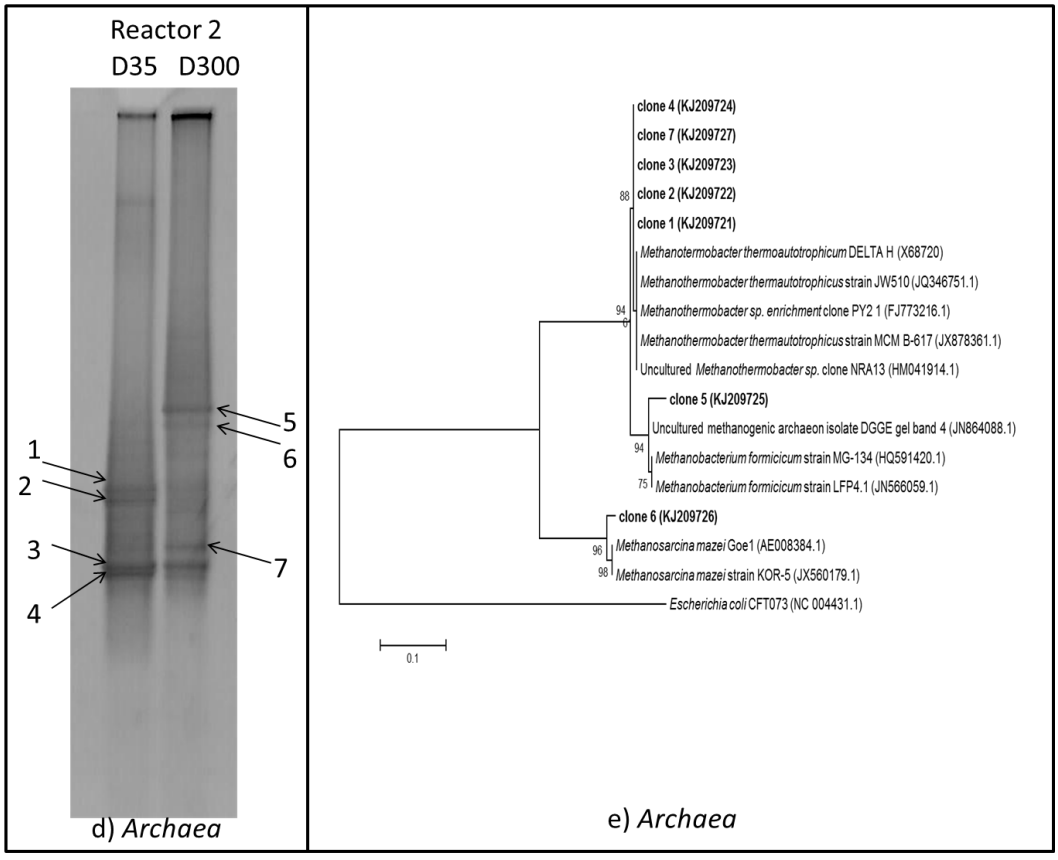


FIGURE 12 DGGE PROFILES USING PRIMERS FOR BACTERIA (A AND B) (R1= H2 REACTOR; R2= CH4-REACTOR) AND THE RELATED NEIGHBOURJOINING TREE (C) USING METHANOSARCINA MAZEI STRAIN KOR-5 AS ROOT. DE: DGGE PROFILES USING PRIMERS FOR ARCHAEA (D) (D35= DAY 35; D300= DAY 300) AND THE RELATED NEIGHBOURJOINING TREE (E) USING ESCHERICHIA COLI CFT073 AS ROOT. SEQUENCED BANDS HIGHLIGHTED BY ARROWS.

For the methanogenic reactor (Figure 12 B, R2) the dominant band (band "f") of the bacterial population reactor belongs to the *Thermotogaceae* family (phylum *Thermotogae*) and it was related to *Defluviitoga tunisiensis* SulfLac1(T) with a 99% similarity. This organism was commonly found in anaerobic digestors (Rivière et al., 2009; Kundu et al., 2012; Gupta et al., 2011; Cardinali-Rezende et al., 2009) since it is able to degrade and utilize complex carbohydrates like xylane and cellulose. The dominance of this organism at 55°C is most likely due to its broad spectrum of substrate, together with its high optimum temperature for growth (45-80°C) (Kundu et al., 2012). The second dominant microorganism of the methanogenic reactor (band "g") showed a 97% similarity to the type strain *Clostridium sporosphaeroides* and to other uncultured *Clostridium spp* (Figure 12 C), belonging to phylum *Firmicutes* (Lee et al., 2010), reported being effective hydrogen producers (Yasin et al., 2011). The bacterial community of both reactors shows substantial stability during the operations since no significant differences can be observed between the DGGE profiles obtained at day 35 and day 300 (Figure 12 A and B). This stability suggests that it is possible to ensure stable persistence of the natural selected bacteria through control of the recirculation flow. On the other hand, a great difference can be observed among the reactors, showing a speciation to specific bacteria with different characteristics.

3.1.3.3. Archaea population diversities

PCR-DGGE analysis was also used to investigate the Archaea population at day 35 and day 300 in the methanogenic reactor (Figure 12 D and E). The profiles of the two samples taken at different times in the methanogenic reactor (Figure 12 D) were very different from each other, indicating that the natural selected thermophilic biomass of the second methanogenic reactor was not stable in this long-term experience.

In fact, the phylogenetic tree (Figure 12 E) showed that methanogenic archaeal community at day 35 was composed only of species of the genus *Methanothermobacter*, with a similarity of 99% to *M. thermautotrophicus* strain JW510 (bands 1, 2, 3, 4). In the second sampling time (day 300) the archeal population shifted, including members of the *Methanobacteriales* families, widely found in thermophilic anaerobic digesters which have very high CH4 production rates using H2 and CO2 as substrate (Kundu etal., 2012). Band 7 showed 99% similarity to *M. thermautotrophicus* whereas bands 5 and 6 were respectively related to *M. formicicum* (95% similarity) and *Methanosarcinales* with a similarity of 97% to *M. mazei* strain Goe1. The microbial structure in the second methamogenic reactor showed that the hydrogen-trophic pathway is the main route for methane formation in thermophilic anaerobic reactors (Banks et al., 2012). This result confirms that the

imbalance of the anaerobic trophic chain observed during RUN 1 was probably due to the free ammonia toxic effect on the hydrogen-trophic metabolism leading to propionate accumulation in the methanogenic reactor.

Due to the difficulty in amplificating any archaeal fragment from the samples taken from the dark fermentation reactor, mainly caused by the acid composition of the sludge, at day 300 samples from the dark fermentation reactor were taken to investigate the presence of methanogenic population by FISH analysis. The hybridization results (images not shown) showed that the 10–15% microbial biomass was composed by *Archaea* (ARCH915). Among the probes used to detect the archaeal community, the only one which showed positive result was MS1414, thus confirming the active presence of *Methanosarcina spp*, which was able to grow even under acetate concentrations of about 2 g COD/L (Table 18). These results are in accordance with the study of Díaz et al. (2003) which reported that *Methanosarcina spp* grows at high acetate concentration. Finally, it can be remarked that, in time, the acclimation of the methanogens microorganisms can occur in the dark fermentation reactor, although strict conditions applied.

3.1.4. CONCLUSIONS

Two-stage thermophilic AD of food waste for H2 and CH4 co-production was studied at pilot scale. The results of this study demonstrated that a stable H2 production in the dark fermentation reactor could be achieved when its pH was controlled by means of methanogenic effluent recirculation at the ratio of 50% of the total influent flow rate. The increasing ammonia concentration determined by recycling was controlled, by means of an evaporation unit, below 2 g N/L, a concentration which determined inhibition on the hydrogenotrophic methanogens since the high level of free ammonia reached in the methanogenic reactor. Microbial analysis showed that the selection of hydrogen producers was achieved by feeding the biowaste directly in the first reactor without any substrate or inoculum pretreatment and that bacterial population in the system was stable over the entire experimentation.

Acknowledgements

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3.2. DEGRADATION OF DIFFERENT LIPID WASTES DURING ANAEROBIC CO-DIGESTION OF PIG MANURE

3.2.1. INTRODUCTION

The production of biogas through anaerobic digestion offers significant advantages over other forms of bioenergy production. It is one of the most energy-efficient and environmentally beneficial technology for bioenergy production and can drastically reduce greenhouse gases emissions compared to fossil fuels by utilization of locally available resources (Weiland, 2010).

Pig manure (PM) can be an excellent base substrate for anaerobic digestion due to its inherent buffering capacity and high content of a wide range of nutrients required for the development of anaerobic microorganisms. However, PM has a low biogas yield, around 20-30 m3/ton (Angelidaki and Ellegaard, 2003), and high ammonium concentrations (2-3 g N-$NH4^{+}$/L). Consequently, PM is preferably co-digested with high carbon content wastes, to improve the C/N ratio and increase the biogas production, essential for the plant's economy. It has been shown that bioenergy production in farm biogas plants could be enhanced by 80-400% by using organic wastes and by-products as co-substrates (Regueiro et al., 2012). Lipid wastes are ideal substrates for methane production, since theoretically their degradation produces more biogas with higher methane content, when compared with proteins or carbohydrates (

Table 19**Errore. L'origine riferimento non è stata trovata.**).

Table 19 Potential biogas production from different classes of substrates (taken from Alves et al., 2009).

Component	Methanogenic reaction	Biogas ($l g^{-1}$)	CH_4 (%)
Lipids	$C_{50}H_{90}O_6 + 24.5H_2O \rightarrow 34.75CH_4 + 15.25CO_2$	1.425	69.5
Carbohydrates	$C_6H_{10}O_5 + H_2O \rightarrow 3CH_4 + 3CO_2$	0.830	50.0
Proteins	$C_{16}H_{24}O_5N_4 + 14.5H_2O \rightarrow 8.25CH_4 + 3.75CO_2 + 4NH_4^+ + 4HCO_3^-$	0.921	68.8

Fat, oil and grease (FOG) collected from the food service industry has been cited to increase biogas production by 30% or more when added directly to the anaerobic digester and the energy value of lipids makes them an ideal co-substrate to increase the economical feasibility of any AD plant based on co-digestion concept, because the net energy production increases significantly if a fraction of waste lipids is mixed in the feedstock.

Despite the reported benefits of co-digestion, studies investigating the anaerobic digestion of high-strength lipid wastes have also reported a wide assortment of operational challenges.

These operational challenges include the inhibition of acetoclastic and methanogenic bacteria, substrate, and product transport limitation, sludge flotation, digester foaming, blockages of pipes and pumps, and clogging of gas collection and handling systems (Hunter Long et al., 2012).

One of the most important concern with anaerobic digestion is that long chain fatty acids (LCFA) may have a detrimental effect on methanogenic bacteria due to sludge flotation and washout; transport limitation from bacteria being coated in a layer of LCFAs thereby hindering the cells access to substrates and its ability to release biogas; or a LCFA toxicity effect on methanogenic bacteria. The mechanism and the nature of the inhibition of methanogenic bacteria is not well understood, however recent studies (Pereira et al., 2003 and 2004) have shown that the exposure to high LCFA concentrations cause an increasing in the lag phase in methanogen activity in witch methane production may initially be decreased, but will not result in a bactericidal affect. A mechanism for LCFA inhibition could be the adsorption of LCFAs to anaerobic sludge due to the transport limitation caused by the hindrance of substrate diffusion and into the sludge as well as transport of methane and other products out of the sludge.

The aims of this project were:

1. To understand the degradation pathways of real wastes containing high percentages of lipids;
2. To determine the optimal conditions to treat lipid wastes in anaerobic co-digestion with pig manure;
3. To characterize the microbial community responsible for specific Long Chain Fatty Acid degradation pathways using PCR-DGGE and FISH analyses.

3.2.2. MATERIALS AND METHODS

3.2.2.1. Pig Manure, Lipid Substrates and Inoculum

Pig Manure (PM) was taken from a sewer of a 150-pig fattener and sow farm, which collects both feces and urine. PM samples were homogenized, sieved to 2 mm and stored at 4 °C until use to minimize decomposition. Different batches of PM were used throughout the experiment (>200 days) due to the impossibility of storing the total amount required.

The following Lipid Wastes (LW) containing different lipid concentrations and LCFA compositions were collected:

- DAF, waste delivered from a canning industry, consisted of lipids and solids obtained from the Dissolved Air Floatation (DAF) system;
- Fish Waste, delivered from a canning industry, consisted of heads, tails, bones and viscera of tuna fish;
- Vegetal Oil waste, mixture of used oil waste before & after solid removal;
- Palm Oil, refined palm oil.

The different Lipid Wastes used in this study were homogenized and stored at 4 °C. These wastes have been analyzed for solid COD content, TS and TVS, Lipid content, LCFA composition.

As non-adapted to lipid inoculum was used an anaerobic biomass from an internal circulation reactor treating brewery wastewater, with an initial in-reactor biomass concentration of about 10 g VSS/L. As adapted to lipid inoculum was used an anaerobic biomass originated by the same biomass, acclimated to lipids during previous experiment.

3.2.2.2. Lipid Degradation Batch Tests

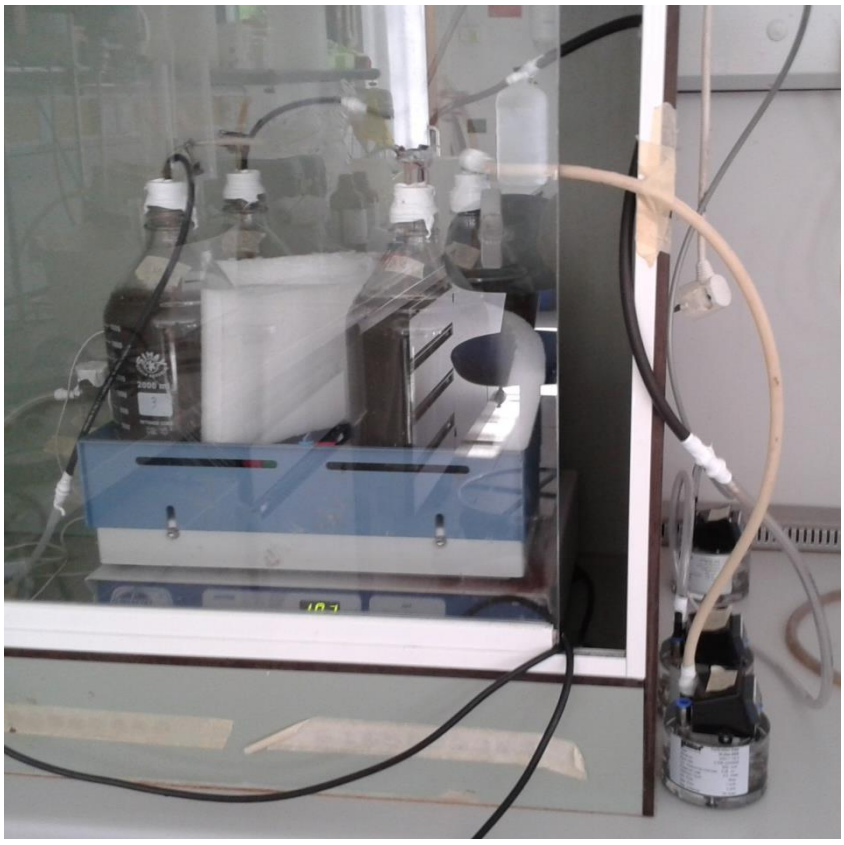

FIGURE 13 ANAEROBIC DIGESTERS USED FOR THE BATCH EXPERIMENTS

Three batch experiments were conducted, adding the characterized lipid wastes as the only carbon source, in order to comprehend the degradation pathway of the different lipids during anaerobic digestion. For that purpose, 2 L reactors (Figure 13) were inoculated with adapted and non-adapted biomasses to lipid substrates degradation. The applied conditions are shown in **Errore. L'origine iferimento non è stata trovata.**:

TABLE 20 CONDITIONS APPLIED IN BATCH TESTS

Parameter	Units	
Volume	L	**2**
T	°C	**~37**
TVS	g/L	**~3.5**
pH		**~7.5**

After two days of temperature acclimatization and consumption of the remaining COD in the inoculum, different lipid wastes at different concentrations were added to the reactors. A list of the conducted tests is shown in Table 21.

TABLE 21 LIST OF THE CONDUCTED BATCH TESTS

Experiment	Biomass	Lipid waste	Concentration
1	Adapted	Vegetal oil	1 g COD/L
1	Adapted	Vegetal oil	3 g COD/L
1	Non-adapted	Vegetal oil	1 g COD/L
1	Non-adapted	Vegetal oil	3 g COD/L
2	Adapted	Vegetal oil	5 g COD/L
2	Adapted	Vegetal oil	10 g COD/L
2	Adapted	DAF	5 g COD/L
2	Adapted	DAF	10 g COD/L
3	Adapted	Palm Oil	5 g COD/L
3	Adapted	Palm Oil	10 g COD/L

3	Non-adapted	Palm Oil	5 g COD/L
3	Non-adapted	Palm Oil	10 g COD/L

Biogas volume was measured all the days and 20 mL-samples were taken every day during the first week and three-times a week later for the analyses of CH_4 percentage, pH, LCFA (both solid and soluble fractions), VFA and COD (soluble fraction). Samples at the start, in the middle and at the end of the experiment were taken for molecular experiments.

3.2.2.3. In Continuous Reactors and Operation Conditions

Experiments were carried out in two continuous stirred tank reactors (Figure 14), a 5 Liter only-PM digester and a 10 Liter co-digester. Reactors were operated at 35 °C by hot water recirculation. The applied feedstock mixtures were prepared every week, diluted with tap water according to the applied organic loading rate (OLR), and stored at 4 °C prior to use. The digesters were fed manually after an equivalent volume of digester mixed liquor was removed.

Stirring speed and biogas production were monitored on-line, while others physico-chemical parameters (pH, temperature, solids content, chemical oxygen demand (COD), alkalinity, volatile fatty acids (VFA) and ammonium concentration) were measured three-times per week.

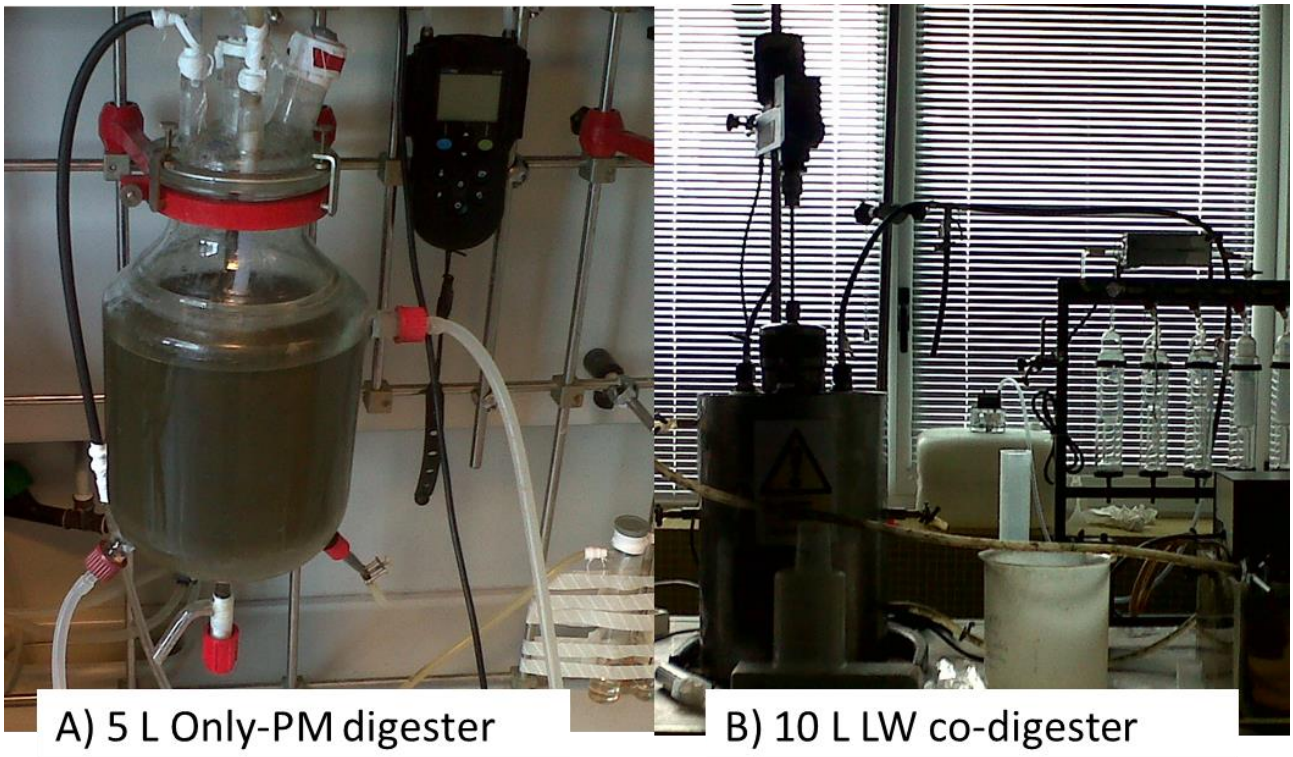

FIGURE 14 THE ANAEROBIC DIGESTERS USED FOT THE CONTINUOUS TESTS

The PM anaerobic digester was operated for 116 days, fed with pig manure with an OLR of 1 g COD/Ld and an HRT of 10 days. The conditions applied are shown in the **Errore. L'origine iferimento non è stata trovata.**.

TABLE 22 OPERATING CONDITIONS APPLIED TO THE PM ANAEROBIC DIGESTER

V	5	L
HRT	10	d
OLR	1	gCOD/Ld
T	37	°C

The co-digester was operated for 256 days, the periods of operation can be divided in:

1. **Start-up**: from day 0 to day 36, when the reactor was fed with only pig manure at an OLR of 2 and HRT of 20 d;
2. **Lipid degradation acclimation**: from day 64 to day 213, when DAF waste was added increasing the OLR;
3. **Influence of the type of lipid waste on AD performances**: when other 2 lipid wastes were tested at the same OLR, from day 214 to day 234 we tested vegetal oil and from day 235 to day 256 we tested palm oil.

From day 64 to 256 the conditions applied are showed in Table 23.

TABLE 23 OPERATING CONDITIONS OF THE ANAEROBIC CO-DIGESTER OF PIG MANURE AND LIPID WASTES

V	10	L
HRT	10	d
OLR	1 PM + LW	g COD/Ld
T	37	°C

From day 64 the lipid fraction of the OLR was increased of 0.5 g COD/Ld while reaching the steady state and waiting 2-3 HRT from each increase. The OLR at the start was 2 g COD/Ld (1 PM and 1 LW) and the last one tested was 4 g COD/Ld (1 PM and 3 LW).

3.2.2.4. Analytical Analyses

pH, COD, total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS), ammonium ($N\text{-}NH_4^+$), TA (total alkalinity) and PA (partial alkalinity) were performed following standard methods (APHA, 1998). FA concentration was calculated using the $NH_4^+\text{-}NH_3$ equilibrium constant (Ka), according to the equations described by Cuetos et al. (2008). Biogas production was measured online by Ritter milligascounters (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany) and biogas composition was analyzed by gas chromatography (HP, 5890 Series II). VFA (acetic, propionic, i-butyric, n-butyric, i-valeric and n-valeric) were analyzed by gas chromatography (HP, 5890A) equipped with a Flame Ionization Detector (HP, 7673A). Total lipids content was determined using the standard Soxhlet method (APHA, 1998) and LCFA were extracted from the samples after an alkaline hydrolysis following the method developed by Neves et al. (2009) and using pentadecanoic acid (C15) as internal standard. The LCFA were analyzed by a CG-FID 6850, equipped with software A.10.02, LCFA were separated using a DB-WAX 30 m x 0.25 mm x 0.25 µm column (Agilent 122-7032E). Temperatures of the injection port and detector were 260°C, initial oven temperature was 130°C for 2 minutes, with a 4°C/min ramp to 250°C and a final isothermal for 10 min.

3.2.2.5. Microbial Analyses

3.2.2.5.1. PCR-Denaturing Gradient Gel Electrophoresys

2 mL aliquots of well-homogenized biomass were stored at -20 °C before analysis and total genomic DNA was extracted using the PowerSoil DNA soil extraction kit (MoBio Laboratories, Inc., Solano Beach, CA) following the manufacturer's instructions. Total nucleic acid diluted in 50 µL of sigma water was quantified and checked for purity at A260/280 and A260/230 nm with Nanodrop (Thermo Scientific 2000c). Genomic DNA was subjected to DGGE analysis as previously described (Regueiro et al., 2012). The 16S rRNA gene hypervariable regions of *Bacteria* and *Archaea* were amplified by PCR using primers U968-f and L1401-r for *Bacteria*, targeting the V6-V8 region (Neubel et al., 1996) and primers A109(T)-f and 515-r for *Archaea* targeting the V2-V3 region (Roest et al., 2005). Primers U968-f and 515-r included a GC clamp at the 5' end. PCR

results were verified in an agar gel. DGGE analysis were performed in 1×TAE buffer using an INGENY PhorU system (Ingeny, Goes, The Netherlands) at 100 V and 60 °C for 17 h. DGGE gels were stained with 1×TAE buffer containing SybrGold (Molecular Probes, Inc., Eugene, OR, USA). The urea/formamide gradient was 40-80% for *Bacteria* and 30-70% for *Archaea*. Predominant DGGE bands were excised with a sterile razor blade, suspended in 50 µL sterilized milliQ water, stored at 4 °C overnight, re-amplified by PCR using the same primers without the GC clamp and sequenced. Sequencing was accomplished using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1) and an ABI PRISM 3700 automated sequencer (PE Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Sequencing results were searched for homology using the BLASTN database (Altschul et al., 1997). Similarity of the sequences with Type Strains is checked using EzTaxon server 2.1 (Chun et al., 2007). The sequences were initially aligned using the multiple alignment program CLUSTAL_X 1.83 (Thompson et al., 1997). Chimeras were checked using DECIPHER database (Wright et al., 2012).

3.2.2.5.2. Fluorescence In Situ Hybridization

Active microbial populations were identified by the FISH technique. Fresh biomass samples were disrupted and fixed with 4% paraformaldehyde according to the procedure described by Amann et al. (1995). Hybridization was performed at 46 °C for 90 min adjusting the formamide concentrations for each probe. The probes used were: Eub338mix (*Bacteria*), Arc915 (*Archaea*), Ms821 (*Methanosarcina*), Mx825 (*Methanosaeta*). All the details of each probe (formamide percentage, sequence and target organism) can be found in the probeBase database (http://www.microbial-ecology.net/probebase/). All probes were 5' labeled with the fluorochromes FITC and Cy3. Fluorescence signals were recorded with an acquisition system (Coolsnap, Roper Sicientific Photometrics) coupled to an Axioskop 2 epifluorescence microscope (Zeiss, Germany).

3.2.3. RESULTS AND DISCUSSION

3.2.3.1. Lipid wastes characterization

From the analyses of the lipid wastes resulted that all the analyzed parameters were very different between the oils (vegetal and palm oil) and the fish-derived wastes (DAF and fish waste). The summarized results of the lipid wastes characterization are shown in Table 24.

TABLE 24 LIPID WASTES CHARACTERIZATION

Waste type	COD	TS	TVS	% TVS	Lipid content
	g COD/kg	g/kg	g/kg	%	g/kg
DAF waste	**1579.6** ± 259.0	189.9 ± 51.4	187.9 ± 51.8	98.9	**382.02** ± 52.57
Fish Waste	**1453.3** ± 216.4	261.4 ± 46.8	249.7 ± 43.1	95.5	**103.74** ± 43.71
Vegetal Oil before treatment	**5248.9** ± 214.6	996.1 ± 3.0	831.7 ± 2.6	83.5	**831.69** ± 27.51
Vegetal Oil after treatment	**5064.8** ± 348.9	991.6 ± 6.9	897.8 ± 6.9	90.5	**959.05** ± 43.65
Palm Oil	**5551** ± 246	999.3 ± 0.4	998.9 ± 0.34	100.0	**992.5** ± 7.5

The oils were characterized by higher values of organic carbon (over 5000 g COD/kg), due to the high lipid content in all the oil samples over 800 g/kg. The main LCFA characterizing Vegetal Oil Waste was Oleic Acid (C18:1, 46.2%), while the main LCFA characterizing Palm Oil was, as we expected, Palmitic Acid (C16, 45.3%). Fish-derived wastes were characterized by lower organic carbon values (1400-1500 g COD/kg) and a lipid content lower than 400 g/kg. The LCFA composition of these wastes shows higher presence of C9 and C10 fatty acids, index of the degradation of these wastes. The main fatty acids characteristics of these kind of waste was Linoleic Acid (C18:2), present for the 38.3% and 37% for DAF and Fish Waste, respectively. All the results from the LCFA characterization of the lipid wastes is shown in Table 25.

TABLE 25 LCFA PERCENTAGES CHARACTERIZING THE TESTED LIPID WASTES

LCFA	N° of C	Vegetal Oils	Palm Oil	DAF Waste	Fish Waste
Pelargonic acid	**C9**	11.4	-	**30.1**	5.2
Capric acid	**C10**	19.8	-	6.8	**30.1**
Lauric acid	**C12**	-	-	7.3	-
Palmitic acid	**C16**	8.1	**45.3**	17.6	27.7
Margaric acid	**C17**	8.0	-	-	-
Stearic acid	**C18:0**	2.2	9.8	-	-
Oleic acid	**C18:1**	**46.2**	32.0	-	-
Linoleic acid	**C18:2**	4.3	12.9	**38.3**	**37.0**

3.2.3.2. Fat degradation batch tests

The first batch test was conducted for 21 days using Vegetal Oil as the only lipid and carbon source at a concentration of 1 and 3 g COD/L. 4 reactors, 2 with adapted biomass and 2 with non-adapted biomass, were inoculated and fed with a single pulse of the lipid at the selected concentration. Methane production of the 4 reactors is shown in Figure 15. The adapted to lipid biomass (ad B) was able to degrade Vegetal Oil with a total COD removal of 43% and 37% for respectively 1 and 3 g COD/L tested. On the contrary the non-adapted biomass (N B) was not able to degrade the lipid source even at the lowest concentration.

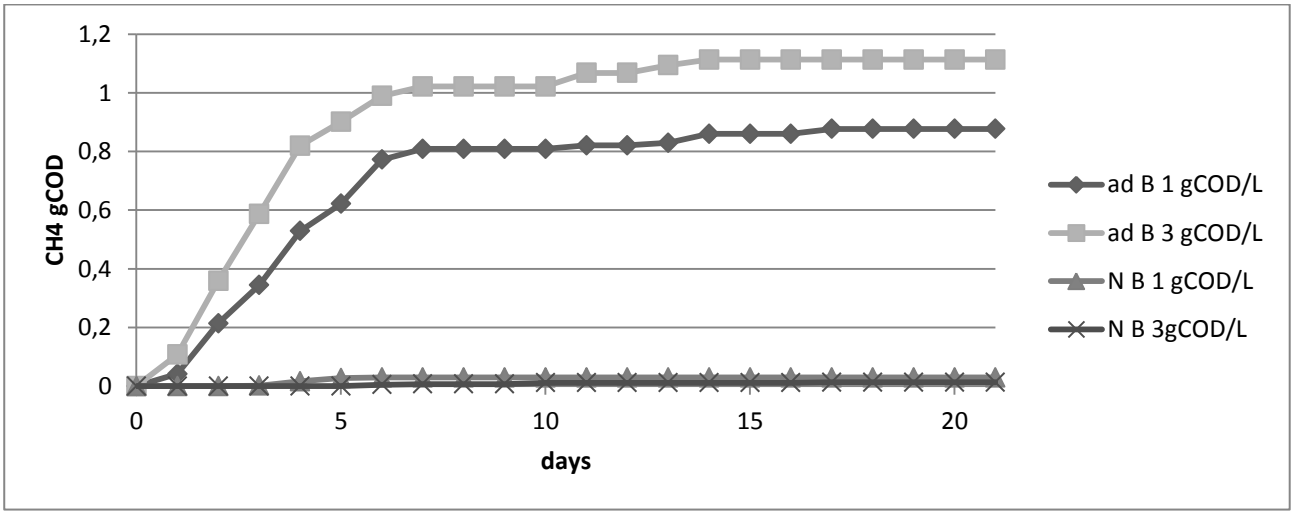

FIGURE 15 METHANE PRODUCTION DURING THE FIRST BATCH TEST

The second batch experiment (Figure 16) was conducted for a total of 46 days, where the highest concentrations of Vegetal Oil and DAF waste were tested only on adapted biomass. 4 reactors were inoculated with the adapted biomass and fed with 5 and 10 g COD/L of the selected lipid. All the samples were able to degrade the added lipid source, but the DAF Waste reached the highest biogas production rates, due to the highest concentration of shorter chain fatty acids.

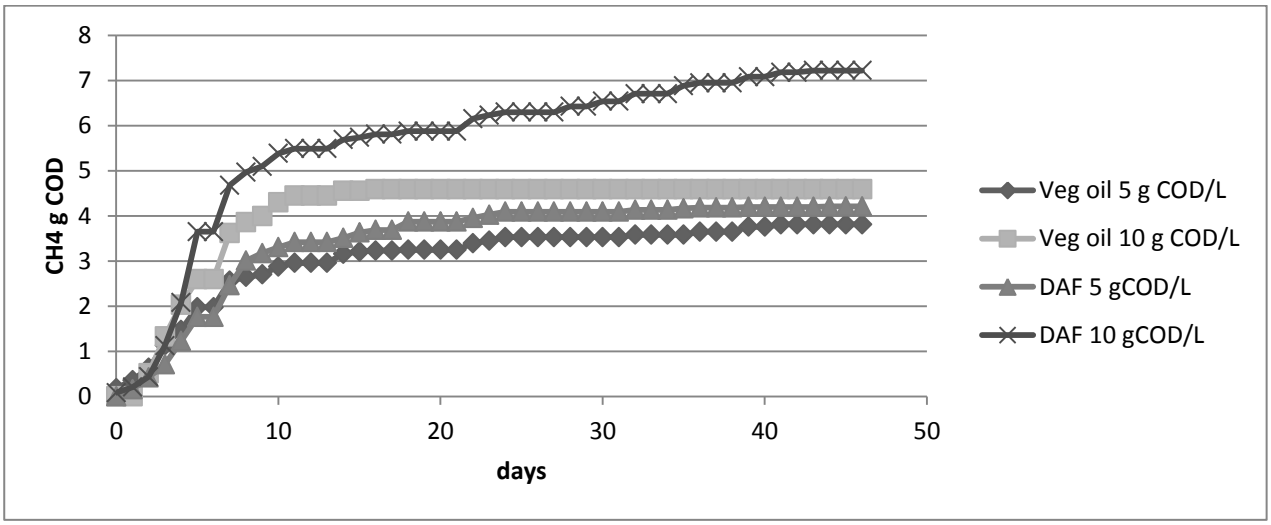

FIGURE 16 METHANE PRODUCTION IN THE SECOND EXPERIMENT

The third experiment (Figure 17) was conducted for 22 days and the analyzed lipid was Palm Oil. We tested the degradation of Palm Oil by adapted and non-adapted biomass, at the concentrations of 5 and 10 g COD/L.

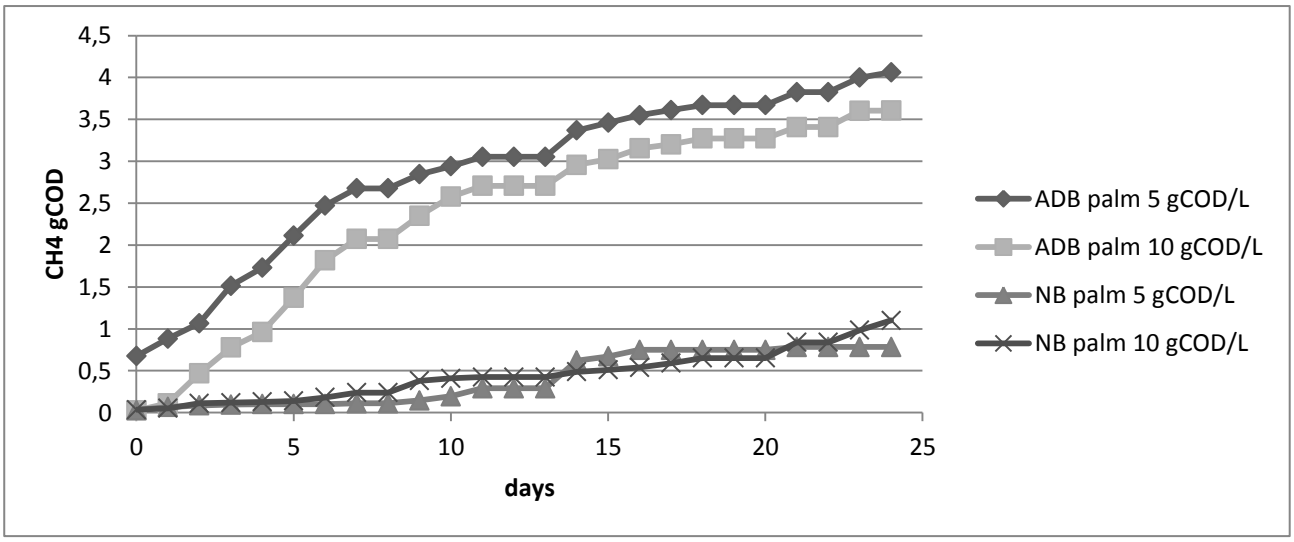

FIGURE 17 METHANE PRODUCTION IN THE THIRD EXPERIMENT

The COD removal of each experiment is summarized in Table 26.

TABLE 26 COD REMOVAL PERCETAGES DURING THE DEGRADATION BATCH TESTS

Waste	Adapted Biomass				Non-adapted Biomass			
	1 g COD/L	3 g COD/L	5 g COD/L	10 g COD/L	1 g COD/L	3 g COD/L	5 g COD/L	10 g COD/L
Vegetal Oil	43.85%	37.10%	40.18%	23.03%	1.48%	0.23%	-	-
DAF	-	-	42.47%	38.39%	-	-	-	-
Palm Oil	-	-	40.63%	18.03%	-	-	7.83%	5.50%

The results of these degradation tests show that is possible to adapt an anaerobic biomass to degrade lipids. The efficiency of degradation of fatty acids depends on the lipid loading, in fact in our case COD removal is near 40% if the concentration of lipid is minor or equal to 5 g COD/L. When the lipid concentration increase to 10 g COD/L the removal efficiency drops significantly (Figure 18).

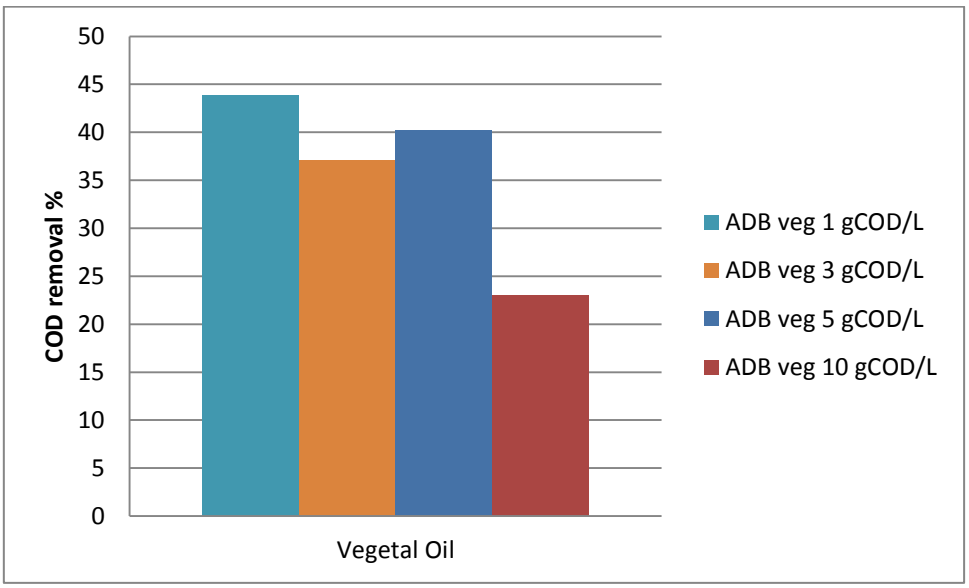

FIGURE 18 COD REMOVAL PERCENTAGE OF ADAPTED BIOMASS USING VEGETAL OIL AS CARBON SOURCE

3.2.3.3. *Continuous digestion of pig manure*

The parameters of the only-PM digester were stable during the operation period.

TABLE 27 CHARACTERIZATION OF THE PIG MANURE USED AS INFLUENT OF THE ONLY-PM DIGESTER

	Influent				
	pH	Par AlK	Tot Alk	COD tot	COD sol
		mgCaCO3/L	mgCaCO3/L	gO2/L	gO2/L
average	7.07	542.57	2069.86	5.12	2.66
st. dev.	0.11	216.49	192.97	0.38	0.39

The produced biogas contain an average percentage of CH_4 of 49.96% ± 6.87. In the following graph (Figure 19) is shown the percentage of methane in the digester related with the applied OLR.

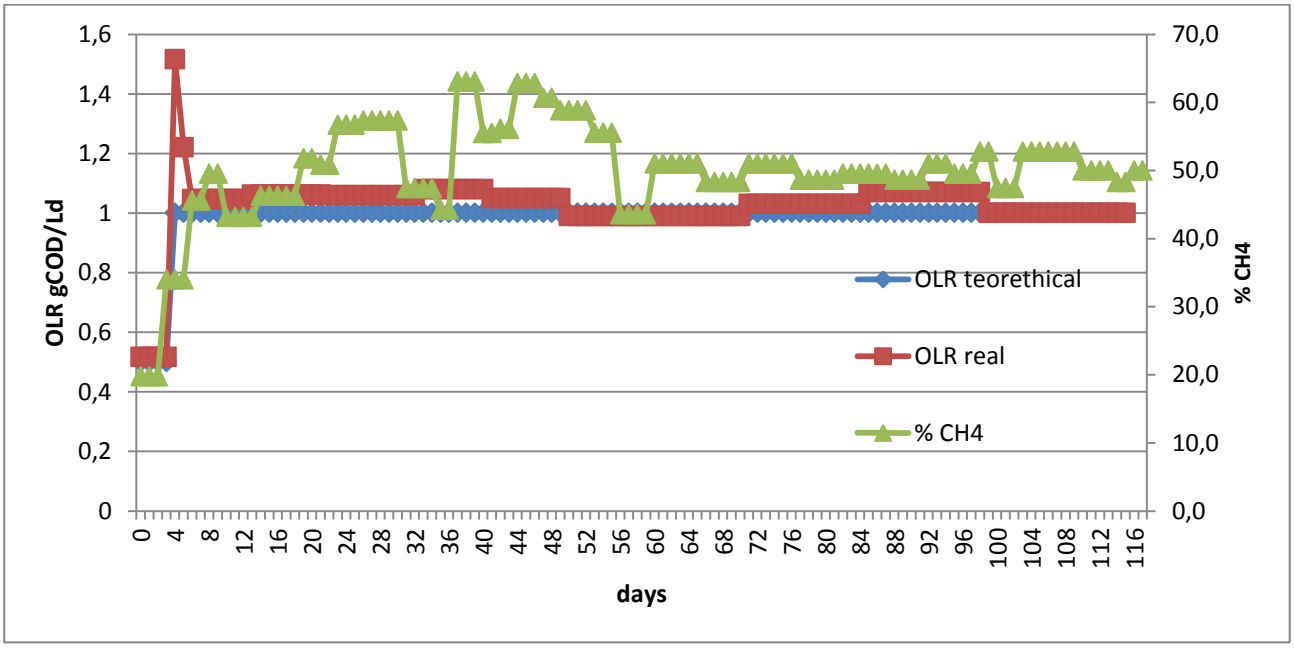

FIGURE 19 METHANE PRODUCTION AND ADDED OLR ON THE ONLY-PM DIGESTER

TABLE 28 DIGESTATE CHARACTERIZATION AND BIOGAS COMPOSITION OF THE ONLY-PM DIGESTER

	Effluent					
	pH	Par AlK	Tot Alk	R alk	COD tot	COD sol
		mgCaCO3/L	mgCaCO3/L		gO2/L	gO2/L
average	7.52	2334.22	3265.54	0.28	2.95	1.45
st. dev.	0.27	913.25	1413.89	0.05	1.76	0.55
	BIOGAS					
	Q	% CH4	% CO2	% N2	CH4	CH4
	L/d				L/d	gCOD/Ld
average	1.03	49.96	8.86	41.31	0.24	0.14
st. dev.	0.13	6.90	2.89	8.19	0.27	0.15

3.2.3.4. Continuous co-digestion of pig manure and lipid wastes

The co-digester was operated for 256 days. the periods of operation can be divided in:

1. **Start-up**: from day 0 to day 36. when the reactor was fed with only pig manure at an OLR of 2 and HRT of 20 d;
2. **Lipid degradation acclimation**: from day 64 to day 213. when DAF Waste was added increasing the OLR from 2 to 4 g COD/Ld;
3. **Influence of the type of lipid waste on AD performances**: when other 2 lipid wastes were tested at the same OLR. from day 214 to day 234 was tested Vegetal Oil and from day 235 to day 256 was tested Palm Oil.

In Table 29 are shown the characteristics of the influent of the reactor, showing the contribution on the OLR of the Pig Manure and the added Lipid Waste.

TABLE 29 INFLUENT CHARACTERISTICS OF THE CO-DIGESTER DURING THE OPERATION PERIODS

			diluted PM					raw PM	LW
	Operation Periods	days	pH	Part Alk mgCaCO3/L	Tot Alk mgCaCO3/L	COD tot gO2/L	COD sol gO2/L	COD tot gO2/L	COD LW gO2/L
start-up	OLR 2	0-36	5.99	1012.62	8553.03	20.89	-	121.33	-
lipid degradation acclimation	OLR 1+1	64-82	6.45	232.53	2003.68	13.34	8.77	83.93	1458.29
	OLR 1+1.5	83-108	6.43	345.62	1525.54	12.35	7.36	75.86	1467.06
	OLR 1+2	109-147	7.01	828.15	2083.82	10.76	5.68	83.85	1557.05
	OLR 1+2.5	148-175	6.98	605.00	2071.00	10.62	4.65	104.50	1592.00
	OLR 1+3	176-213	7.11	303.53	2149.24	10.59	4.89	102.26	1700.00
influence of the type of lipid waste	veg oil	214-234	7.18	650.00	2068.00	11.72	6.88	101.33	6250.00
	palm oil	235-156	7.15	680.00	2327.00	11.55	6.95	107.90	6875.00

Methane production during the operation periods and the applied OLRs are shown in the graph below (Figure 20), where the blue arrow highlight the increase in the CH_4 production due to the increase in the LW addition. The reactor reached a stable biogas production up to the OLR of 4 g COD/Ld. During the "lipid degradation acclimation" phase the reactor increased the degradation efficiency reaching over 90%. The main parameters of the reactor were stable (pH, FA, COD) while biogas production increased to 12 L/d and the CH_4 percentages reached over 73%.

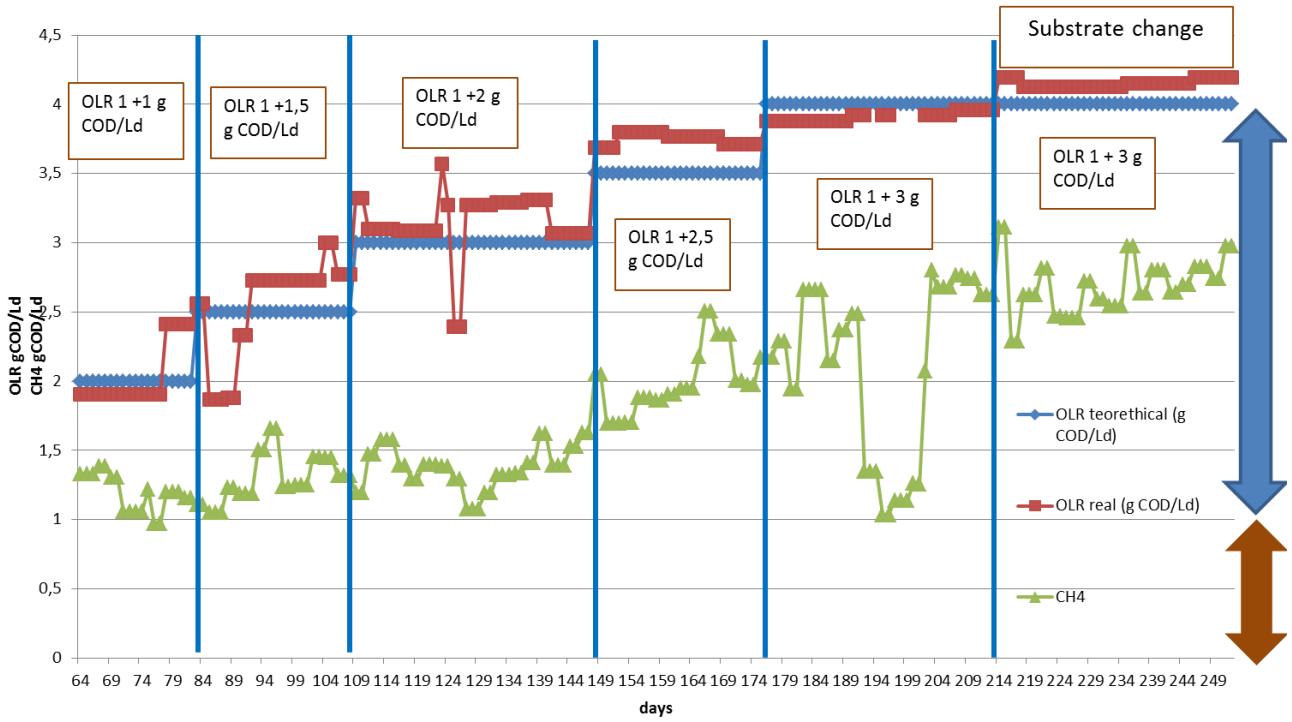

FIGURE 20 OLR AND METHANE PRODUCTION OF CO-DIGESTOR

Lipid composition of the DAF Waste used to acclimate the biomass to degrade lipids was characterized by a 38.3% of Linoleic Acid (C18:2) and an 30.1% of Pelargonic Acid (C9).

During the change of substrate in the "influence of the type of lipid waste" period the tested lipid wastes were :

- Vegetal Oil, characterized by a 46.2% of Oleic Acid (C18:1);
- Palm Oil, characterized by a 45.3% of Palmitic Acid (C16).

The reactor performances and in particular the biogas production were stable as shown in Table 30 and Table 31. This result is a clue that the lipid acclimation reached by the biomass in our experiment is not specific for a particular lipid type or lipid chain length and the organisms involved in the lipid degradation can metabolize different lipid mixtures.

TABLE 30 DIGESTATE OF THE CO-DIGESTER CHARACTERIZATION DURING THE OPERATION PERIODS

			pH	Par AlK	Tot Alk	R alk	COD tot	COD sol	N-NH4+	FA
	Operation Periods	days		mgCaCO3/L	mgCaCO3/L		gO2/L	gO2/L	g/L	g/L
start-up	OLR 2	0-36	7.77	5941.81	7541.84	0.2	11.6	1.61	1.21	0.08
lipid degradation acclimation	OLR 1+1	64-82	7.78	3809.47	4828.63	0.2	12.4	1.23	1.25	0.10
	OLR 1+1.5	83-108	7.57	2472.92	3185.15	0.2	6.0	0.85	0.48	0.02
	OLR 1+2	109-147	7.5	2276.17	3136.82	0.2	2.9	0.51	0.74	0.03
	OLR 1+2.5	148-175	7.40	2068	2903.92	0.2	3.2	0.76	0.48	0.02
	OLR 1+3	176-213	7.23	1781.05	2730.94	0.3	4.2	1.10	0.41	0.01
influence of the type of lipid waste	veg oil	214-234	7.29	1723.33	2314.19	0.2	2.6	1.05	0.42	0.01
	palm oil	235-156	7.33	1812.85	2390.28	0.2	3.2	1.20	0.49	0.01

TABLE 31 BIOGAS COMPOSITION AND COD ELIMINATION OF THE CO-DIGESTER DURING OPERATION PERIODS

			BIOGAS						Elimination
	Operation Periods	days	Q	CH4	% CO2	% N2	CH4 real	CH4 gCOD/Ld	Elim. COD
			L/d	%			L/d		%
start-up	OLR 2	0-36	7.14	69.52	27.61	2.86	4.94	1.41	42.56
lipid degradation acclimation	OLR 1+1	64-82	5.68	73.43	22.78	3.80	4.17	1.19	36.20
	OLR 1+1.5	83-108	6.15	73.70	22.25	3.92	4.53	1.30	76.20
	OLR 1+2	109-147	6.65	72.63	24.52	3.02	4.82	1.38	90.68
	OLR 1+2.5	148-175	9.61	72.71	24.30	3.90	6.99	2.00	91.49
	OLR 1+3	176-213	12.25	73.05	24.83	2.12	7.49	2.14	92.13
influence of the type of lipid waste	veg oil	214-234	13.06	70.00	26.37	3.64	9.14	2.61	93.75
	palm oil	235-156	13.83	71.01	25.41	3.58	9.82	2.81	92.36

3.2.3.5. Microbial Community

The *Bacteria* community of the adapted and non-adapted to lipid biomass of the first and second batch test was analyzed using general primers for the V6-V8 region of the 16S rDNA.

The main band (L1.1, L2.1, L3.1, L5.1 in Figure 21 A) is present in all the profiles (both adapted and non-adapted biomass), and showed a similarity of 99% with the uncultured bacterium clones ATB-KM1327, ATB-KM1280, ATB-KM1238 and ATB-KM1394, found in the work of Klocke et al. (2007), where these clones are recognized as members of the phylum *Firmicutes*, and classified as *Bacilli*. The same band has the 95% of similarity with the uncultured bacterium clones 4BacR8 and 12BacR8 found in the work of Bengelsdorf et al. (2013), where are classified in the order *Ruminococcaceae*, members of the phylum *Firmicutes*. In another work (Dang et al., 2013) a clone with the 95% of similarity was found (clone A50) that is recognized as member of the phylum *Firmicutes*, order *Clostridiales*.

The non-adapted sludge is characterized by the presence of only this band as the dominant *Bacteria*, while the adapted to lipid biomass is characterized by the presence of other bands. In particular the band L1.4 and L5.2 (in Figure 21 A) and 1.5 and 6.2 (in Figure 21 B) are present in all the adapted to lipid biomass profiles and are related to *Pseudomonas caeni* (EU620679) with the 100% of similarity. The same band show a 99% of similarity with other non-published sequences related with the genus *Pseudomonas*. In various works (Prasad and Manjunath, 2010; Amara and Salem., 2009; Okuda et al., 1991) members of the genus *Pseudomonas* (in most cases *Pseudomonas aeruginosa*) show high lipid degradation capacity and high level of lipase production.

Another band (L1.5 in Figure 21 A and 1.7 and 6.3 in Figure 21 B), present only in the adapted biomass is related with the uncultured *Peptostreptococcaceae* bacterium clone De192 (Liu et al., 2011).

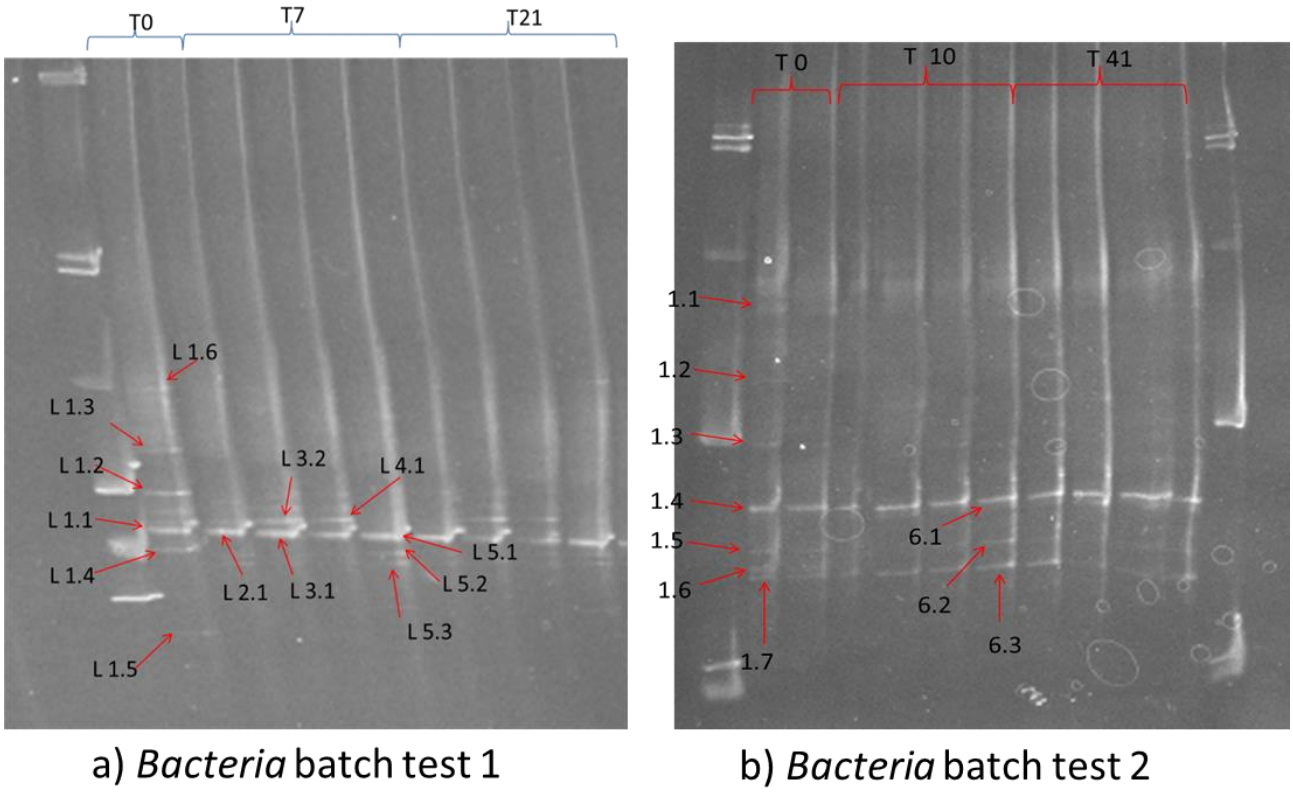

FIGURE 21 DGGE PROFILES OBTAINED USING BACTERIA PRIMERS. IN A) ARE SHOWN THE RESULTS OF THE FIRST BATCH TEST AND IN B) ARE SHOWN THE RESULTS OF THE SECOND BATCH TEST

Analyzing the Archaeal community in Figure 22 we can observe that the *Archaea* composition is not influenced by the lipid addition and there are few differences between adapted and non-adapted biomass. The community is characterized by the presence of an uncultured archaeon clone (band 7 and 17) that has 96% of similarity with the uncultured *Methanomicrobiales* archaeon clone QEEC1BF111 found in Riviere et al. (2009) and the same similarity with the uncultured *Methanomicrobiales* archaeon clone MADSarc23 (Ito et al.,). Bands 3, 8 and 18 showed a similarity of 95% with uncultured *Methanoculleus sp.* clone ACD6, found in Chen et al. (2012). Bands 4, 9 and 19 are related with the order of the *Methanosarcinales* and shows a similarity of 98% with Uncultured *Methanosaeta sp*. clone arc6.5-F6, found in Ziganshin et al. (2011). These bands are present with more intensity in the adapted to lipid biomass and are absent in the non-adapted profiles.

The presence of members of the *Methanosarcinales* order in both the biomasses is confirmed by FISH analyses (data not shown), that highlight the differences in the archaeal composition among this order:

- adapted biomass showed the presence of members of the *Methanosaetaceae* family;
- while non-adapted biomass showed the presence of *Methanosarcinaceaea* family.

Other bands that has significant results were band 10 and 12, that showed a 100% of similarity with uncultured *Methanospirillum sp.* clone KA7 (DQ085319) and uncultured archaeon clone LB18F11 found in Barret et al. (2013), where is recognized as a *Methanospirillum*. Bands 21, 23 and 25 are highly similar (100%) with the uncultured archaeon clone GZK39, found to be a member of the *Methanosarcinales* order in Huang et al. (2003).

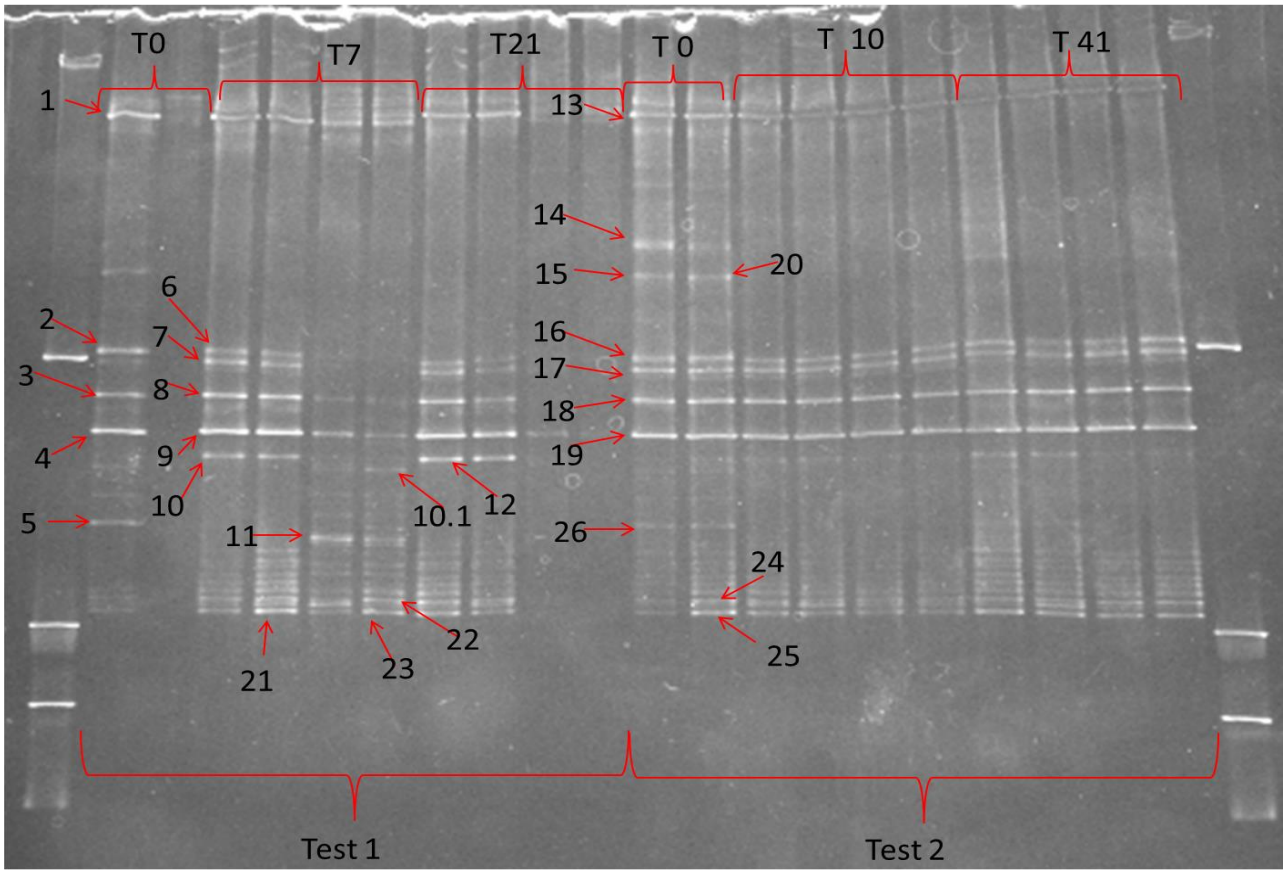

FIGURE 22 DGGE PROFILES OBTAINED USING ARCHAEAL PRIMERS

3.2.3. CONCLUSIONS

In this work we demonstrated that anaerobic biomass can be adapted to high lipid concentration using a continuous feeding strategy in co-digestion with pig manure as a source of humidity and alkalinity and the adaptation is not related to the type of LCFA composing the lipid substrate.

In our case the maximum reached OLR was 4 g COD/Ld, where the lipid OLR was 3 g COD/Ld.

A further attention of this study was to identify the dominating community members involved in the anaerobic degradation of lipids. The Archaeal community did not change between adapted and non-adapted biomasses, while the Bacterial community is different: the adapted biomass shows more biodiversity. As in other mesophilic biogas plants, the bacterial community was dominated by the phyla *Firmicutes* and members of the *Pseudomonas* genus are present in all the adapted to lipid biomass, probably responsible of the LCFA degradation.

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4. POST-TREATMENT OF SWINE DIGESTATE VIA SHORT-CUT NITRIFICATION

4.1. ROLE OF EXTERNAL CARBON SOURCES IN THE SPECIATION OF A MICROBIAL COMMUNITY IN A SHORT-CUT BIOLOGICAL NUTRIENTS REMOVAL

Abstract:

Biological nutrients removal via-nitrite has gained interest recently due to several advantages over the conventional processes. In this processes the role of certain short chain fatty acids as carbon source is crucial for the nutrients removal. but a gap of knowledge about their influence on the speciation of heterotrophic denitrifying microbial populations. including denitrifying phosphorus accumulating organisms. The purpose of this study is to investigate the microbiological composition of a via-nitrite process when different carbon sources for nutrients removal are used.

A demonstration sequencing batch reactor treating supernatant from anaerobic co-digestion of sewage sludge and OFMSW was stably operated via-nitrite pathway. In long term operation different external carbon sources were tested. In particular. microbial community of the sludge during acetic acid and fermentation liquid of the organic fraction of municipal solid waste (OFMSW) dosage was analyzed through PCR-DGGE technique using primer set for the universal eubacterial V3 region within 16S rRNA gene.

4.1.1. INTRODUCTION:

Presence of high concentration of nutrients. especially nitrogen and phosphorus in anaerobic digestion effluents and their impacts on natural water bodies are of major concern. Biological nutrients removal via-nitrite has gained interest recently due to several advantages over the conventional via-nitrate pathway (Ji et al.. 2010 and Peng et al.. 2006). Compared to complete autotrophic nitrogen removal. partial nitrification and heterotrophic denitritation may be more robust and reliable as it is less sensitive to environmental and operating parameters (Gustavsson et al.. 2010). The current understandings of the microbial ecology of nutrient removal activated sludges showed the high phylogenetic diversity of the microorganisms involved in the process. Such diversity could partly be attributed to the complex nature of the treated wastewater and the operational conditions applied to the system (Liu et al.. 2005). In addition. the heterotrophic

processes may contemporary allow for the Short-Cut Nitrification Denitrification and Denitrifying Phosphorus Removal via-Nitrite as long as they are coupled to certain short-chain carbon sources (Zhang et al.. 2010). The purpose of this study is to investigate the microbiological composition of start-up and long-term operation of a via-nitrite process in complete aeration using different carbon sources for the denitrification: acetic acid and fermented Organic Fraction of Municipal Solids Waste (OFMSW).

4.1.2. MATERIALS AND METHODS:

Sampling was done in duplicates using the sampling port of the reactor. Samples were taken when the system reached steady state under the particular OLR (NLR) and were stored at -20°C until DNA extraction. Total DNA extraction from activated sludge was carried out using the Fast DNA Spin Kit for Soil (MO BIO. Carlsbad. CA) according to the manufacturer's instructions. Approximately 0.3 mL of material was used per extraction and the extracted DNA was checked by electrophoresis.

The 16S rRNA genes were amplified by PCR using Taq DNA polymerase with primers targeting conserved domains. Bacterial 16S rRNA genes were selectively amplified using F8/R11 primers (Weinsburg et al.. 1991) with the following thermocycling program: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 45 s. annealing at 50°C for 30 s and extention at 72°C for 2.5 min; final extention at 72°C for 5 min. Afterwards a nested PCR was performed starting from 16S rDNA amplicons obtained from the first amplification. In this second PCR reaction. the iper-variable V3 region of the 16S rRNA gene was amplified using primers P3 (with a GC clamp) and P2 (Muyzer et al.. 1993). conditions were as above. except for number of cycles. 35. the annealing temperature. 57°C. and extension time. 35 s. For the β-subgroup ammonia oxidizers bacteria (AOB) the second PCR reaction for DGGE were conducted with an equimolar mixture of the three forward primers CTO189fA-GC. CTO189fBGC. and CTO189fC-GC. each with a GC clamp and the reverse primer CTO654r containing a single ambiguous base (Kowalchuk et al.. 1997). The PCR was conducted under the conditions described by Hirooka et al.. (2009). All primers were purchased from Sigma-Genosys (Milan. Italy). The PCR products were quantified using Low DNA Mass Ladder (Celbio. Italy) in a 2.0% agarose gel. DGGE analyses were performed on amplicons obtained both for bacterial V3 and archaeal V2-V3 regions. Gels (8% acrylamide/bisacrylamide 19:1. BioRad) were cast using a denaturing gradient of 30–60%. with 100% denaturant defined as 7 mol L^{-1} urea and 20% (v/v) formamide. Electrophoresis was performed at 45 V for 18 h at 65◦C with the Dcode® Universal Detection System (Biorad) and gels

were stained with EtBr (1 mgL^{-1}). Representative DGGE bands were excised and incubated for 4 h in 50 mL of sterile water. DGGE bands containing DNA to be sequenced were re-amplified. PCR amplification was carried out as described before. except for the use of non-GC-clamped primers. PCR products were transformed in Escherichia coli DH5α using the pGEM-T vector system according to the manufacturer's instructions (Promega. Italy). sequenced on both strands. and finally searched for homology using the BLASTN database (Altschul et al.. 1997). Similarity of the sequences with Type Strains is checked using EzTaxon server 2.1 (Chun et al.. 2007). The sequences were initially aligned using the multiple alignment program CLUSTAL_X 1.83 (Thompson et al.. 1997). A phylogenetic tree was constructed using the neighbor-joining method with the MEGA version 5.1 software package (Tamura et al.. 2011). Bootstrap analysis was performed from 1000 bootstrap replications.

4.1.3. RESULTS AND DISCUSSION:

Microbial community of the activated sludge during start-up. long-term operation and the anaerobic supernatant was analyzed previously using PCR-DGGE of the universal eubacterial V3 fraction of the 16S rDNA gene. In Figure 23 are visible the DGGE profiles of the samples. where S is the supernatant feeding the reactor. T0 is the inoculum sludge. T1 is the sludge after start-up. T2 is the sludge on polyelectrolyte granules and T3 is long-term sludge.

Considering the DGGE profiles of the activated sludge and the supernatant we can observe that the inoculum sludge is formed by a totally different population compared to the population of the sludge in the subsequent times. index of a speciation of the biomass inside the reactor. In t1 (after start-up) is possible to see that the microbial community is influenced by the supernatant used to feed the reactor: in fact the band S8. that showed a similarity of 98% with the Type Strain *Decloromonas agitata* CKB (Bruce et al.. 1999). is present in both the profiles and in t1 is one of the band with the major intensity. This band is not present in the subsequent times because other organisms become dominant in the sludge.

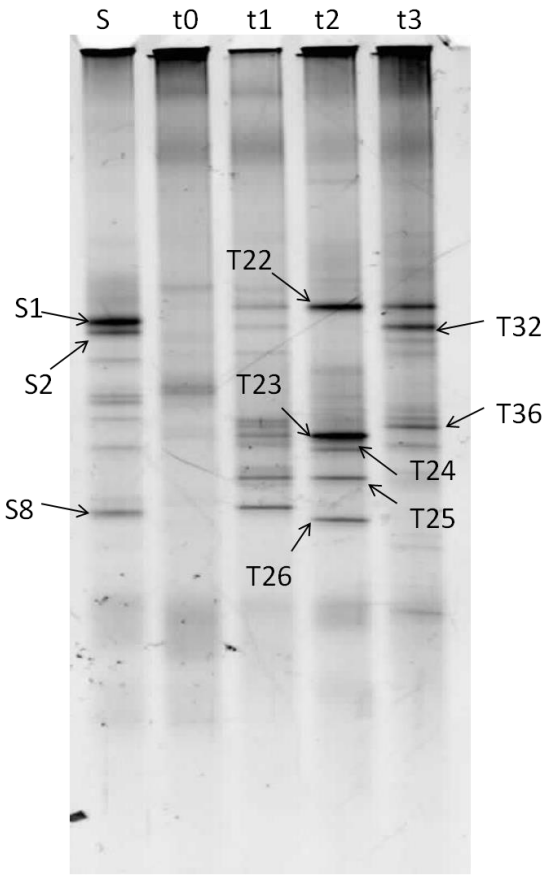

FIGURE 23 . PROFILES OF THE DGGE OF THE EUBACTERIAL V3 REGION. ARROWS SHOW THE SEQUENCED BANDS

We can observe that the profiles of t1 and t2 and t3 are very similar in terms of bands. that mean the stabilization of the biomass to a certain group of microorganisms. In t3 profile some bands become dominant over others. probably due to the change of organic carbon. In Figure 24 is shown a dendrogram of similarity of the profiles obtained with the program UVIBandMap ® (UVISoft). that confirm that t1 and t3 (called 3 and 5 respectively in the dendrogram) profiles has the 80% of homology and the inoculum t0 (sample 2 in the dendrogram) has 0% of homology in common with the other profiles.

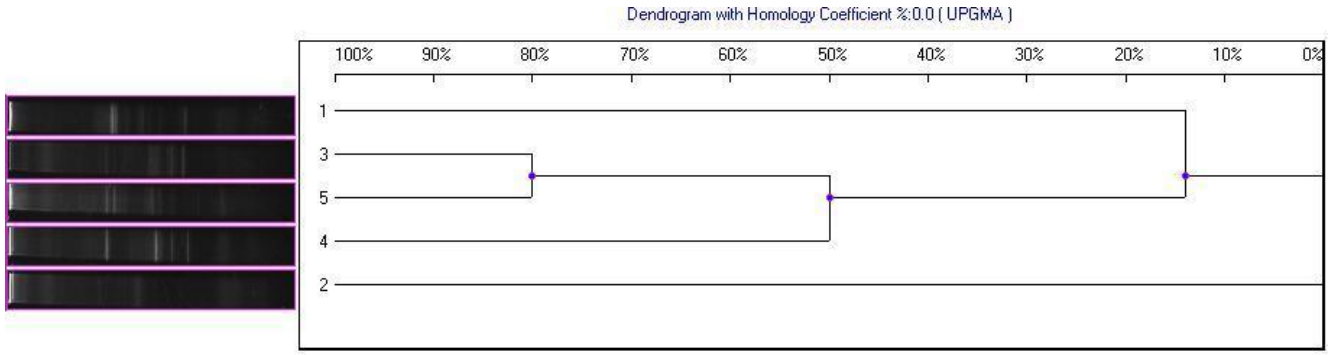

FIGURE 24. DENDROGRAM OF HOMOLOGY BETWEEN DGGE PROFILES

According to the sequencing results of the bands obtained from the PCR-DGGE the composition of the microbial community of the activated sludge was mainly composed of *Bacteroidetes*. including *Sphingobacteriaceae*. and *Proteobacteria*. and showed a strong speciation to a few species of denitrifying bacteria commonly present in reactors with high F/M loading and able to form biofilm on inorganic surfaces. The sludge composition is very different from the anaerobic supernantant population. composed mainly of other species of *Proteobacteria*. showing that the conditions applied to the system and the electron donor used influenced the speciation of the biomass.

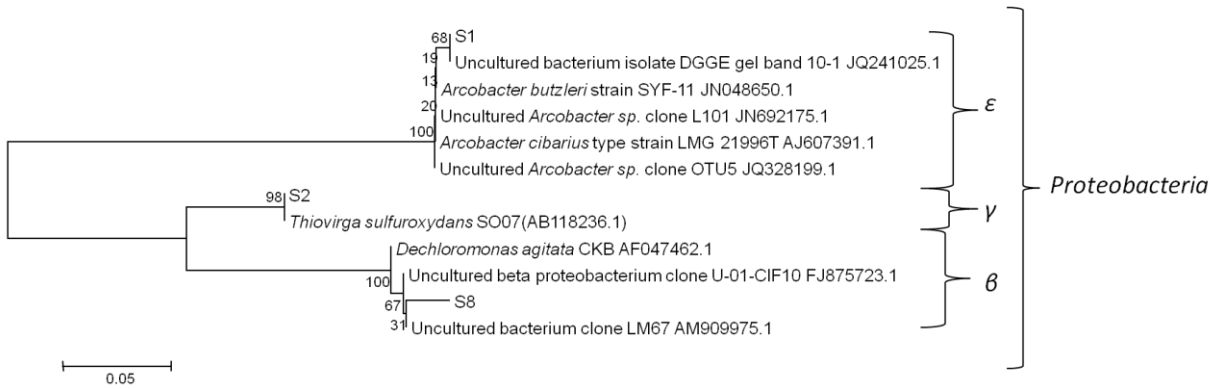

FIGURE 25. NEIGHBOURJOINING TREE OF THE CLONES OBTAINED FROM THE SUPERNATANT

We observed that in the last sampling time (**T3**). fed with fermented OFMSW as electron donor. the process showed a high Nitrite Uptake Rate (up to NUR 0.65÷1.14 $kgNO_2\text{-}N\ kgVSS^{-1}day^{-1}$) and a high Phosphorus Uptake Rate of 0.37±0.09 $kgPO_4\text{-}P\ kgVSS^{-1}day^{-1}$ allowing a complete nutrient removal in the effluent of the reactor.

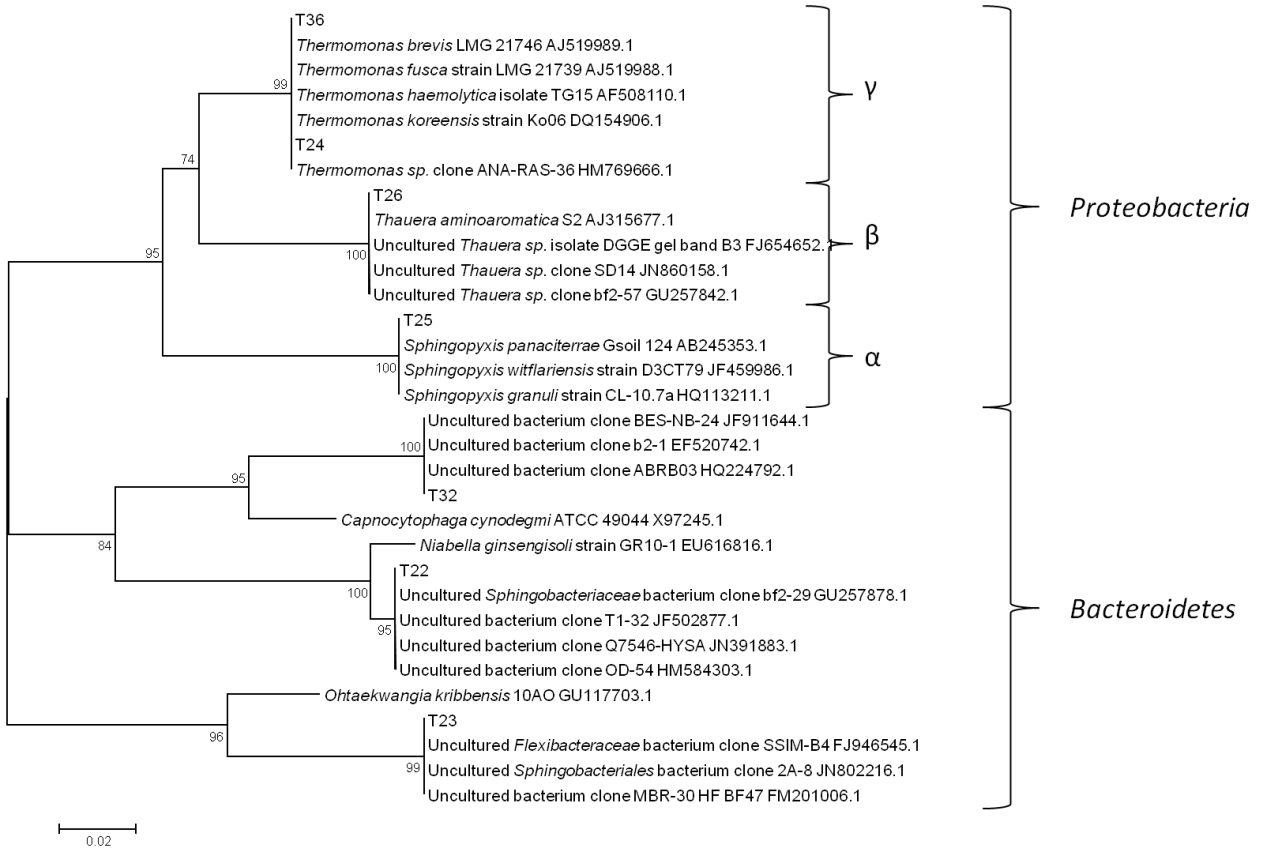

FIGURE 26. NEIGHBOURJOINING PYLOGENETIC TREE OF THE CLONES OBTAINED FROM THE ACTIVATED SLUDGE

In particular a strong speciation to a few species of denitrifying bacteria commonly present in reactors with high F/M was observed. This result is in accordance with the operating conditions and observed denitrifying activity of the biomass (NUR 0.65÷1.14 $kgNO_2$-N $kgVSS^{-1}day^{-1}$). Using the OFMSW fermentation liquid as external carbon source. the highest biological phosphorous removal was observed. with a Maximum Phosphorus Uptake Rate of 0.37±0.09 $kgPO_4$-P $kgVSS^{-1}day^{-1}$.

In this specific case. a high NLR (1.14 $gN\text{-}NH_3/d$) and F/M. coupled with OFMSW fermentation liquid were the driving forces to the speciation of a biomass composed of members of the CFB group related to the genus *Cytophaga* and *γ-Proteobacteria* (genus *Thermomonas*). which are the major population of enhanced phosphorus biological removal via-nitrite reactors (Ahn et al.. 2007; Liu et al.. 2005 and Dabert et al.. 2001). These organisms are likely to be denitrifiers. judging from the high NUR associated to the process in the sampling period and their close phylogenetic affiliation with known denitrifiers and could be the responsible for the coupled Nitrogen and Phosphorus removal. However. there is insufficient evidence to conclude whether these organisms are truly PAOs and/or denitrifiers. For this reason other analysis using PAO-specific probes for FISH are in progress. with the aim of understand the relationship between the denitrifying biomass and the phosphorus accumulation in this specific sludge.

Using non-specific primers for DGGE we do not have information about the nitrifying bacteria. because they are present in less concentration compared to denitrifying bacteria. For this reason we used the three forward primers CTO189fA-GC. CTO189fB-GC and CTO189fC-GC and the reverse primer CTO654r specific for the β-subgroup ammonia oxidizers bacteria (AOB). Figure 27 shows the profiles, positioned in the same order of the previous figure.

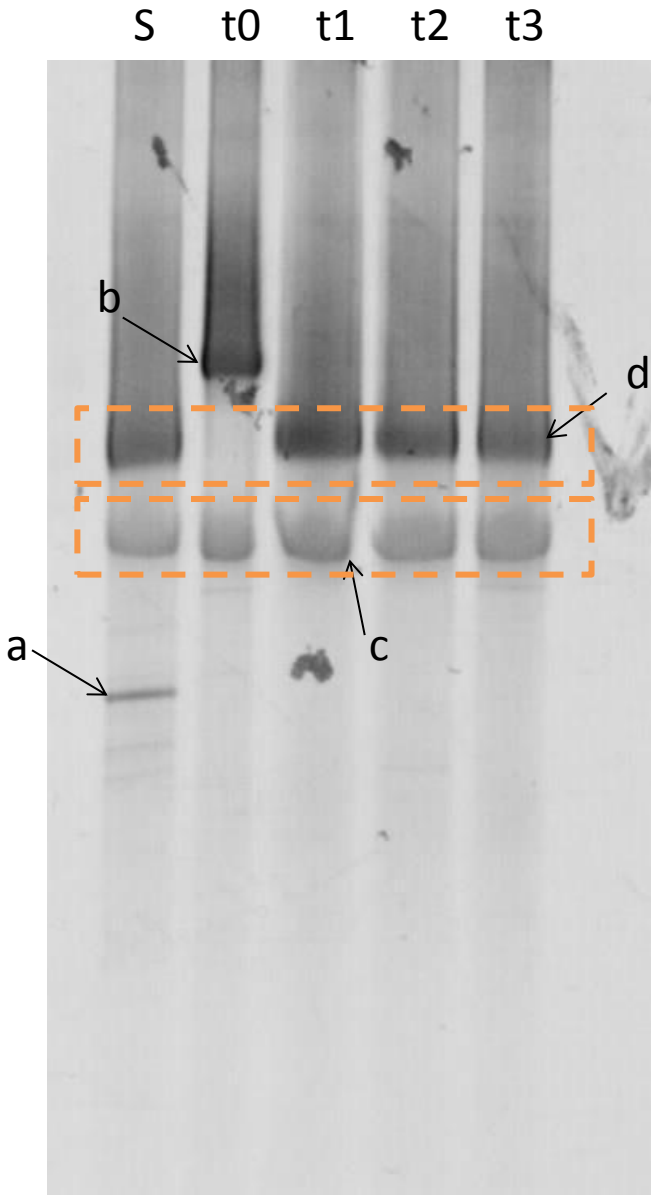

FIGURE 27. PCR-DGGE PROFILES WITH AOB-SPECIFIC PRIMERS. ARROWS SHOW THE SEQUENCED BANDS.

DGGE results shows that the AOB community is formed mainly by the band **c** corresponding to the clone with the 98% of similarity with the Type Strain *Nitrosospira multiformis* ATCC 25196 (Norton et al., 2008) an AOB very widespread in the environment present in environment with low oxygen concentrations and the band **d**, corresponding to the clone with the 99% of similarity with the Type Strain *Nitrosomonas europaea* ATCC 25978 that is metabolically versatile and is distributed over a wider range of ammonia concentrations. ranging from activated sludge reactors to

highly N-loaded reactors. *N. europaea*-related clones were found to be the dominant AOB in partial nitrification for treating high-strength nitrogen wastewaters by Ahn et al. (2011).

The inoculum profile differs from the other profiles because a *N. europaea*-related clone is not present. showing another time that the microbial community present in the activated sludge during the long-term operation is not influenced by the inoculum sludge population.

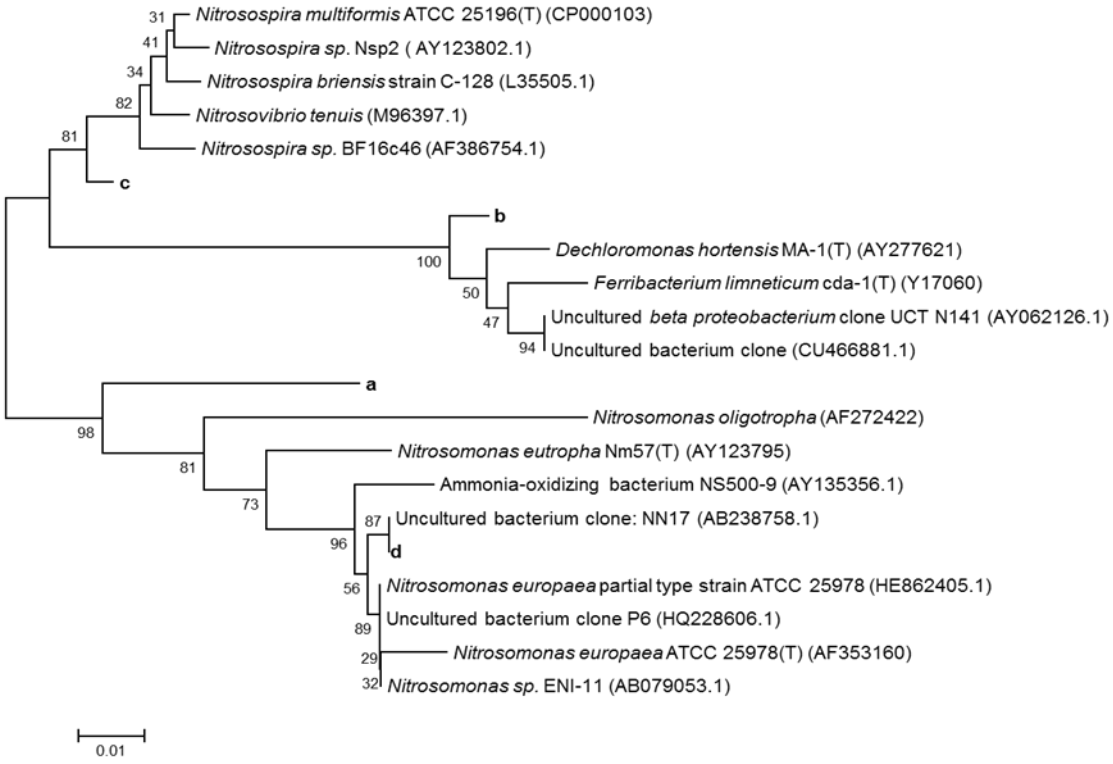

FIGURE 28. NEIGHBOURJOINING TREE OF THE CLONES OBTAINED FROM THE SLUDGE USING AOB SPECIFIC PRIMERS

4.1.4. CONCLUSIONS:

Results showed that the microbial community was mainly composed of *Bacteroidetes*. including *Sphingobacteriaceae*. and *Proteobacteria*. In particular a strong speciation to a few species of denitrifying bacteria commonly present in reactors with high F/M was observed. This result is in accordance with the operating conditions and observed denitrifying activity of the biomass (NUR 0.65÷1.14 $kgNO_2$-N $kgVSS^{-1}day^{-1}$). Using the OFMSW fermentation liquid as external carbon source. the highest biological phosphorous removal was observed. with a Maximum Phosphorus Uptake Rate of 0.37±0.09 $kgPO_4$-P $kgVSS^{-1}day^{-1}$. In this specific case. a high NLR (1.14 gN-NH_3/d) and F/M. coupled with OFMSW fermentation liquid were the driving forces to the speciation of a biomass composed of members of the CFB group related to the genus *Cytophaga*

and *γ-Proteobacteria* (genus *Thermomonas*). which are related to the enhanced phosphorus biological removal via-nitrite.

4.1.5. REFERENCES

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