MICROBIAL COMMUNITY ANALYSIS, INFLUENCE OF REACTOR HYBRIDATION AND EFFECT OF THE PROPORTION OF GLYCOL ETHERS/ETHANOL MIXTURES IN EGSB REACTORS
P. Ferrero
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Programa de Doctorado en Ingeniería Química, Ambiental y de Procesos

Memoria que, para optar al Título de Doctor por la Universitat de València, presenta **PABLO FERRERO AGUAR**

Valencia, octubre de 2018

D. VICENTE MARTÍNEZ SORIA, Catedrático del Departament d'Enginyeria Química de la Universitat de València, y

D. JOSEP MANUEL PEÑARROCHA OLTRA, Profesor Titular del Departament d'Enginyeria Química de la Universitat de València.

CERTIFICAN: Que **D. Pablo Ferrero Aguar**, con los títulos de Graduado en Biotecnología y Máster en Ingeniería Ambiental, ha realizado bajo su dirección el trabajo que bajo el título de: **"MICROBIAL COMMUNITY ANALYSIS, INFLUENCE OF REACTOR HYBRIDATION AND EFFECT OF THE PROPORTION OF GLYCOL ETHERS/ETHANOL MIXTURES IN EGSB REACTORS"** presenta en esta Memoria y que constituye su Tesis para optar al Título de Doctor por la Universitat de València, en el Programa de Doctorado en Ingeniería Química, Ambiental y de Procesos

Y para que conste a los efectos oportunos firman el presente certificado en Valencia a 19 de Octubre 2018.

Fdo.: D. Vicente Martínez Soria

Fda.: D. Josep Manuel Peñarrocha Oltra

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Summary

Volatile organic compound (VOC) emissions can cause different problems in the public health and in the environment, acting as a primary pollutant and allowing the formation of secondary pollutants as tropospheric ozone. Due to these problems VOC emissions are regulated in many countries, such as USA and the European Union, in this case by the Council Directive 2010/75/EU. Because of the use of solvents in its productive process, flexographic industry is one of the major contributors to the emissions of these compounds, and biological techniques have been considered as one of the best available technologies for the treatment of VOC emissions in this industrial sector. Among these processes, a new technology the anaerobic bioscrubber is emerging as a feasible technology (patent number WO2015114436A1). In this process, VOC are transferred from the gas phase (air emission) to the liquid phase (water) and then transformed into biogas in an anaerobic expanded granular sludge bed (EGSB) reactor. So, the VOC emissions can be converted into bioenergy.

However, the use of EGSB reactors for this process has some inherent barriers as the lack of information about the anaerobic degradation of some of the compounds typically used in the flexographic industry or the loss of biomass in the effluent due to the use of a high up flow liquid velocity. For this last aspect, an alternative configuration to the EGSB reactors should be studied in order to avoid the biomass leakage. In this regard, an anaerobic hybrid reactor configuration, which consists in the installation of a filter of polypropylene rings inside the gas-liquid-solid separator in the upper zone of the reactor, seems to be a good alternative to improve the biomass retention capacity of the EGSB reactors. Furthermore, the flexographic industry uses synthetic organic solvents as glycol ethers, such as 1-ethoxy-2-propanol and/or 1-methoxy-2-propanol, but the anaerobic biodegradation of mixtures of these compounds remain unknown yet. In addition, in the literature there is also barely information available about the possible negative or toxic effect of these glycol ethers on the microbial population responsible of the biotransformation of VOC emission into biogas. In this regard, different molecular techniques, as denaturing gradient gel electrophoresis (DGGE), quantitative polymerase chain reaction (qPCR) and high throughput sequencing technologies, are available to characterize the microbial community and to analyse the microbial evolution in biological systems, such as anaerobic reactors, which can be helpful to check these negative or toxic effects by the presence of some of these solvents.

In this context, the main objectives of this PhD thesis are: i) to evaluate an alternative reactor to the EGSB reactor to improve the biomass retention capacity and the performance of a reactor treating glycol ethers and ethanol mixtures; ii) to study the pathways for the anaerobic degradation of glycol ethers as 1-ethoxy-2-propanol (E2P) and 1-methoxy-2-propanol (M2P) used in the flexographic industry and the possible impact on the microbial community; iii) to evaluate the effect of the ethanol / glycol ethers ratio in an EGSB reactor treating mixtures of these compounds; and iv) to compare the performance and microbial communities from laboratory scale reactors and an industrial prototype reactor.

An alternative configuration based on the modification of the EGSB with a filter of polypropylene rings, called anaerobic hybrid reactor (AHR), has been compared with the conventional EGSB (control reactor). Both reactors were operated at the same conditions and the experiment was divided in seven stages (from S-I to S-VII). First (S-I), the organic loading rate (OLR) was increased step by step up to 45 kg chemical oxygen demand (COD) m^{-3} d^{-1} using a readily biodegradable substrate such as ethanol, then E2P was introduced (S-II and S-III), resulting in a binary mixture of ethanol and E2P, and the total OLR was maintained around 45 kg COD $m^{-3}d^{-1}$. After that, M2P was also introduced as a new substrate in the reactor feed (ternary mixture of ethanol, E2P and M2P) maintaining the same total OLR (S-IV). Later on, the feeding was switched off to simulate a long-term shutdown of production process, which typically occurs in the printing facilities, and so to check the influence of a long starvation period (S-V). Then, reactors were restarted again by increasing the OLR, step by step with the ternary mixture, and finally, in the last stage the proportion of M2P was slightly increased (S-VI and S-VII). Proportion of glycol ethers in this experiment was always lower than 30% in weight. Results showed a high performance of both reactors with global removal efficiencies (RE) higher than 92% even treating OLR of 54 kg COD m^{-3} d^{-1} and RE only decreased below 90% in both reactors during the first days after the feeding of E2P, indicating that biomass was not adapted to this solvent and an adaptation period was needed to be able to metabolize it. The adaptation period was also observed in the evolution of RE of E2P, as only around 20% RE was achieved in both reactors when this compound was introduced, but after 40 - 50 days the RE of E2P increased to 80% and maintained during all the experimental period. Regarding M2P, RE was almost complete (100%) immediately after its introduction in the reactor feed and no adaptation period was needed, which would suggest that both glycol ethers have the same mechanism of degradation. Furthermore, the removal of M2P was practically complete along all the experimental period, even after the increased in its proportion in the feed (S-VII). In addition, during the first days of exposure to the

glycol ethers, some intermediate products of their degradation (methanol, acetone and isopropanol) were detected and identified, allowing the clarification of the anaerobic degradation pathways of these compounds. Regarding the biomass retention capacity, the accumulated solids in the effluent (563.2 g in the EGSB reactor and only 293.7 g in the AHR) showed that the AHR had a higher biomass retention capacity than the EGSB reactor. However, the filter installed in the AHR was finally clogged and the rings of the filter had to be replaced by new polypropylene rings to maintain its higher biomass retention capacity. Despite the higher biomass concentration in the AHR, both reactors performed similarly.

These results, included in the Chapter 4 of this PhD thesis, have been published in the Journal of Environmental Management (Ferrero, P., San-Valero, P., Gabaldón, C., Martínez-Soria, V., Penya-roja, J.M., 2018. Anaerobic degradation of glycol ether-ethanol mixtures using EGSB and hybrid reactors: Performance comparison and ether cleavage pathway. J. Environ. Manage. 213, 159–167).

The dynamics of the microbial community of both reactors was also analysed using different molecular tools, such as denaturing gradient gel electrophoresis (DGGE), quantitative polymerase chain reaction (qPCR) and high throughput sequencing technologies. These analyses revealed an important impact in the microbial populations caused by the introduction of both glycol ethers (E2P and M2P). DGGE technique showed an evolution in the bacterial community from the beginning to the end of the experiment. So, some initially predominant bands (inoculum and/or early stages) decreased their intensity in later stages, and the opposite happened with other bands, which appeared or increased their intensity even becoming predominant with the evolution of the experiment. In addition, DGGE also evidenced that sludge bed of both reactors behaved as a mixed reactor, as the same microbial community structure was found in the bottom and in the top ports of both reactors. qPCR results indicated a toxic effect of the glycol ethers over the bacterial and archaeal populations, as both population concentrations decreased after the introduction of E2P. Later, the population concentration values were recovered after 30 days of exposure to this solvent, which also indicated the necessity of an adaptation period of the microorganisms to degrade anaerobically E2P. Besides, when M2P was afterwards introduced into the feed, no negative influence in the concentration of the bacterial population was found, demonstrating that M2P was anaerobically degraded in the same pathway that E2P, so the bacterial population was already adapted to this substrate (M2P). In contrast, archaeal populations as *Methanobacteriales* and *Methanomicrobiales* showed a decreased in their population concentrations after the introduction of M2P and also after the increased in the OLR of this substrate, which suggest a toxic effect of M2P over these

archaeal populations. Additionally, the results of high throughput sequencing technique emphasized the importance of the type of substrate over the predominance evolution of the microbial communities along the experimental period. Through this technique, *Proteobacteria* phylum was observed as the predominant phylum when reactors were fed only with ethanol as carbon source, and the predominance of this phylum was replaced by *Firmicutes* when glycol ethers, especially M2P, were introduced as substrates.

This study, included in Chapter 5, was developed in collaboration with the '*Laboratoire du Génie de l'Environment Industriel, IMT Mines Alès, Université de Montpellier*' during a research stay carried out under the supervision of Professor Luc Malhautier. Recently, it has been sent for its publication to the journal Bioresource Technology (Ferrero et al., 2018. Link between the anaerobic degradation of glycol ether-ethanol mixtures using EGSB and hybrid reactors and the dynamics of the microbial community structure, Bioresour. Technol. Submitted for decision).

To complete the previous study an experiment with high proportion of glycol ethers was carried out using an EGSB reactor. This experiment was divided in 8 stages. First, from stage I to stage III, the start-up of the reactor was carried out using ethanol as only carbon source, increasing the OLR in these stages up to a value of 45 kg COD m^{-3} d^{-1}. Then, in the following stages (IV-VII), ethanol was progressively replaced by E2P and M2P maintaining the OLR constant at 45 kg COD m^{-3} d^{-1}, until the last stage (VIII) where only the glycol ethers were fed into the reactor. Reactor had a high performance (RE >95%) during the start-up period, and when glycol ethers were introduced global RE dropped initially to 80% and then it was recovered to 90%, showing again the corresponding adaptation period. Later, when ethanol was progressively replaced by glycol ethers in the feed the global RE slightly decreased, and in the last stage (VIII) where only E2P and M2P (without ethanol) were fed into the reactor, global RE diminished to 80% indicating that the removal of glycol ethers could not be complete. Regarding the individual RE of each compound, a low RE was registered during the first days of exposure to each compound (≈20%), but thereafter their maximum RE were achieved. The maximum RE of E2P was around 70% and it was achieved after 15 days of exposure, while the maximum RE of M2P was 100% and it was achieved after 24 days of exposure. Furthermore, in this last stage (VIII) without ethanol in the feed, a partial degranulation of the sludge bed was observed, which could suggest, among other things, that ethanol is a necessary substrate to maintain the granular structure of the sludge. Extracellular polymeric substances (EPS) content and the microbial community of both kind of final sludges (granulated and degranulated) were

studied, revealing that the microbial community of both sludges was almost identical, which indicate that fast changes in the physico-chemical properties of the granular sludge did not cause any change in the structure of the microbial populations. However, the EPS content analysis showed a lower concentration of protein and polysaccharide in the degranulated biomass than in the granulated one, which indicates that the loss of these compounds should be related to the degranulation process. Additionally, specific methanogenic activity (SMA) of each sludge was determined using as substrate both only ethanol or a mixture of ethanol and glycol ethers. Results of SMA analysis demonstrated that: 1) an adaptation period to degrade glycol ethers is needed; and 2) the anaerobic degradation of ethanol carried out by the granular sludge was faster than obtained with the degranulated sludge.

This study, included in Chapter 6, has been prepared to be sent for its publication to the journal Applied Microbiology and Biotechnology (Ferrero et al. 2018. Behaviour, stability, and microbial community analysis of EGSB reactor at hihg content of glicol ether solvents in mixtures with etanol. Appl. Microbiol. Biotechnol, ready to be submitted).

In the last part of this document, a comparison of the microbial community structure of laboratory reactors and a prototype scale reactor has been shown. For this purpose, samples of biomass of an industrial prototype reactor installed and operated in a printing facility were collected, analysed and compared with those obtained in a laboratory control reactor. The on-site prototype reactor treated mainly ethanol, ethyl acetate and E2P. Typical fluctuations in the OLR derived from changes in the production process of the facility were observed. In spite of these fluctuations stable performance of the reactor was achieved (average RE of 93%) and volatile fatty acids were only accumulated when the organic load was higher than 3 kg COD h^{-1}, which suggested a limit OLR that can be used for assuring the stable operation of the reactor. The microbial community structure was analysed through the DGGE technique and it showed an evolution during the first months of operation in the domains *Archaea* and *Bacteria*. Taking into account that the initial inoculum was obtained from a brewery treatment plant, the limitation of the carbon source to only a few organic solvents inherent to the flexographic emissions was suggested to be the cause of this shift. Archaeal populations were the most affected by this change in the carbon source, resulting in a diminution of the biodiversity as the Shannon index showed (from 1.07 to 0.41 in the first 123 days of experiment). *Methanosaeta* was the dominant microorganism in this domain, and its dominance persist during all the experimental period. The proportion of the archaea *Methanospirillum* and *Methanobacterium* increased along the experimental period,

which was related to variations in the temperature and in the load, respectively. Regarding the domain of *Bacteria*, species from *Geobacter* and *Pelobacter genera,* which are microorganisms specialised in the ethanol degradation, were the predominant microorganisms. Besides, *Methanosaeta* and *Geobacter* are syntrophic partners able to use direct interspecific electron transference for methane production, so the predominance of these species indicated that this phenomenon occurred in the prototype reactor. Comparing laboratory and industrial prototype reactors, both performed similarly with high RE (>90%), showed a similar adaptation period for E2P degradation, and the only difference was the limit value for the organic load associated to the volatile fatty acid accumulation. The microbial community structure showed similar trends in both reactors, with an evolution from the initial sludge during the beginning of the operation in *Archaea* and in *Bacteria*. A stable community in the domain of *Archaea* was finally obtained with the predominance of *Methanosaeta*. In the domain of *Bacteria* the predominance of the microorganisms showed a dependence on the used substrate.

These results have been published in the Journal of Environmental Management (Bravo, D., Ferrero, P., Penya-roja, J.M., Álvarez-Hornos, F.J., Gabaldón, C., 2017. Control of VOCs from printing press air emissions by anaerobic bioscrubber : Performance and microbial community of an on-site pilot unit. J. Environ. Manage. 197, 287-295).

TABLE OF CONTENTS

1 THESIS DOCUMENT STRUCTURE

The document of this PhD Thesis has been structured into 12 chapters. The present chapter, Chapter 1, is a brief guide where the structure of the thesis document is described. In Chapter 2 a general introduction to the thematic of the thesis is presented and the issue of the emissions of volatile organic compounds as well as their importance in the flexographic industry and the possible ways to control and prevent these emissions is briefly exposed. Finally, the techniques for microbial identification that can be used in biological treatments are also described. Then in the Chapter 3 the general and the specific objectives are presented.

The following 4 chapters (Chapters 4, 5, 6 and 7) comprise the core part of this thesis, where the research studies carried out can be found. Every one of these chapters represents an adaptation of an original manuscript which has already been published or has been proposed to be submitted for its publication. Every chapter has been individually structured as a research paper journal publication, including all the characteristic items such as abstract, introduction, materials and methods, results and discussion, conclusions and references. In this way, each of these chapters could be read and understood separately.

Chapter 4 is an adaptation of the original manuscript that was finally published in Journal of Environmental Management (Ferrero, P., San-Valero, P., Gabaldón, C., Martínez-Soria, V., Penya-roja, J.M., 2018. Anaerobic degradation of glycol ether-ethanol mixtures using EGSB and hybrid reactors: Performance comparison and ether cleavage pathway. J. Environ. Manage. 213, 159–167).

Chapter 5 has been generated from the pre-review manuscript that has been sent for its publication in Bioresource Technology (Ferrero et al., 2018. Link between the anaerobic degradation of glycol ether-ethanol mixtures using EGSB and hybrid reactors and the dynamics of the microbial community structure, Bioresour. Technol. Submitted for decision).

Chapter 6 is the former version of the manuscript that has been prepared to be sent for its publication in Applied Microbiology and Technology (Ferrero et al. 2018. Behaviour, stability, and microbial community analysis of EGSB reactor at hihg content of glicol ether solvents in mixtures with etanol. Appl. Microbiol. Biotechnol, ready to be submitted).

Chapter 7 is an adaptation of the original manuscript that was finally published in Journal of Environmental Managment (Bravo, D., Ferrero, P., Penya-roja, J.M., Álvarez-Hornos, F.J., Gabaldón, C., 2017. Control of VOCs from printing press air emissions by anaerobic bioscrubber : Performance and microbial community of an on-site pilot unit. J. Environ. Manage. 197, 287-295.) Only the part

of this paper which is involved in the original work carried out by the author of this PhD thesis has been discussed in this document.

In case of already published papers, permission for the use in this document of published material have been obtained from the Editor of the Journal.

After that, the general conclusions of the thesis are presented together with the future perspectives of this research field in the Chapter 8. Then, the list of figures (Chapter 9) and tables (Chapter 10), and a glossary of abbreviation and terms (Chapter 11) for a better understanding of the reader can be found.

Finally, in the last chapter (Chapter 12), in order to meet the regulations of the University of Valencia an extended summary of the PhD thesis, written in Spanish, has been included as an appendix (Apéndice A: Resumen extendido). Additionally, the original published papers can be found in this Chapter (Appendix B)

2 INTRODUCTION

2.1 VOLATILE ORGANIC COMPOUND EMISSIONS AND THEIR ENVIRONMENTAL CONCERNS IN THE FLEXOGRAPHIC INDUSTRY

Volatile organic compounds (VOCs) have been defined as "any organic compound as well as the fraction of creosote, having at 293.15 K a vapor pressure of 0.01 kPa or more, or having a corresponding volatility under the particular conditions of use" (Council Directive 2010/75/EU). VOCs emission can be linked to the use of solvents in several activities and sectors, such as, road transport or the agricultural sector, but the industrial processes contribute a far greater share of these emissions (EEA, 2016). Surface treatment activities using solvents, and particularly the flexographic industry, are among the largest sources of non-methanogenic volatile organic compound (NMVOC) emissions in Spain, exceeding 13000 Tn in 2016 (PRTR-2016).

Inks based on solvents are utilized in the flexographic industry, and are characterized by its rapid drying. One of the most common solvents in the flexography is ethanol, which acts as cleaner agent and as solvent for the inks. Ethyl acetate, with those same properties, is also frequently used. Other organic solvents as isopropanol, n-propanol and glycol ethers as 1-ethoxy-2-propanol (E2P) and 1-methoxy-2-propanol (M2P) can also be found in the inks, but in lower quantity (EC, 2007). Glycol ethers can also act as retarder agents. The use of these compounds in the productive processes provides their presence in the atmospheric emissions of the flexographic industry, as can be observed in the Table 2.1, where the emission percentage of each solvent is shown.

Table 2.1. Typical composition of emissions in flexographic industries. (Passant, 2002)

Solvent	weight% of NMVOC
1-propanol	8
2-propyl acetate	4
2-propanol	5
Ethanol	60
Ethyl acetate	12
Propyl acetate	3
1-methoxy-2-propanol	4
1-ethoxy-2-propanol	4

All these compounds are primary pollutants, which present diverse negative effects in the environment and in the public health. Furthermore, they are also responsible for the development of secondary pollutants as the tropospheric ozone, because these compounds can affect the equilibrium between the nitrogen oxides and the oxygen in the Champan cycle, thus generating ozone at tropospheric level. As a result, in the European Union and other geographic zones as USA, the industrial atmospheric emissions of these type of compounds has been regulated and limited (Council Directive 2010/75/EU and USEPA 1978, 2006).

To fulfill regulatory requirements, control techniques for VOCs emissions must be applied. Before using end-pipe control techniques, the potential for implementing other techniques as substitution, reduction and the recovery of the compounds should also be evaluated. However, the achievement of these preventive techniques is usually not enough to meet the current regulatory specifications. Therefore, the industrial facilities should resort to control technologies, as thermal or catalytic oxidation, adsorption, condensation and biological treatments. The selection between the different control technologies depends on the characteristics of the emission such as air flowrate and pollutant concentration. Figure 2.1 shows the recommended technique in function of these characteristics.

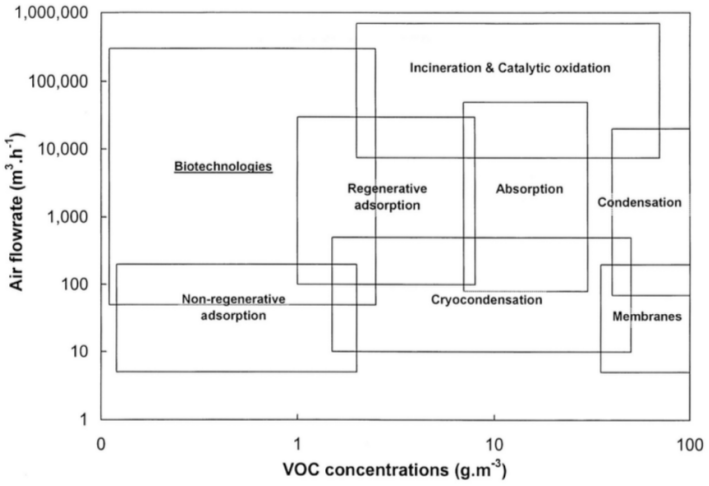

Figure 2.1. Recommended end-of-pipe technologies for VOC treatment as a function of pollutant concentration and air flowrate. (Adapted from Singh et al., (2005)).

Emissions from the flexographic industry are characterized by a high flowrate and low pollutant concentration (Sempere et al., 2012), so taking into account the most favorable operational conditions of each technology (Figure 2.1), biological treatments are considered as one of the optimal available technology. In the biological treatments we can distinguish between biofilters, biotrickling filters and bioscrubbers. In these last systems, the pollutant has to be transferred from the gas phase to the liquid phase in an initial stage, and then the pollutants can be transported to the biofilm developed in the packing material that is located inside the bioreactor. Later on, the pollutants are biologically removed by the microorganisms developed in the biofilm. The bioscrubbers, as can be observed in Figure 2.2, have two operational units, the first one is an absorption column (scrubber) where the transference of the pollutant from the air to the water is carried out, and the second one is a bioreactor where the biological degradation occurred.

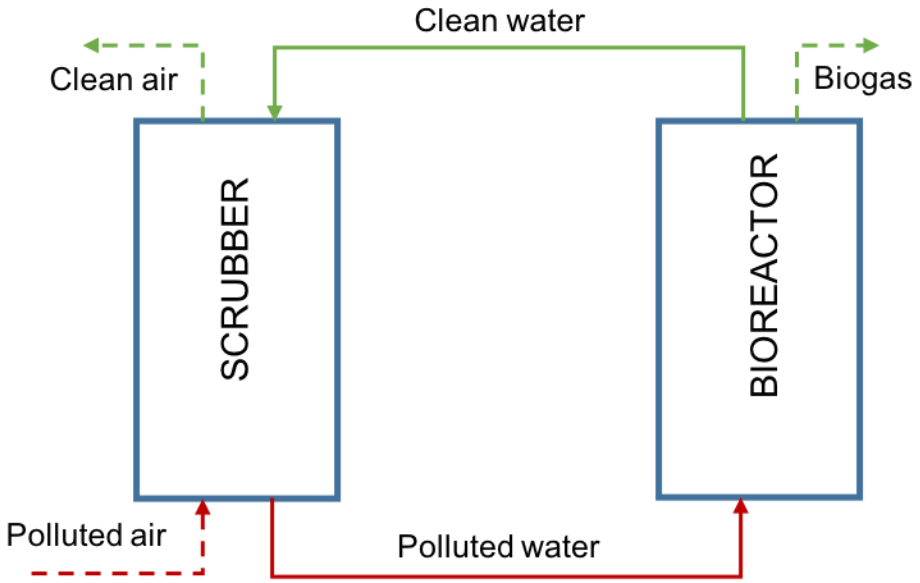

Figure 2.2. Bioscrubber scheme.

Therefore, as bioscrubber usually comprise two units, and furthermore conventional aerobic degradation needs a process of aeration to carry out the degradation of pollutants, higher operational costs than the other biological treatments are needed. So, the implementation of biofilters and biotrickling filters has currently predominated in the industry field. In this regard, during the last years a new alternative, based on an anaerobic bioscrubber which overcomes these

disadvantages, has been developed by the GI^{2}AM research group in collaboration with Pure Air Solutions B.V. (Waalkens et al., 2015). In this case, the degradation of pollutants is produced in an anaerobic bioreactor, and the need of aeration of conventional bioscrubbers has been eliminated. Additionally, this technology has a great advantage, due to the fact that in the anaerobic degradation the pollutants are biotransformed into a valuable product, as biogas, which can be useful in thermal and/or electrical applications, thereby resulting in a positive net energy balance and contributing to the circular economy that promotes the European Union.

2.2 ANAEROBIC PROCESSES

Anaerobic treatments are technically simple and relatively inexpensive technologies which consume less energy, space and produce less excess of sludge in comparison to the conventional aerobic treatment technologies. Net energy production from biogas makes the anaerobic treatment technology an attractive option over other treatment methods (Erssahin et al., 2011; van Lier, 2008).

The next sections intend both to cover brief essential information on the fundamentals of anaerobic technology and to describe the reactor configurations that can be used in the anaerobic bioscrubber.

2.2.1 Fundamentals of anaerobic degradation

The anaerobic degradation can be defined as the process by which complex microbial communities break down organic matter in absence of electron acceptors as oxygen or nitrate, resulting in two main final products: biogas (mainly CH_4 and CO_2) and sludge. In this process different stages can be distinguished depending on the generated products in each stage. Figure 2.3 shows each stage of the anaerobic process with the generated products. A brief description of each stage can be found below.

Hydrolysis

The first stage that takes place is the hydrolysis, where the complex substrates as carbohydrates, proteins and lipids are transformed by enzymes into simpler compounds as sugars, amino acids, and long chain fatty acids. The velocity of the hydrolysis is affected by parameters such as temperature (Mahmoud et al.,

2004), pH (Chen et al., 2014), cellular retention time (CRT) and the nature and type of substrate (Pavlostathis and Giraldo-Gomez, 1991). For the anaerobic degradation of complex substrates this stage is usually the limiting step (Miron et al., 2000).

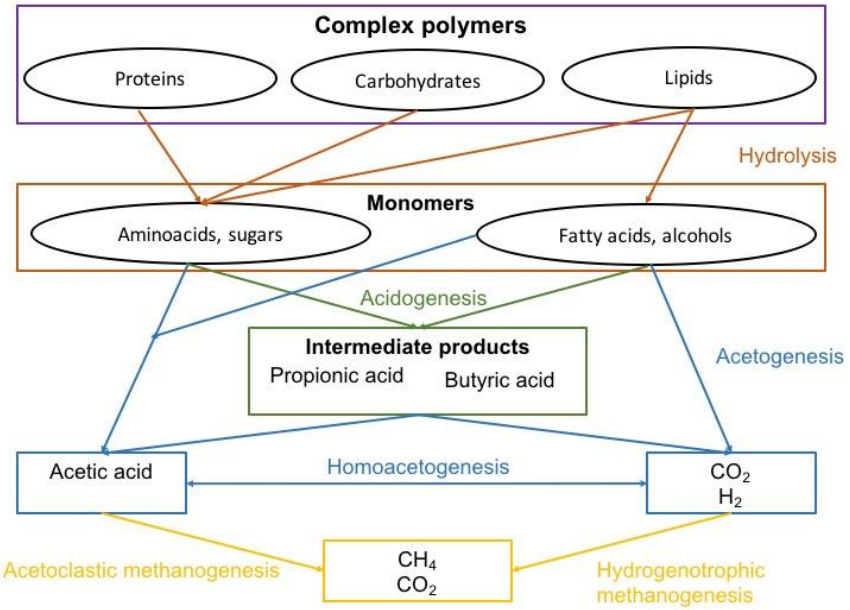

Figure 2.3. Stages of anaerobic degradation and generated products.

Acidogenesis

In this stage acidogenic bacteria transform the products generated in the previous stage in more simple products such as short chain volatile fatty acids as the acetic acid, propionic acid or butyric acid. Some byproducts as alcohols, ammonium, carbon dioxide and hydrogen can also be generated. The bacteria involved in this stage are also involved in the previous stage, as these bacteria are the producers of extracellular hydrolytic enzymes. Besides, the predominant acidogenic bacteria belong to the phyla *Clostridia*, *Bacili*, *Bacteroidetes* and *Actinobacteria* (Krause et al., 2008).

Acetogenesis

During the acetogenesis step the products generated in the acidogenesis stage are converted into acetic acid, carbon dioxide and hydrogen. These compounds can be directly converted to methane and carbon dioxide in the last stage of the anaerobic degradation. Besides, there is also the feasibility of producing acetic acid through the process denominated homoacetogenesis. This process

consists in the formation of acetic acid from hydrogen and carbon dioxide. Most of the microorganisms involved in the acetogenesis are syntrophic microorganisms. Bacteria belonging to the *Clostridia* class and the *Syntrophomonas* genera, *Clostridium* (Kallistova et al., 2014), *Acetobacterium* (Balch et al., 1977), *Deltaproteobacteria*, *Syntrophobacter*, *Geobacter* or *Pelobacter* have been described as responsible microorganisms for acetogenesis processes (Nevin et al., 2005; Schink et al., 1987).

Methanogenesis

This is the last stage of the process of the anaerobic degradation, where the formation of methane from the products generated in the previous stages takes place. This stage is carried out by methanogenic archaea, which can be distinguished depending on its metabolism associated to the methane production. On the one hand, we can find the acetoclastic methanogenic archaea which are the archaea that use acetic acid as substrate for the methane formation. In this group there are two main genera, *Methanosaeta* (Patel and Sprott, 1990) and *Methanosarcina* (Sowers et al., 1984). Besides, these genera have different maximum growth rates and different affinity for the substrate, *Methanosarcina* shows a higher maximum growth rate while *Methanosaeta* has a higher affinity for the acetic acid (Huser et al., 1982; Schönheit et al., 1982). The reactor conditions such as the substrate concentration (acetic acid) determine the predominance of one or other genera inside the reactor (Jetten et al., 1990). On the other hand, we can also find the hydrogenotrophic archaea, which are the archaea that use hydrogen and carbon dioxide as substrates for the methane formation. In this group we can find species belonging to the genera *Methanobacterium* (Cadillo-Quiroz et al., 2014; Mori and Harayama, 2011), *Methanomicrobium* or *Methanospirillum* (Iino et al., 2010). Even though methane production is mainly carried out by acetoclastic and hydrogenotrophic pathways, there are some archaea able to produce methane from other substrates, for instance, species from the genus *Methanomethylovorans* are able to produce methane from methanol (Cha et al., 2013), or some species from the genus *Methanospirillum* are able to use secondary alcohols as 2-propanol and 2-butanol, which are used as hydrogen donors (Zellner and Winter, 1987).

2.2.2 Anaerobic degradation of solvents

As the previous subchapter describes (Section 2.2.1), the anaerobic degradation may involve several processes depending on the compounds degraded and generated by the microorganisms in each stage. The flexographic industry emissions are composed basically by organic solvents (Table 2.1), which do not

usually present a complex structure that needs stages as hydrolysis. Taking into account that most of these solvents are alcohols, ethers or glycol ethers, only acetogenesis and methanogenesis stages are required in their anaerobic degradation. Below, a brief description of state-of-the-art of the knowledge about the anaerobic degradation of some of the most usual solvents in the emission of the flexographic industry and/or of interest in this work is provided.

Anaerobic degradation of ethanol

The anaerobic degradation of ethanol is well documented in the literature. It has been shown that the anaerobic degradation of ethanol has to be done syntrophically (Bryant et al., 1967). The reaction of production of acetic acid from ethanol (Equation 1.1) at standard conditions has a Gibbs free energy positive (+17 kJ mol^{-1} of ethanol) (Thauer et al., 1977). As can be seen, in this reaction hydrogen is also produced (Equation 2.1). This compound can be used by the hydrogenotrophic methanogens, finally obtaining methane (Equation 2.2). This last reaction has a Gibbs free energy at standard conditions of -131 kJ mol^{-1} of methane, so the Gibbs free energy for the whole process (Equation 2.3) is -112 kJ mol^{-1} of ethanol (Thauer et al., 1977), and therefore the process is favorable energetically, but is important to highlight that the acetogenic bacteria are not able to grow with ethanol in absence of their hydrogenotrophic partner at standard conditions, and the first reaction can occur only if the partial pressure of hydrogen remains low enough for the hydrogenotrophic methanogens. So, the anaerobic degradation of ethanol is linked to the presence of these hydrogenotrophic archaea.

$$2\,CH_3CH_2OH + 2\,H_2O \rightarrow 2\,CH_3COO^- + 2\,H^+ + 4\,H_2 \quad (2.1)$$

$$4\,H_2 + \,CO_2 \rightarrow CH_4 + 2\,H_2O \quad (2.2)$$

$$2\,CH_3CH_2OH + CO_2 \rightarrow \,2\,CH_3COO^- + \,2\,H^+ + \,CH_4 \quad (2.3)$$

A large number of enzymes such as the ethanol dehydrogenase, acetaldehyde ferredoxin oxidoreductase, phosphotransacetylase and the acetate kinase, are involved in the reaction of oxidation of ethanol to acetate, giving a performance of a molecule of ATP per molecule of oxidized ethanol through phosphorylation at substrate level (Schink, 1997).

Anaerobic degradation of glycol ethers

Even though the mechanisms of anaerobic degradation of glycol ethers such as M2P and E2P have not been completely determined, some studies about the anaerobic degradation of these compounds can be found (Lafita et al., 2015; Vermorel, 2017; Torres et al., 2018). Although the mechanism for the cleavage of the ether bond of this type of compounds has not been identified with certainty, the postulation with wider acceptation for the cleavage of these compounds include a double H/OH interchange (*trans*hydroxylation), giving place to a *gem*-diol that rapidly collapse to a cetonic group or to a carbonyl group (Speranza et al., 2002). Lafita et al. (2015) proposed this mechanism by analogy for the anaerobic degradation of 1-methoxy-2-propanol, leading in this case to methanol and acetone or propionaldehyde as intermediate products. While in the case of the degradation of 1-ethoxy-2-propanol their intermediates products should be ethanol and acetone or propionaldehyde. Even though the enzymes involved in the process of the anaerobic degradation of glycol ethers are not exactly known, some authors have identified the diol dehydratase enzyme as the responsible to carry out the cleavage of this type of bonds (Schramm and Schink, 1991; Speranza et al., 2002).

Anaerobic degradation of acetone

The anaerobic degradation of acetone has been widely studied, as occurs with the degradation of ethanol. Mazé (1915) was the first to observe the anaerobic degradation of this compound, which was confirmed several years later by Symons and Buswell (1933). In presence of oxygen, acetone is hydroxylated by oxigenases to an acetol and later oxidized through metylglioxal and pyruvate (Taylor et al., 1980). However, in absence of oxygen the degradation of this compound is completely different. Basically, there are three possible pathways for the anaerobic degradation of acetone: 1) the methanogenic archaea could directly use acetone (Equation 2.4); 2) one bacterium ferments the acetone to carbon dioxide and hydrogen (Equation 2.5) or to acetate, carbon dioxide and hydrogen (Equation 2.6); or 3) fermentative bacteria could produce only acetate from acetone in a coculture with an acetoclastic methanogenic archaea (Equation 2.7).

$$CH_3COCH_3 + H_2O \rightarrow 2\,CH_4 + CO_2 \quad (2.4)$$

$$CH_3COCH_3 + 5\,H_2O \rightarrow 3\,CO_2 + 8\,H_2 \quad (2.5)$$

$$CH_3COCH_3 + 3\,H_2O \rightarrow CH_3COO^- + H^+ + CO_2 + 4\,H_2 \quad (2.6)$$

$$CH_3COCH_3 + CO_2 + H_2O \rightarrow 2\,CH_3COO^- + 2\,H^+ \quad (2.7)$$

Platen and Schink (1989) showed that the anaerobic degradation of acetone can be carried out in a coculture between a fermentive bacterium and an acetoclastic methanogenic archaea as *Methanotrix*, also known as *Methanosaeta*. Besides, these authors showed through radioactively labelled carbon that the first step for the anaerobic degradation of acetone is a carboxylation (Equation 2.8) that has a Gibbs free energy positive, so this carboxylation should be coupled to the production of methane from acetate.

$$CH_3COCH_3 + CO_2 \rightarrow CH_3COCH_2COO^- + H^+ \quad (2.8)$$

Anaerobic degradation of isopropanol

The anaerobic degradation of isopropanol (also called 2-propanol) has also been studied by different authors (Widdel, 1986; Zellner and Winter, 1987). In these studies it was observed that isopropanol can be degraded anaerobically by some methanogenic populations that are able to use this compound as hydrogen donor (deshydrogenation of isopropanol), which can be coupled to the reduction of carbon dioxide, producing finally methane and acetone. This has also been observed in other study where a continuous stirred tank reactor (CSTR) was used in order to treat a mixture of ethanol and isopropanol (Vermorel et al., 2017). Authors observed that after the introduction of isopropanol as substrate, acetone was detected in the effluent of the reactor as an intermediate compound of degradation of isopropanol. This process can be reverted in determined conditions, for instance, in presence of high concentration of hydrogen, forming again isopropanol from acetone. Then, acetone is degraded anaerobically as it has been described previously. So, there are different microbial populations involved in the anaerobic degradation of isopropanol. First, the methanogenic populations, which are able to use isopropanol as hydrogen donor in the methanogenesis stage. Second, the fermentative bacteria of the acetone that is produced in the reaction of deshydrogenation of isopropanol. Third, the hydrogenotrophic methanogens that couple the process of acetone oxidation to acetic acid, and finally the acetoclastic methanogens that use the acetic acid produced in the whole process to produce methane. Nevertheless, not all the hydrogenotrophic methanoges are able to use isopropanol as hydrogen donor, and only a few species have been described in the literature as competent to carry out this reaction as *Methanobacterium formicum*, *Methanobacterium bryantii*, *Methanogenium marisnigri*, *Methanomicrobium paynteri*, *Methanocorpusculum parvum*, *Methanocullus chikugoensis* (Zellner and Winter, 1987) y *Methanocullus palmolei* (Tonouchi, 2004). Furthermore, Ince et al. (2011) showed that isopropanol has negative effects in the acetoclastic methanogenic populations as *Methanosaeta* species, revealing a negative impact at concentrations higher than 0.1 M in the level

of the gene expression of the acetyl-CoA synthetase, a key enzyme in the methane production in these species. Therefore, the presence of methanogens able to use isopropanol as hydrogen donor is needed for its complete degradation since in this way it is possible to obtain low levels of isopropanol concentration.

Anaerobic degradation of methanol

The anaerobic degradation of methanol has been also widely studied. Due to the different possible pathways for the degradation of this compound, some authors have been defined its degradation as unique. In the Figure 2.4 the different pathways that can follow the anaerobic degradation of methanol can be observed.

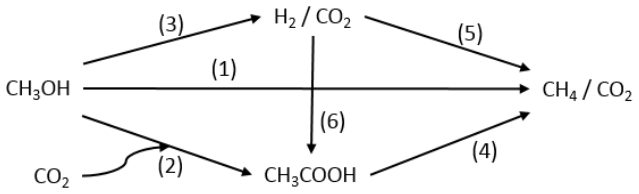

Figure 2.4. Scheme of the possible pathways of the anaerobic degradation of methanol.

Methanol can be converted directly in methane by some methanogenic populations named methylotrophic as *Methanosarcina barkerii* (Smith and Mah, 1978; Weimer and Zeikus, 1978) ((1) in the Figure 2.4). Other option is that methanol could be transformed by acetogenic bacteria to acetic acid (Adamse and Velzeboer, 1982; van der Meijden et al., 1984) ((2) in the Figure 2.4) and then the acetic acid would be used by acetoclastic methanogens ((4) in theFigure 2.4). Methanol can be also converted to hydrogen and carbon dioxide (Cord-Ruwisch and Ollivier, 1986) ((3) in the Figure 2.4), and these compounds can be directly utilized by hydrogenotrophic methanogens ((5) in the Figure 2.4) or can be used by homoacetogens bacteria ((6) in the Figure 2.4) and to form acetic acid, which later can be used in the methanogenesis by acetotrophic methanogens. In the literature a large number of anaerobic reactors have been described treating satisfactorily wastewater that contained methanol (Cerrillo et al., 2016; Lu et al., 2015). Kobayashi et al. (2011) showed that treating an industrial wastewater containing methanol at concentrations of 10 g COD L^{-1} in an UASB reactor, a degranulation process of the sludge bed occurred, while when the UASB reactor treated a mixture of methanol and starch the granule integrity of the sludge bed was maintained, revealing the addition of another source of carbon as the starch a key factor for the stability of the process. In this regard, Nishio et al. (1993) also observed that the

granulation of the sludge bed was not produced when wastewater containing methanol as sole source of carbon was treated, but the opposite was produced when acetate was added to the feed.

However, there are no studies in the literature about the anaerobic degradation of mixtures of glycol ethers, such as E2P and M2P, and the possible interaction or influence in the proportion of each one of the solvents in the degradation of each solvent and in the performance and stability of the process.

2.2.3 Types of high rate anaerobic reactors

In the anaerobic bioscrubber, high rate anaerobic reactors are used. Because of the special incidence that anaerobic bioscrubber have both on the objectives and on the development of this thesis, a brief description of the different high rate reactor configurations that can be used in the anaerobic bioscrubber is required.

These bioreactors are based on the use of the granular biomass that has a greater density than the suspended biomass, which allows a much quicker and more effective separation by settling. So, the hydraulic residence time (HRT) and the cellular retention time (CRT) can be decoupled, enabling these reactors to operate with relatively low HRTs and much higher CRTs (van Lier et al., 2015). Depending on the reactor characteristics, such as to whether or not there is an effluent recirculation, the upflow velocity or the configuration of the gas-liquid-solid (GLS) separator, the values of these retention/residence times can vary widely (Grady et. al 2011). These types of bioreactors were developed in the 1970s by Lettinga and his coworkers (Lettinga, 2014). During that decade some of these reactors were installed, but not much attention was paid to them. However, nowadays there is a great number of this type of reactors implemented across the world. In fact, in 2001 there were 1215 of this type of anaerobic reactors in operation (Frankin, 2001), mostly treating wastewater from diverse industries as for instance, brewery, distillery, textile and paper mill (Lim and Kim, 2014). The different types of anaerobic reactors have different properties, with their advantages and disadvantages, which are described in the next section.

2.2.3.1 *Upflow Anaerobic Sludge Bed (UASB) reactor*

The upflow anaerobic sludge bed (UASB) reactor was developed in the Netherlands, thanks to the observations that were made years before about the granulation of biomass in an anaerobic filtration system. The first UASB reactor was utilized for the treatment of an industrial wastewater of sugar beet in the

Netherlands (Lettinga et al., 1980). Since then, this reactor technology has proved able to treat very different industrial effluents, even those that can contain toxic and/or inhibitory compounds (Lettinga, 1995).

In these reactors the influent is fed by the bottom part of the reactor and ascends through the sludge bed that is placed inside the reactor. The ratio height/width range from 0.2 to 0.5. A GLS separator is located in the upper part of the reactor, allowing a proper retention of the biomass inside the reactor. The biogas and the treated liquid effluent leave the reactor by the upper part. Besides, these reactors also have a flow distribution system which favors an uniform dispersion of the wastewater, because the typical upflow velocities that can reach these reactors are not very high (from 1 to 3 m h^{-1}) (Lettinga and Hulshoff Pol, 1991).

In addition, it should be recalled that the biomass used in this reactor is granular with spherical or lenticular shapes, and presents higher density than water. These characteristics are very useful, facilitating its separation from the effluent and avoiding the need for an additional clarifier or a final settler. This kind of reactors can be used for the treatment of high organic loading rates (OLR), and are usually designed to treat OLR between 10 and 15 kg COD m^{-3} d^{-1} (Lim and Kim, 2014), although it has been shown that is possible to treat higher OLR (Torres et al., 2018).

The principal advantages of these reactors is related to the production of biogas with a high content in methane, which can be use to generate energy and as a consequence optimizing operational costs (van Lier, 2008). Another advantage is that they can be operate at room temperature and therefore there is no need to use auxiliary streams, which reduce investment costs and simplify its implementation. Furthermore, they are less sensitive to fluctuations of environmental parameters, such as acidity, and the sludge production can be reduced up to 8 times in comparison with aerobic processes, thus reducing the treatment and management costs associated to the excess of sludge (Kato, 1994). Nevertheless, these reactors has also some drawbacks and limitations as the relative long start-up periods because of the need to form granular biomass. Even though new techniques, as chitosan addition, has been developed to accelerate the formation of granules, a period ranging from 3 to 8 months is required to achieve the start-up (Lim and Kim, 2014). Even if the reactor is inoculated with granular sludge biomass, an acclimatization period to the new substrates is need, and not high OLR are recommended in the early days of the operation. A diagram of an UASB reactor can be observed in the Figure 2.5.

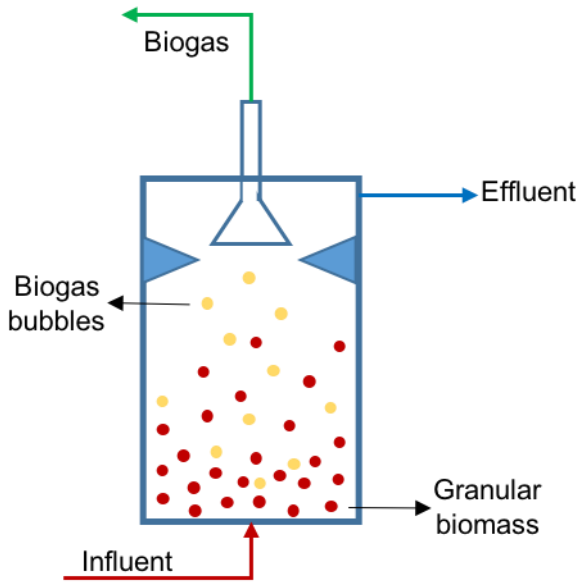

Figure 2.5. Squematic diagram of an UASB reactor.

2.2.3.2 *Expanded Granular Sludge Bed (EGSB) reactor*

The expanded granular sludge bed (EGSB) reactor is quite similar to the UASB reactor, which has been described previously (Section 2.2.3.1). The main difference between both types of reactors is the greater grade of expansion of the sludge bed in the EGSB reactor. This greater expansion is produced by the higher hydraulic rate applied to the system that provokes also higher upflow velocities of the liquid in the EGSB reactor. These facts intensify the hydraulic mixture, which allows a better contact between the biomass and the substrate (Lim and Kim, 2014). The range of upflow velocities utilized in the EGSB reactors varied from 5 to 10 m h^{-1}, these high upflow velocities are achieved through the use of both high height/width ratios (around 20) and a recirculation stream from the effluent (Kato, 1994; Lettinga, 1995).

In the Figure 2.6 a scheme of an EGSB reactor is shown. In these reactors, as in the UASB reactors, the influent is introduced by the bottom of the reactor with the recirculated effluent. In this way, both high up flow velocities and efficient hydraulic mixture are achieved, and this, combined with the produced biogas, provokes the expansion of the sludge bed. In the upper part of the reactor the GLS separator is placed. In this part the produced biogas is collected, and the treated water is discharged out throughout the outlet/effluent.

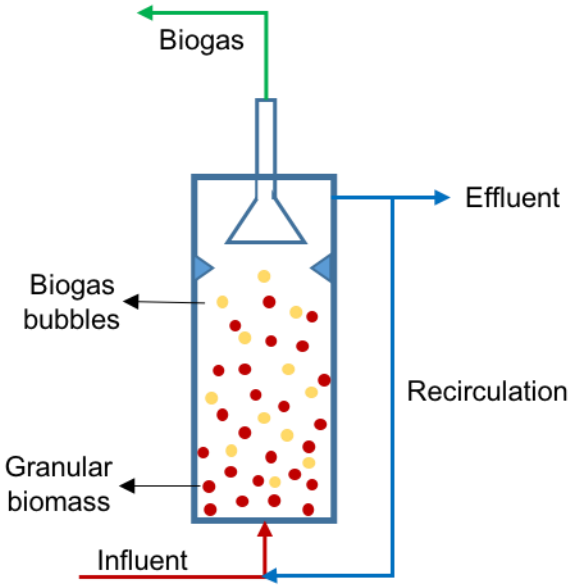

Figure 2.6. Scheme of an expanded granular sludge bed (EGSB) reactor.

With regard to the implementation of these reactors, because the high upflow velocities of the liquid inside, these reactors do not allow the removal of particulate organic matter; and only the treatment of soluble organic matter is advisable. Besides, the excessive presence of suspended solids in the influent can deteriorate the granular sludge inside the reactor (Chernicharo, 2007).

In addition, and as consequence of the high upflow velocities used, these reactors are tall and narrow which results in a significantly small footprint for their installation. In contrast, the use of high upflow velocities involves that biomass cannot be efficiently retained inside the reactor and can be lost through the effluent (Dries et al., 1998). Several technical solutions have been studied to improve the biomass retention in this type of reactors. One of them consists in the reconfiguration (hybridation) of the anaerobic reactor through the introduction of modifications in the GLS separation.

2.2.3.3 *Anaerobic Hybrid Reactor (AHR)*

The first anaerobic hybrid reactor (AHR), developed to improve the biomass retention in an UASB reactor, was described by Guiot and van den Berg (1985). They modified the UASB reactor, which was used to treat synthetic wastewater containing sugar, replaced the conventional GLS separator by an anaerobic filter in

the upper part of the reactor. The results showed that the anaerobic filter provides a notably improvement in the biomass retention and the solution was not too much expensive.

In addition to the improvement in the biomass retention, these reactors offer other advantages. So, Shivayogimath and Ramanujam (1999) observed that these reactors showed a final polishing effect in the effluent (thus improving its performance) due to the accumulated biomass in the filter. McHugh et al. (2006) observed that these reactors had a beneficial buffering effect against OLR shocks. Due to these advantages, the AHR is presented as an alternative of improvement in the treatment of wastewaters from different industries, in particular in situations with high OLR.

In this regard, Shivayogimath and Ramanujam (1999) showed that this type of reactors were able to treat a wastewater from a distillery with OLR as high as 36 kg COD m^{-3} d^{-1}. Their efficiency and high performance (>90%) was also proved in the treatment of effluents from the dairy industry (Belançon et al., 2010). McKeown et al. (2009) showed that the AHR was able to carry out the treatment of acidified wastewaters at low temperatures, while Oktem et al. (2008) revealed that this type of reactors showed great stability against changes in the OLR.

Taking into account the advantges and characteristics outlined above, the AHR configuration could improve the biomass retention in the EGSB reactor in the anaerobic bioscrubber, so the production of bioenergy in form of biogas could be potentially improved by the use of these reconfigurated reactors.

2.3 TECHNIQUES FOR MICROBIAL IDENTIFICATION

For a long time, bioreactors have been operated and studied like they would be a black box, measuring their inputs and their outputs, without analyze, study and understand what is happening inside the reactors. However, to optimize these systems results necessary to have a better understanding of them and how the variations and parameters can affect to the predominant microorganisms. At the beginning some conventional microbiological techniques were used, based on the isolation of pure cultures and morphologic, metabolic and genetic assays that gave a great information about the microbial communities that exist in these engineered systems (Bitton, 2005). Nevertheless, these techniques have some inherent inconvenients as an incomplete knowledge about the physiological requirements, both nutritionals and physical-chemical, and about the syntrophic and symbiotic relationships that are frequently found in the nature. So this incomplete knowledge

makes no possible to achive pure cultures of most of the microorganisms present in the nature (Su et al., 2012). Therefore, if we restrict ourselves only to the use of culture-depending techniques our understanding about the microbial ecology and the physiology associated with the anaerobic digestion would be incomplete and biased (Vanwonterghem et al., 2014).

However, during the last decade the use of culture-independent techniques for microbial identification in anaerobic processes have been provided a new sight in the community composition and in the function of the predominant populations (Vanwonterghem et al., 2014). These techniques have improved the knowledge of how the feed or substrate, the reactor configuration and the operational conditions can affect to the structure of the microbial community and its link with the global performance and the stability of the process (Ho et al., 2013; Nelson et al., 2011; Werner et al., 2011). Besides, these techniques have also shown that the anaerobic digesters are typically dominated by microorganisms previously uncharacterizated (Sundberg et al., 2013). Many techniques culture-independent are based in the analysis of conserved genes, being the 16S rRNA gene the most widely utilized and with the most extense database (Su et al., 2012). Through studies of the 16S gene a functional sight of the process of anaerobic digestion can be inferred through the search of closely related cultured species (Li et al., 2009; Ziganshin et al., 2013), but this has to be done carefully because some close microorganisms can be functionally differents (Ghoshal et al., 2004). Below, a description of some techniques base on the analysis of the 16S gene is shown.

2.3.1 Denaturing Gradient Gel Electrophoresis (DGGE)

The increment of the popularity of the denaturing gradient gel electrophoresis (DGGE) technique was reflected in the increment of the number of studies where this technique has been used. This technique is based on the different mobility of DNA fragments with the same size, but different sequence in their nucleic acids, in a gel with denaturing conditions. This generates a fingerprint with bands that reflects directly the genetic biodiversity of the sample. The number of bands corresponds to the number of predominant species that are present in the reactor. This technique coupled to the sequencing and phylogenetic analysis of the bands could give significant information about the composition of the microbial communities. In the Figure 2.7 a scheme of the methodology used in the DGGE is shown.

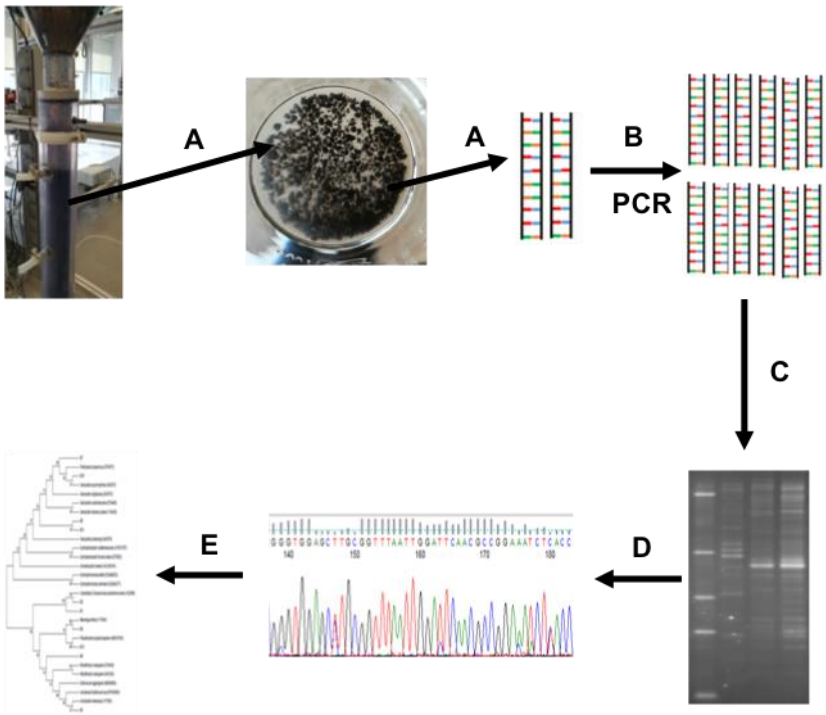

Figure 2.7. Squeme of the methodology used for microbial identification by the DGGE (Adapted from (Sanz and Köchling, 2007)).

Following the scheme of the Figure 2.7 the first step in the DGGE technique is to extract the DNA of the sample (A). Then the 16S rRNA gene of the DNA is amplified through the polymerase chain reaction (PCR) (B). Later the electrophoresis in a gel that contains a denaturing gradient is run (C), and the predominant bands are selected for their further sequencing (D). When the bands are sequenced a phylogenetic analysis (E) and/or an alignment through bioinformatics tools as the software Basic Local Alignment Search Tool (BLAST) is carried out to identify and to assign a microorganism to each predominant band. Furthermore, the primers that are used in the PCR to do the DGGE contain a GC clamp, a consecutive sequence of guanines and cytosines that gives more stability to the amplified fragment and also allows that the amplicon is not completely denatured during the electrophoresis.

Since the first time that this technique was used in the study of complex microbial populations (Muyzer et al., 1993). It has been used in the characterization of a wide range of habitats, as soil, continental waters, bacteriplancton, etc. The technique was less used in the anaerobic treatment of waters, although in the last

years the DGGE technique has increased its popularity in this sector. For example, it has been used to evaluate the microbial diversity of the granular sludge from UASB reactors installed in alcohol distilleries (Akarsubasi et al., 2006), in brewery industries (Chan et al., 2001) and in the treatment of wastewater of paper mill industries (Buzzini et al., 2006).

The most important application of the DGGE is to monitor dynamic changes in the microbial communities. In this regard, there are multiple studies related with processes of anaerobic treatment: studies in the differences between mesophilic and thermophilic reactors, showing a lower biodiversity in the termophilic reactors utilized in the treatment of wastewater of a pharmaceutical industry (Lapara et al., 2000); analysis of the bacterial diversity changes observed in an anaerobic digester that was treating unsorted municipal solid waste (Silvey et al., 2000). Nevertheless, the major drawbacks of this technique are the high time consuming and its relatively low high-throughput (Delmont et al., 2012).

2.3.2 Quantitative Polymerase Chain Reaction (qPCR)

Other molecular technique that allows the study of the dynamics of the microbial populations in bioreactors is the quantitative polymerase chain reaction (qPCR). This technique is based on the monitoring of the amplification reaction at real time, finally, allowing quantifying the initial DNA concentration of one group of species of the sample. The monitoring at real time of the qPCR is carried out through the fluorescence registration, so the more DNA concentration of the target group in the sample is, the more the fluorescence signal will be. For the utilization of this technique the following elements are necessaries: template DNA, dNTPs, specific primers for the region to amplify and to quantify, buffer solution, DNA polymerase, which in case of the use of some probes as TaqMan probes must be an special polymerase, probes or fluorochromes able to interlock with double stranded DNA (dsDNA) and a thermocycler that allows to register the fluorescence. It is important to remark that in case of the use of flourochromes able to interlock to the dsDNA, the specificity of the reaction exclusively depends on the suitable design of the primers, while if the probes are used the specificity of the quantification depends on both, on the primer and on the probe used.

A probe is a DNA molecule of single strand DNA fluorescently labelled, which perfectly hybridizes with a region of the DNA. There are multiple probes depending on the type of qPCR that is going to be done. One of the most frequently used probes are the TaqMan probes. These probes are based on the phenomenon called fluorescence resonance energy transfer (FRET), in which transference of non-

radioactive energy from a donor molecule (excited fluorophore) to an acceptor molecule (another fluorophore) is produced, resulting in a fluorescence emission of the acceptor molecule. These probes are labelled by a fluorochrome at the 5' terminal and with a quencher at the 3' terminal. Therefore, the fluorescence produced by the fluorochrome is cancelled by the quencher when they are close. So, even though the fluorochrome is excited by the light at a determined wavelength, as the quencher is close to the fluorochrome, the fluorochrome transferred the energy by FRET but without fluorescence emission as the acceptor molecule is the quencher. As can be observed in Figure 2.8 during the qPCR, in the probe hybridization step, the probe hybridizes with a region of the gene that is going to be amplified. Then in the extension step, as the DNA polymerase progress with the extension it will found the attached probe that block the polymerization, so the extension would be impeded, and to finalize the extension the DNA polymerase needs to have also an exonuclease activity to degrade the probe. Therefore, the fluorochrome will be free and far from the quencher and it will emit fluorescence, being the level of fluorescence proportional to the number of copies amplified.

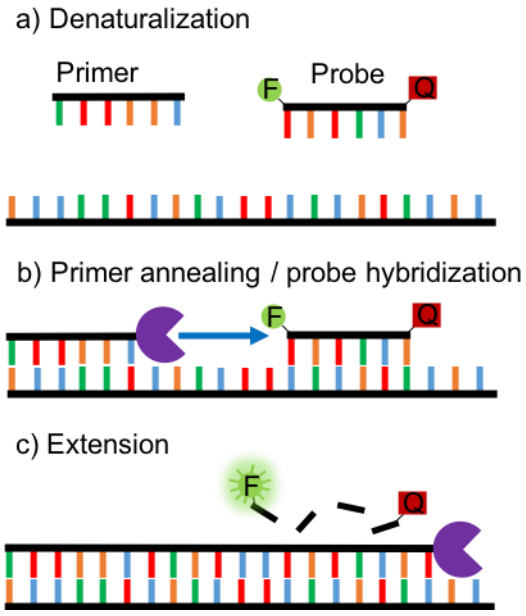

Figure 2.8. Squeme of the different stages of the qPCR through the TaqMan probes.

The quantification is done through a parameter called cycle threshold (C_t), which is defined as the fractional cycle number at which the fluorescence emission crossed an arbitrarily defined threshold within the logarithmic increase phase. The

threshold correspond to a level low enough for the amplification curves would be in exponential phase, but high enough to be above of the background noise. So, the smaller is the C_t the higher would be the concentration of the quantified gene. Besides, the quantification can be carried out in absolute or in relative methods, being the absolute quantification the most utilized, where a standard curve is created with known concentrations of the gene to quantify, so the samples with unknown concentration can be calculated from the standard curve.

As it has been mentioned before, the qPCR allows the specific quantification of a gene through the design and development of primers and probes. These properties were utilized by Yu et al. (2005) to design specific primers and probes to quantify the different methanogenic populations as the orders *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales* among others. These authors used the small differences in the 16S rRNA gene of the archaea of these orders for the design of the primers and probes for each population. The primers designed by these authors have allowed the quantification of these populations in bioreactors and anaerobic digesters, providing valuable information about the population dynamics of these microorganisms in function of the substrates used, organic load applied or the conditions of the reactor. For example, Aydin et al. (2015) showed how the introduction of antibiotics can affect to the microbial community of an anaerobic reactor, revealing a significant reduction in the number of active genes during the first stages in presence of antibiotics. While in later stages, the number of active genes recovered their concentration thanks to an adaptation of the biomass to the antibiotics. Siggins et al. (2011) also studied the dynamic of the methanogenic populations in the anaerobic digestion of a wastewater that contained trichloroethylene through qPCR, indicating that the temperature of the process affected more to the methanogenic populations than the presence of trichloroethylene.

2.3.3 High throughput sequencing

The high throughput sequencing is another technique that has been recently applied to the identification of species of samples from complex environments as the bioreactors. These studies are also called metagenomic studies, and they use the 16S rRNA gene, which contains variable and conserved regions that facilitate the sequencing and the phylogenetic classification. The metagenomics studies of the 16S can achieve efficiently levels of identification and classification to specie of microbial populations. The workflow includes the DNA extraction, the preparation of the libraries, sequencing and the bioinformatics

analysis of the obtained data. The metagenomics studies provide data about the physiological potential of the microbial community (Shakya et al., 2013; Su et al., 2012).

At the beginning of the apparition of this technique, it was barely used in the field of the bioreactors due to the high costs. However, the continuous improvement in the chemistry and the reduction of the costs has allowed the implantation of this technique as one of the most used to analyze the microbiology of the bioreactors for the treatment of wastewater and to have a better understanding of their performance (Vanwonterghem et al., 2014).

One of the first metagenomic studies in anaerobic digestion was carried out by Jaenicke et al. (2011). They observed in a reactor treating a mixture of maize silage, green rye and chicken manure, that the members of the phyla *Firmicutes* and *Bacteroidetes* seemed to be the responsible microorganisms of the hydrolysis of the polysaccharides and of the fermentation, and besides they suggested that *Clostridia* populations formed a syntrophic relationship with hydrogenotrophic methanogens. Later, another study about other biogas plant fed periodically with a mixture of maize silage and pig manure, showed the importance both of the regulation of the metabolism of hydrogen and of maintaining an equilibrium between producers and hydrogen consumers to have an stable and efficient biogas production (Wirth et al., 2012). Besides, these studies can also help to understand the influence that operational conditions, pretreatments or influent composition have over the structure of the community and their function (Rademacher et al., 2012; Wong et al., 2013).

The analysis of the microbial stratification in anaerobic reactors through this technique is also possible, for instance, Antwi et al., (2017) found in an UASB reactor a microbial stratification where *Firmicutes* was the predominant phylum in the bottom and in the upper part of the reactor, while *Euryarchaeota* was the predominant phylum in the medium part of the UASB reactor. Ambuchi et al. (2016) observed similar findings working with an EGSB reactor where *Chloroflexi, Euryarchaeota* and *Firmicutes* were the predominant phyla in the bottom, medium and the upper part of the reactor, respectively. Due to this fact, the authors suggested the possibility to do a compartmentalized design of the anaerobic reactor for an optimum performance.

Furthermore, sometimes is not possible to assign a functionality to the 16SRNA reads due to the limited information of the sequence data, because most of the metagenomic reads are unidentified yet, due to the lack of reference genomes in this type of applications (Jaenicke et al., 2011; Rademacher et al., 2012).

Nevertheless, the number of microbial sequences entered into public databases has increased exponentially, and with the current rate of sequencing, it is predicted that most microbial taxa will have been described by the end of this decade (Yarza et al., 2014).

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3 AIMS

The main aims of this thesis are the analysis of an alternative hybrid reactor to the EGSB reactor to improve the biomass retention, the evaluation the performance of reactor treating glycol ethers and ethanol mixtures, the identification of the pathways for the anaerobic degradation of solvents used in the flexographic industry, the determination of the possible impact that these compounds could cause in the microbial community and the comparison of performance and microbial communities between laboratory and industrial prototype scale reactors.

Each chapter has also more specific objectives. Chapter 4 tittled "Anaerobic degradation of glycol ether-ethanol mixtures using EGSB and hybrid reactors: Performance comparison and ether cleavage pathway" has the following specific objectives:

- To evaluate the feasibility of the anaerobic degradation of a typical mixture of solvents present in the emission of the flexographic industry, especially glycol ethers as M2P and E2P in an expanded granular sludge bed reactor.
- To determine the behavior of an hybridized expanded granular sludge bed reactor modified with a bed of polyethylene rings in the upper zone of the reactor where the separation gas-liquid-solid is carried out and to compare the biomass retention capacity of this reactor with the control expanded granular sludge bed reactor.
- To deepen in the study of the mechanisms involved in the anaerobic ether cleavage of glycol ethers as M2P and E2P.

Chapter 5 entitled "Anaerobic degradation of glycol ether-ethanol mixtures using EGSB and hybrid reactors: Microbial community study" has the following specific objectives:

- To investigate the dynamics of the microbial community by using molecular techniques to achieve a better comprehension of the behavior of the process.
- To evaluate the microbial stratification in high rate anaerobic reactors with high upflow velocities (≈10 m h^{-1}).
- To determine the possible impact that can cause the introduction of glycol ethers as M2P and E2P in the microbial populations.

Chapter 6 entitled “Behavior, stability and microbial community analysis of EGSB reactor at high content of glycol ether solvents in mixtures with ethanol” has the following specific objectives:

- To determine the influence of the diminution of the proportion of ethanol in the stability of the anaerobic granular sludge and in the microbial communities.
- To evaluate the performance and the elimination capacity of the reactor where glycol ethers are present at high proportion.
- To investigate the microbial community structure in the reactor treating high proportion of glycol ethers such as M2P and E2P, in mixtures with ethanol.

Chapter 7 entitled “Microbial community analysis of an on site pilot anaerobic bioscrubber treating VOCs from printing press air emissions” has the following specific objectives:

- To evaluate the microbial communities in a prototype reactor installed in a flexographic industry.
- To link the microbial evolution to the operational conditions and carbon source utilized at the flexographic industry.
- To compare the performance reactor and the microbial populations that proliferate in the laboratory scale reactor with the industrial prototype reactor.

4 ANAEROBIC DEGRADATION OF GLYCOL ETHER-ETHANOL MIXTURES USING EGSB AND HYBRID REACTORS: PERFORMANCE COMPARISON AND ETHER CLEAVAGE PATHWAY

4.1 ABSTRACT

The anaerobic biodegradation of ethanol-glycol ether mixtures as 1-ethoxy-2-propanol (E2P) and 1-methoxy-2-propanol (M2P), widely used in printing facilities, was investigated by means of two laboratory-scale anaerobic bioreactors at 25 °C: an expanded granular sludge bed (EGSB) reactor and an hybrid reactor (AHR) which incorporated a packed bed to improve biomass retention. Despite AHR showed almost half of solid leakages compared to EGSB, both reactors obtained practically the same performance for the operating conditions studied with global removal efficiencies (REs) higher than 92% for organic loading rates (OLRs) as high as 54 kg of chemical oxygen demand (COD) m^{-3} d^{-1} (REs of 70% and 100% for OLRs of 10.6 and 3.3 kg COD m^{-3} d^{-1} for E2P and M2P, respectively). Identified byproducts allowed clarifying the anaerobic degradation pathways of these glycol ethers. Thus, this study shows that anaerobic scrubber can be a feasible treatment for printing emissions.

4.2 INTRODUCTION

Solvents are commonly used in packaging printing industries as ink components that are dried through evaporation, which are the sources of volatile organic compounds (VOCs) in air emissions throughout the printing process. These emissions show ethanol as the main solvent, which is used in many solvent-based inks, but other solvents such as glycol ethers are also predominantly identified (Bravo et al., 2017; Sempere et al., 2012). The glycol ethers that are present in these emissions are 1-ethoxy-2-propanol (E2P) and/or 1-methoxy-2-propanol (M2P) that are frequently used as retarders in the printing process (Cheremisinoff, 2003). Several regulations and guidelines have been established in many countries for the control of the emissions of VOCs in printing industry. In the European Union, VOC concentrations of these emissions must comply with limit values ranging from 20 to 100 mg C Nm^{-3} (Council Directive 2010/75/EU), depending on printing activity (offset, rotogravure, flexography, etc.) and solvent consumption threshold (>15 or >25 tonnes per year). USA Environmental Protection Agency also recommends levels of control for these emissions ranging from 65 to 80% for overall control efficiency and from 90 to 95% for control device efficiency, depending on installation date of equipment and printing activity. Many states and local agencies have adopted regulations for controlling emissions from printing industry consistent with these control levels (USEPA, 1978, 2006). To fulfill these regulations and guidelines, the printing industry frequently needs end-of-pipe treatments of these emissions. In this regard, biological treatments such as biofiltration have been studied to remove volatile organic compounds from air emissions (Malhautier et al., 2005). A new alternative treatment based on an anaerobic bioscrubber has recently been described (Bravo et al., 2017; Waalkens et al., 2015). In this process, solvents are initially transferred from the gas phase to the liquid phase and subsequently degraded in an anaerobic reactor, where solvents are transformed into bioenergy in the form of biogas. The anaerobic biodegradability of M2P was demonstrated by Lafita et al. (2015) working with an expanded granular sludge bed (EGSB) reactor at 25°C. The removal efficiency (RE) of M2P reached up to 87% and showed a long lag phase that took 34 days to remove more than 50% of M2P. A first evidence of anaerobic degradation of M2P was reported by the European Chemicals Bureau (2006). In this study, a batch test using inoculum from a municipal digester, only a removal efficiency of 38% was observed after 81 days of operation.

Regarding the mechanism for the ether cleavage, it is not yet well understood, but the most accepted mechanism for glycol ether cleavage includes a double H/OH interchange (*trans*hydroxylation), resulting in the gem-diol

intermediate (Speranza et al., 2002; White et al., 1996), which rapidly collapses to the carbonyl or the keto group. In the anaerobic degradation of polyethylene glycol (PEG), migration of hydroxyl group generates an intermediate hemiacetal leading to acetaldehyde. Thus, degradation of the polymer occurs by successive eliminations of acetaldehyde units catalyzed by a cobalamin-dependent intracellular enzyme (PEG acetaldehyde lyase), according to the following reaction (Frings et al., 1992):

$$R\text{-}O\text{-}CH_2\text{-}CH_2\text{-}OH \rightarrow R\text{-}O\text{-}CH(OH)\text{-}CH_3 \rightarrow R\text{-}OH + CH_3\text{-}CHO$$

Kawai (2002) hypothesized that the metabolism of polypropylene glycol (with identical terminal OH group as M2P and E2P) is the same as that of PEG. By analogy, Lafita et al. (2015) proposed the same mechanism for the degradation of M2P, in this case leading to methanol and acetone or propionaldehyde.

Among these different biological treatments, anaerobic reactors based on granular sludge technology have become interesting options for the cost-effective and sustainable treatment of industrial wastewaters (Petta et al., 2017; Delforno et al., 2016). In this regard, the high contact between wastewater and biofilm that is promoted by expanding the sludge bed, makes the EGSB a suitable technology to treat high stress, less biodegradable or toxic components in wastewater. Expanding the granular sludge requires high upflow velocity (v_{up}) that can produce the wash-out of granular biomass from the reactor (Dries et al., 1998). This wash-out phenomenon can also be caused by a low-concentrate substrate (Puñal et al., 2003); wastewaters containing fat, oil, and grease (Jeganathan et al., 2006); or the combination of organic overload and daily interruption in the substrate supply (Lafita et al., 2015).

To improve biomass retention, Guiot and van den Berg (1985) used an anaerobic hybrid reactor (AHR) in treating synthetic soluble sugar wastewater, where an upflow anaerobic sludge bed (UASB) reactor was modified by adding an anaerobic filter in the upper zone to replace the gas-liquid-solid (GLS) separator. The results showed the filter as an inexpensive method to enhance biomass retention. This type of AHR offers different advantages, such as higher biomass retention capacity, a polishing effect to the effluent due to the biomass accumulated in the filter (Shivayogimath and Ramanujam, 1999), and/or a higher buffering effect against shock loading (McHugh et al., 2006). Because of these characteristics, the AHR seems a promising and emerging alternative that can be considered feasible for the treatment of (especially high-strength) wastewaters from different industries. Thus, for wastewater from a distillery spent wash, Shivayogimath and Ramanujam (1999) demonstrated that a hybrid reactor, combining a UASB reactor

in the lower part with polypropylene pall rings filter media in the upper part, could treat high organic loading, such as, 36 kg of chemical oxygen demand (COD) m^{-3} d^{-1}, which was a very efficient GLS separator. The AHR also showed high REs (up to 90.3%) in the treatment of a dairy industry effluent (Belançon et al., 2010). Treating a chemical synthesis-based pharmaceutical wastewater, Oktem et al. (2008) revealed that the AHR presented strong stability to changes in organic loadings. McKeown et al. (2009) showed that the AHR had a suitable configuration for long-term low-temperature treatment of acidified wastewaters.

The main purposes of this work were (i) to evaluate the feasibility of the anaerobic biodegradation of wastewater containing solvents from a flexographic printing industry by working with an AHR, including common glycol ethers such as M2P and E2P, and (ii) to compare the operation performance and biomass retention capacity between the AHR and the EGSB reactor. Furthermore, (iii) the study of the degradation of two different glycol ethers such as E2P and M2P might be useful for an in-depth understanding of the mechanisms involved in the anaerobic ether cleavage. Additionally and to the best of the authors' knowledge, this research is the first study about E2P degradation in this type of anaerobic reactor.

4.3 MATERIALS AND METHODS

4.3.1 Reactors' setup

Most of the experiments were performed in two anaerobic bioreactors, an EGSB reactor (R1) and an AHR (R2). Figure 4.1 shows a schematic diagram of both systems. The EGSB diagram reactor was equivalent to the AHR but without the presence of the packing material (Figure 4.1, part 4 in R2). The upper part of the AHR, from 1.40 m to 1.57 m in height, was randomly packed with polyethylene rings (0.0254 m in diameter). Both reactors had the same dimensions, with a sludge bed (Figure 4.1, part 3) of 4 L (height of 1.205 m and internal diameter of 0.065 m) and a total volume of 19 L. In this study, organic load rate was calculated on the basis of this sludge bed effective volume (4L). The supernatant reactor samples were taken at the recirculation port installed at a 1.60-m height. The gas was conducted to a gas seal (Figure 4.1, part 8) with a solution of NaOH to remove H_2S and CO_2. The reactors' temperature was maintained at 25°C, with an external jacket with water recirculation connected to a thermostat system (Polyscience SD15R-30, USA). Each anaerobic system was equipped with two peristaltic pumps that provided feeding

and recirculation flows (Figure 4.1, part 7) through the reactor. The feeding (Figure 4.1, part 1) and the recirculation were maintained at constant flows of 10 L day^{-1} and 32 L h^{-1}, respectively, to provide a v_{up} of 9.80 m h^{-1} in the sludge bed and a v_{up} of 0.98 m h^{-1} in the fixed film section. The increment in the organic loading rate (OLR) was carried out by increasing the COD concentration, keeping the feeding flow constant. A second EGSB (R3), identical to R1, was also utilized in the last part of this study to elucidate the reaction mechanism. For this purpose, an ethanol-acetone mixture was used as substrate.

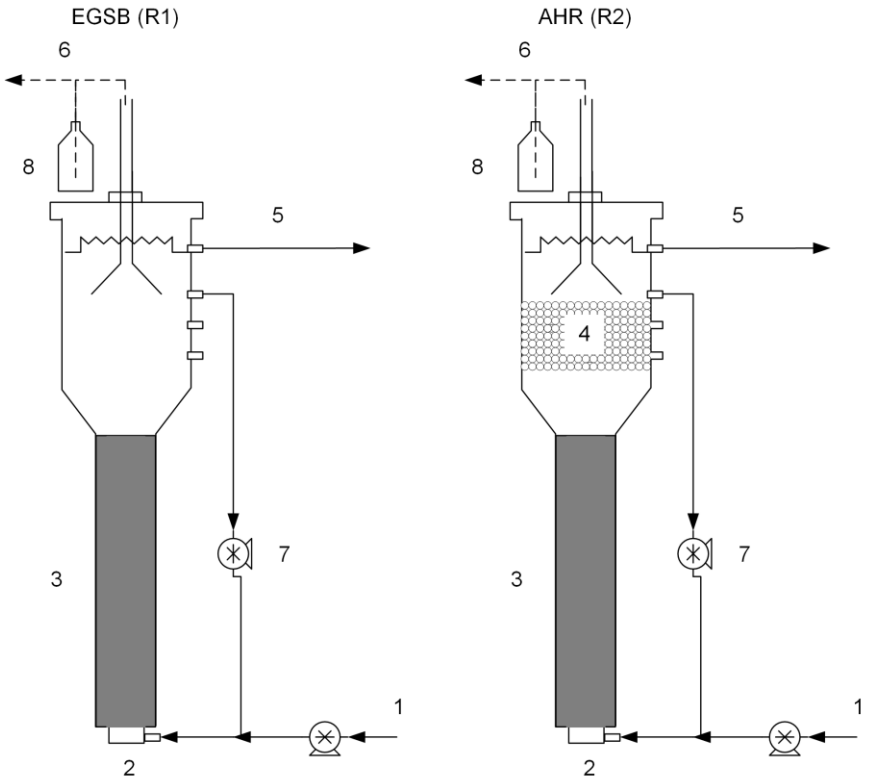

Figure 4.1. Diagram of the EGSB reactor (R1) and AHR (R2) with numbered parts: 1) Feed, 2) Glass balls, 3) Effective volume, 4) Packing material, 5) Effluent, 6) Biogas, 7) Recirculation, 8) Gas seal.

4.3.2 Reactor operation

The reactors were started up by using anaerobic granular sludge (4 L) taken from a local brewery wastewater treatment plant (Font Salem S. L., El Puig, Spain).

Both R1 and R2 were operated for more than 300 days (Table 4.2), with influent buffered with 5 g of $NaHCO_3$ L^{-1}; magnesium and calcium were maintained at 40 mg L^{-1}, and macronutrients and micronutrients were added in proportion to the COD (Table 4.1). All chemicals used were of analytical grade.

Table 4.1. Macronutrient and micronutrient supplementation influent.

Compound	Dose, mg g^{-1} COD	Compound	Dose, mg g^{-1} COD
NH_4Cl	15.7	H_3BO_3	0.1143
Yeast extract	7.5	EDTANa	0.100
KCl	6.1	$(NH_4)_2Mo_7O_{24}{\cdot}4H_2O$	0.0625
$(NH_4)_2HPO_4$	5.6	Al_2O_3	0.0595
$FeCl_3{\cdot}6H_2O$	0.4208	$NiSO_4{\cdot}6H_2O$	0.0447
$CoCl_2{\cdot}6H_2O$	0.1615	$CuCl_2{\cdot}2H_2O$	0.0134
$MnCl_2{\cdot}4H_2O$	0.1441	$ZnSO_4{\cdot}7H_2O$	0.132

The startup (stage S-I) of the reactors was carried out in the first 90 days of the experimental period (Table 4.2). At this stage, the OLR was increased from 9.1 to 47.0 kg COD m^{-3} d^{-1}, with a readily biodegradable substrate such as ethanol. From day 91 to day 202 (stage S-II), the influent composition was changed to a binary mixture of ethanol and E2P with a mass ratio of 9:1, and the average OLR was kept at 47.4 kg COD m^{-3} d^{-1}. At stage S-III, from day 203 to day 221, the OLR of E2P was increased to an ethanol-E2P mass ratio of 4:1, resulting in an OLR of 54.1 kg COD m^{-3} d^{-1}. At stage S-IV, from day 222 to day 230, the composition of the influent was changed to a ternary mixture of ethanol, E2P, and M2P with a mass ratio of 8:1:1 to check if there would be no need for a period of adaptation for M2P degradation. At this stage, the average OLR was 48.8 kg COD m^{-3} d^{-1}. At stage S-V, from day 231 to day 271, no feeding and no recirculation were applied to evaluate the effect of a long starvation period that typically occurs in industrial facilities. At stage S-VI, from day 272 to day 305, a restartup was carried out with the same ternary mixture used at stage S-IV and with the OLR range from 15.9 to 40.7 kg COD m^{-3} d^{-1}. Finally, at stage S-VII, from day 306 to day 335, the average OLR was 46.2 kg COD m^{-3} d^{-1}, and the mass ratio of the mixture of ethanol, E2P, and M2P was changed to 7:1:2.

Table 4.2. Operational conditions for the EGSB reactor (R1) and AHR (R2). Average values and standard deviations

Stage	S-I	S-II	S-III	S-IV	S-V	S-VI	S-VII
Time, d	0-90	91-202	203-221	222-230	231-271	272-305	306-335
COD, g L^{-1}	3.9 to 18.8	18.9 ± 3.0	21.6 ± 3.8	19.5 ± 2.1	-	6.3 to 16.3	18.5 ± 1.9
OLR, kg COD m^{-3} d^{-1}	9.1 to 47.0	47.4 ± 7.4	54.1 ± 9.5	48.8 ± 5.1	-	15.9 to 40.7	46.2 ± 4.8
E2P, g COD L^{-1}	0	2.0 ± 0.3	4.2 ± 0.3	2.4 ± 0.9	-	0.7 to 2.1	1.9 ± 0.2
E2P OLR, kg COD m^{-3} d^{-1}	0	5.1 ± 0.8	10.6 ± 0.7	6.1 ± 2.3	-	1.7 to 5.3	4.8 ± 0.6
M2P, g COD L^{-1}	0	0	0	1.9 ± 0.1	-	0.6 to 1.9	3.3 ± 0.4
M2P OLR, kg COD m^{-3} d^{-1}	0	0	0	4.8 ± 0.2	-	1.5 to 4.7	8.3 ± 1.1

The influent of the R3 reactor was buffered and the micronutrients and the macronutrients were added, in the same way as in the previously described reactors. The startup was carried out with ethanol until reactor reached almost complete substrate degradation for an OLR of 25.0 kg COD m^{-3} d^{-1}. Then, maintaining the OLR, the feeding composition was changed to an ethanol-acetone mixture with a mass ratio of 1:1, and the reactor was operated for 27 days under these conditions.

4.3.3 Effluent and biogas analyses

The COD, as well as volatile fatty acid (VFA), total suspended solid (TSS), and volatile suspended solid (VSS) concentrations were analyzed according to Standard Methods (American Public Health Association, 1999). Total RE was determined from inlet and outlet COD values. The NH_4^+, Ca^{2+}, Mg^{2+}, K^+, PO_4^{3-}, and SO_4^{2-} concentrations were measured by ionic chromatography (883 Basic IC Plus, Metrohm, Switzerland). The effluent's byproducts and solvents were identified by gas chromatography-mass spectrometry (5973 N MS/GC Agilent Technologies, Spain). The solvents in the effluent were determined by gas chromatography (Agilent GC 7890A, Spain) equipped with flame ionization detector; the solvents and the byproducts of the solvent degradation were separated on a Restek Rtx-VMS (USA) column (30 m long x 0.25 mm i.d. x 1.4 µm particle size), and helium was used as the carrier gas. Compound RE was obtained from these values. The biogas composition before the gas seal was determined by using a portable biogas analyzer (COMBIMASS® GA-m, Binder, Germany). The methane flow rates of both reactors were measured after H_2S and CO_2 in the gas seal were removed by a gas meter (MGC-10 PMMA, Ritter, Germany). Conductivity and pH were determined by a precision handheld meter (Multi 340i, WTW, Germany).

The monitoring of bioreactors was carried out from individual determinations, average values and standard deviation have been considered for overall operational conditions for each stage (Table 4.2).

4.4 RESULTS AND DISCUSSION

4.4.1 Reactor performance

The evolution of the performance of both reactors, R1 and R2, is plotted in Figure 4.2, where stages S-I to S-VII are indicated. Total REs higher than 95% were

achieved during all startup periods (S-I), including when the OLR reached the maximum value of 47.0 kg of COD $m^{-3}d^{-1}$, as can be observed in Figure 4.2. This was a predictable behavior since the organic substrate was ethanol, and the sludge came from a brewery wastewater treatment plant, so the biomass was well adapted to this substrate.

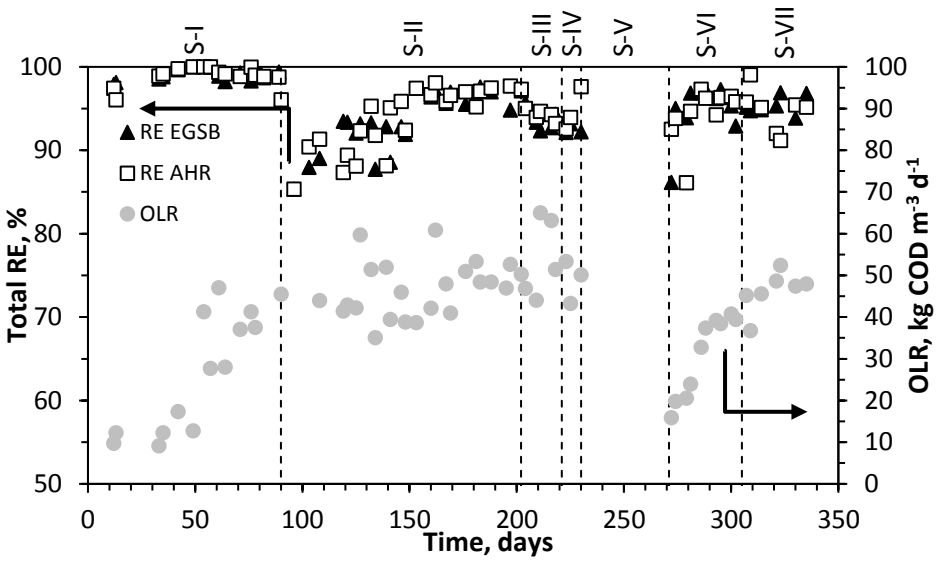

Figure 4.2. Evolution of total OLR and COD removal efficiency of EGSB reactor (R1) and AHR (R2).

The evolution of the VFA concentration in the effluent of each reactor can be observed in Figure 4.3. The VFA concentrations were below 70 mg L^{-1} in the first 50 days, but when the OLR was increased to 47.0 kg COD m^{-3} d^{-1}, the VFA concentrations reached values of 221.9 and 172.0 mg CH_3COOH L^{-1} for R1 and R2, respectively (Figure 4.3). The minimal accumulation of VFA can be attributed to the faster metabolism of acidogenic bacteria compared to methanogenic archaea, but the VFA concentrations decreased when the high OLR was maintained over time, reflecting the methanogenic population's adaptation to these operational conditions. In any case, the VFA concentrations were always relatively low (< 250 mg CH_3COOH L^{-1}) in both reactors, indicating no significant kinetic decoupling between the acidogenic and the methanogenic communities.

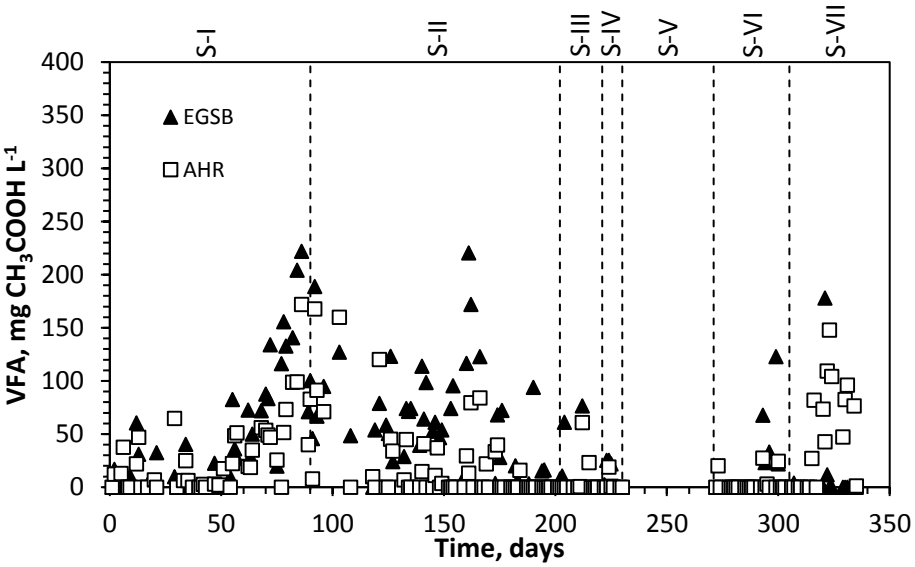

Figure 4.3. Evolution of volatile fatty acid (VFA) concentration in the effluent of the EGSB reactor (R1) and AHR (R2).

When E2P was included as a component of the influent at stage S-II (Table 4.2), the total RE in both reactors dropped from 99% to 85%, but the RE was progressively recovered to more than 95% (Figure 4.2). This behavior can be explained by assuming that the biomass was not adapted to E2P, so the biomass needed an acclimatization period to be able to metabolize this substrate. This period could be related to the development of ether-cleavage enzymes to degrade this substrate as suggested by Lafita et al. (2015). This approach is shared by Chen et al. (2008) who point the induction of specific enzymes as one of the mechanisms through which adaptation can occur. Various anaerobic bacteria yielding rearrangements of carbon-carbon bonds via transhidroxylation and depending on cobalamin were described for the degradation of polyethylene glycol (Schink et al., 1992; Frings et al., 1992). It would seem plausible that this same catalytic system governs the degradation of ether glycols as E2P, M2P, but no empirical evidences have been reported so far.

Figure 4.4 illustrates the evolution of the OLR and the RE of E2P in both reactors. The RE of E2P was very low at the beginning, 21% and 16% for R1 and R2, respectively, but gradually increased to around 80% for both reactors after 40-50 days of exposure to E2P. Generally, longer adaptation periods had been reported for similar solvents. Traverso-Soto et al. (2016) needed 169 days to reach 99.7% of

anaerobic biodegradation of alcohol ethoxylates in anaerobic degradation assays with marine sediments. A longer adaptation period for another glycol ether such as M2P was also observed by Lafita et al. (2015), who reported a 34-day acclimatization period for degrading more than 50% of M2P. In a recent study (Lafita et al., 2018), the time required to obtain 80% of removal of M2P was shortened to 22 days when chitosan was supplemented. In the present study, the acclimatization period for removing over 50% of E2P lasted only 11 and 16 days for R1 and R2, respectively. This shorter acclimatization period could be explained by taking into account the different substrate and the slightly lower OLR of the glycol ether used in comparison with previous studies. Besides, during these first weeks of exposure to E2P, acetone and isopropanol were transitorily observed in the effluents of both reactors. The appearance of these byproducts indicates the amenability of E2P to anaerobic treatment and sheds some light on the mechanism of degradation as discussed further in this chapter.

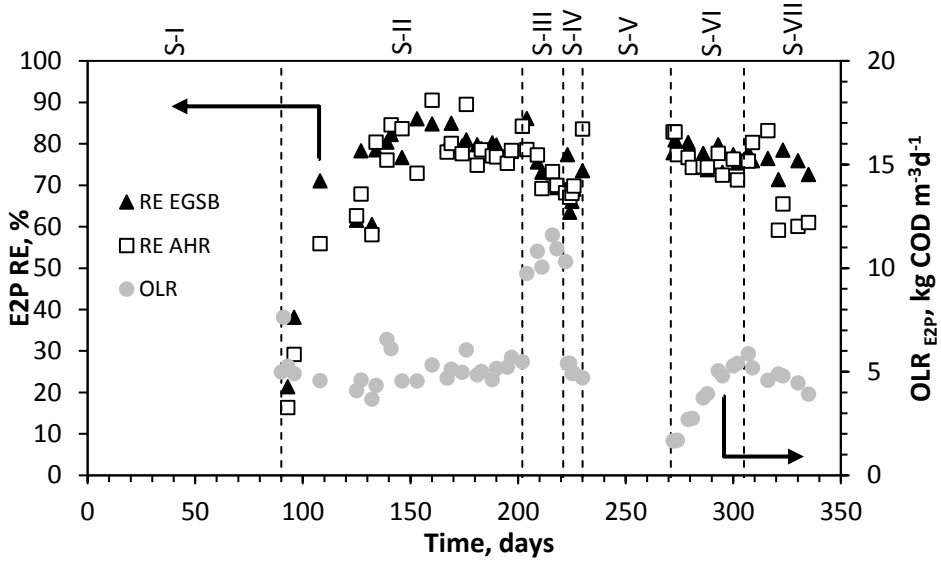

Figure 4.4. Evolution of E2P OLR and E2P removal efficiency of EGSB reactor (R1) and AHR (R2).

The OLR of E2P was doubled to 10.6 kg COD m^{-3} d^{-1} at stage S-III (Table 4.2) reaching an average total load of 54.1 kg COD m^{-3} d^{-1}, which resulted in a decrease of the global RE to around 92% for both reactors (Figure 4.2). This decline can be

essentially attributed to the decrease in the RE of E2P down to 70%, as shown in Figure 4.4, indicating that the presence of E2P in the system has no observable effect on the ethanol's RE.

At stage S-IV, the average of the total OLR was maintained at around 50 kg COD m^{-3} d^{-1} (similar to stages S-II and S-III), but a ternary mixture of ethanol, E2P, and M2P, with a mass ratio of 8:1:1, was used as the organic substrate, in Figure 4.5 can be observed the OLR and RE of M2P in both reactors. At this stage, the performance of both reactors barely changed in terms of the global RE (Figure 4.2), as well as the RE of E2P in comparison to the previous stage (S-III). The RE of M2P at the end of S-IV was practically complete as shown in Figure 4.5. Even from the first day of exposure to this compound, the RE of M2P was very high (> 85%) in both reactors, indicating no significant adaptation period for metabolizing this compound. This result could be attributed to the fact that both glycol ethers are degraded through the same mechanism; therefore, the adaptation period for M2P degradation is negligible for a system degrading E2P. As in the case of E2P, degradation byproducts were detected in the effluent during the first days of exposure to M2P (acetone and methanol in this case).

Regarding the biodegradability of E2P and M2P, the RE is greater for M2P (almost 100%), which could be related to the substrate's accessibility to the active center of the enzyme, as an M2P molecule is smaller than an E2P molecule. Therefore, the results suggest that the use of M2P would be preferable to E2P as a retarder additive if an anaerobic system is considered for the effluent treatment configuration of the packaging printing facility.

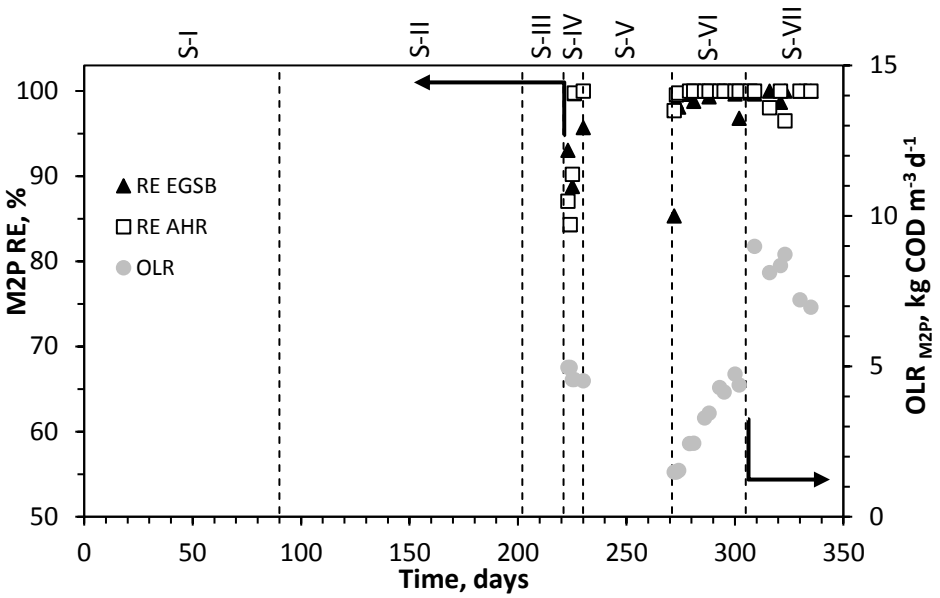

Figure 4.5. Evolution of M2P OLR and M2P removal efficiency of EGSB reactor (R1) and AHR (R2).

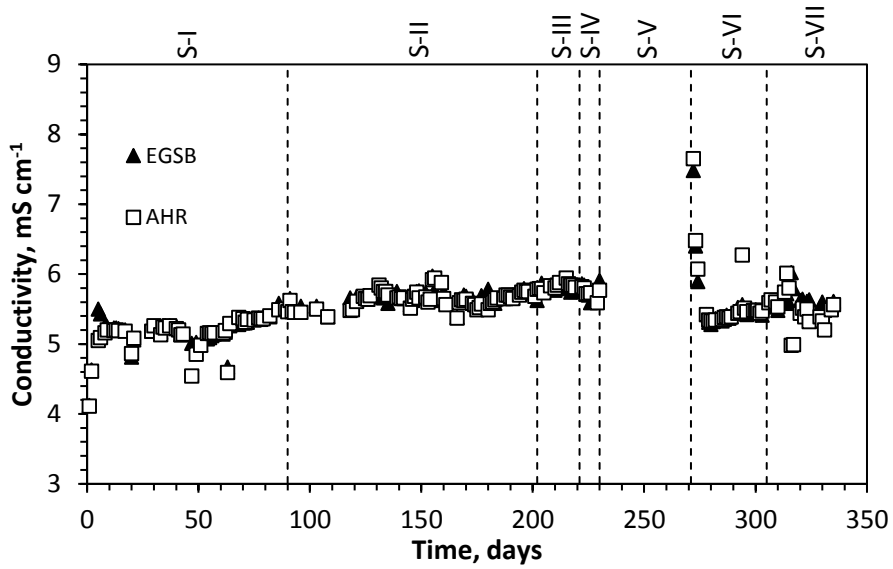

Figure 4.6. Evolution of contuctivity in the effluent of the EGSB reactor (R1) and AHR (R2).

Evolution of conductivity along all the experimental period in both reactors is depicted in Figure 4.6. A noticeable conductivity increase to 7.48 mS cm^{-1} and 7.65 mS cm^{-1} for R1 and R2, respectively, was observed at the end of S-V, the stage in which no feeding and no recirculation were applied to the system. This conductivity increase above the typical value in both reactors, ≈ 5.50 mS cm^{-1} (Figure 4.6), was caused by the increase in ammonium and phosphate concentrations as a consequence of a lysis phenomenon during the starvation period.

From a mass balance and considering the cell content of nitrogen and phosphorus, the total biomass decay during this period can be estimated: 52.2 g and 66.0 g for R1 and R2, respectively. This higher increase in nitrogen and phosphorus concentration in R2 than in R1 reflects the AHR system's (R2) greater biomass retention capacity. Because during this period (S-V), no feeding was applied, the cell growth can be neglected, and the concentration of microorganisms (X) can be related to the microorganism concentration before the starvation period (X_0) from a mass balance, as shown in the following equation:

$$X = X_0 \exp(-bt)$$

where b is the decay coefficient of biomass, and t denotes the duration of the starvation period. The calculated decay coefficients were 0.0024 d^{-1} and 0.0022 d^{-1} for R1 and R2, respectively. These values are quite similar to the decay coefficients obtained by Wu et al. (1995) with methanogenic granules that previously degraded VFA at 22 ˚C (between 0.0015 and 0.0028 d^{-1}).

During the restartup, at stage S-VI, conductivity decreased after 4 days to the habitual values, and the OLR was gradually increased from 15.9 to 40.7 kg COD m^{-3} d^{-1}, with the same feeding composition as in stage S-IV. Both reactors showed a high total RE, 95% for R1 (EGSB) and 94% for R2 (AHR), indicating that a typical industrial long starvation period (> 30 days) does not negatively affect the biomass activity. The glycol ethers' REs were high since the beginning of stage S-VI, 100% for M2P (Figure 4.5) and 77% for E2P (Figure 4.4) in both reactors, so no adaptation period was observed despite the biomass not being exposed to these compounds for more than 40 days.

At stage S-VII, the average total OLR was 45.9 kg COD m^{-3} d^{-1} (Table 4.2), but the feeding composition was changed to a ternary mixture of ethanol, E2P, and M2P with a mass ratio of 7:1:2. In both reactors, the total RE was maintained at a high value, similar to the previous stage (Figure 4.2). Moreover, M2P was completely removed despite its increase in OLR, indicating that the levels of M2P concentration

used in the experiment did not affect the performance of the reactors. Regarding the REs of E2P (Figure 4.4), these reached 76% and 80% for R1 and R2, respectively, until day 315 when the pall rings in the hybrid reactor (R2) were replaced by clean ones to avoid clogging problems in the filter media. After the replacement, the RE of E2P for R2 dropped to 65%, and the average concentration of VFA in the effluent increased from negligible values to 68.5 mg CH_3COOH L^{-1}, while in the EGSB reactor, only amounted up to 14.6 mg CH_3COOH L^{-1} (Figure 4.3). The decrease in the E2P's RE and the increase in VFA after the replacement of the packaging material showed that a not quite significant part of the R2 activity was related to the active biomass associated with the packed fraction of the reactor.

The methane average and standard deviation (SD) yield was 0.324 ± 0.051 m^3 CH_4 kg^{-1} $COD_{degraded}$ and 0.318 ± 0.049 m^3 CH_4 kg^{-1} $COD_{degraded}$, in R1 and R2 respectively, indicating no significant difference between both reactors in the methane production. The evolution over time of methane production can be found in the Figure 4.7. The average and SD proportion of methane in the biogas was 82.4 ± 2.6 % and 83.0 ± 2.9 % for R1 and R2, respectively, with a presence of H_2S below 100 ppmv.

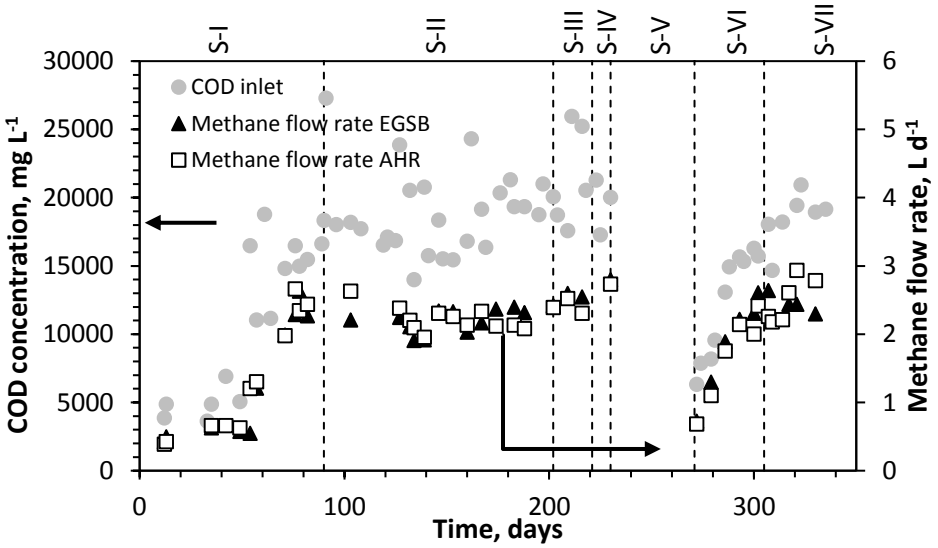

Figure 4.7. Evolution of the methane flow rate of the EGSB reactor (R1) and AHR (R2) and COD inlet concentration for both reactors.

Narra et al. (2014) reported a methane yield between 0.12 and 0.16 m^3 CH_4 kg^{-1} $COD_{degraded}$ when working with anaerobic hybrid reactors that treated the

wastewater of mild alkali-treated rice straw in ethanol fermentation process. This low yield could be due to the different substrate used. Treating more similar substrates such as solvents that included ethanol, acetone, propanol, and methanol, Enright et al. (2009) obtained methane yields between 0.11 and 0.35 m^3 CH_4 kg^{-1} $COD_{degraded}$, within the range of those obtained in the present study for both reactors.

4.4.2 Intermediated and anaerobic mechanism of ether cleavage

Since acetone was detected as a key intermediate product of anaerobic biodegradation of glycol ethers, an additional EGSB reactor (R3) was fed with this solvent and ethanol (1:1 mass ratio) to shed light on the mechanism. Figure 4.8 shows the evolution of the organic compounds detected in the effluent of R3. The acetone concentration was quite high in the beginning of the trial, but since day 9, the concentration gradually decreased, and the system practically reached complete degradation (98%) of this compound on day 21. These results showed that the biomass needed an adaptation period to remove acetone, as what occurred with E2P, but in this case, the adaptation period was shorter than in the case of E2P, indicating that acetone could be more readily biodegradable than E2P. Thus, experimental evidence seems to point out that the first step of the cleavage to alcohol and acetone could be considered the controlling step for the biodegradation of glycol ethers. This hypothesis would be reinforced, considering the detection of negligible amounts of acetone and/or isopropanol in the effluent found in the experimental series with R1 and R2 (these compounds were only briefly detected when the glycol ethers were first fed into the bioreactors). Furthermore, isopropanol was identified in the effluent of bioreactor R3, as shown in Figure 4.8. The appearance of isopropanol in a system treating acetone was explained by Tonouchi (2004) and Zellner and Winter (1987), who concluded that isopropanol could be produced from acetone in the presence of hydrogen, with a reversible reaction. Additionally, Vermorel et al. (2017) observed a significant presence of acetone, besides isopropanol, in the effluent of a laboratory-scale anaerobic continuous stirred tank reactor (CSTR) that treated synthetic wastewater with ethanol and isopropanol as organic substrates. This observation would support the hypothesis of the reversibility of the transformation between isopropanol and acetone.

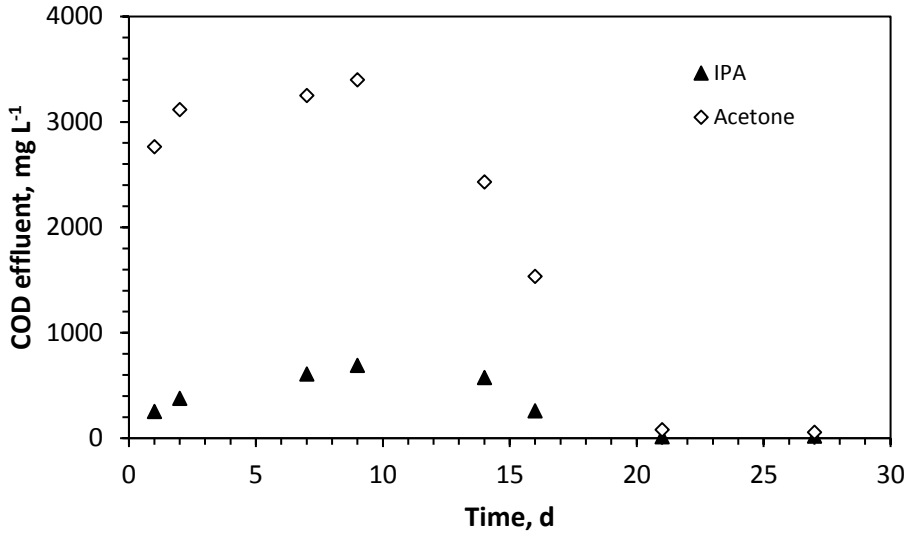

Figure 4.8. Evolution of intermediate compounds' concentration found in the effluent in the reactor R3 treating the ethanol-acetone mixture with an average inlet COD concentration of 11.2 ± 1.5 g L^{-1}.

The mechanism of the anaerobic ether cleavage is not well established yet, although intracellular enzymes depending on cobalamin seems to be involved in this process (Frings et al., 1992). The most accepted mechanism implies a double H/OH interchange (hydroxyl shift) resulting in the gem-diol that rapidly collapses to a carbonyl or a keto group (Speranza et al., 2002). As previously described, the identified byproducts during the first days of the biomass exposure to E2P were acetone and isopropanol, while acetone and methanol were identified in the case of M2P. By analogy with the hydroxyl-shift mechanism of ether excision for anaerobic ether cleavage (White et al., 1996), the theoretical byproducts should be ethanol and acetone from E2P degradation and methanol and acetone from M2P degradation (Lafita et al., 2015). In the case of E2P, no ethanol was observed in the effluent as a readily biodegradable substrate. In any case, as a main substrate, ethanol could not have been attributed as an intermediate product of degradation. On the other hand, the presence of isopropanol could be related to the formation of acetone as it was corroborated with the R3 reactor performance, as previously described. Thus, acetone together with the hydrogen contained in the biogas can make the conversion to isopropanol possible, explaining the appearance of this compound in the effluent.

In the case of M2P, the presence of methanol was briefly detected in the effluent of both bioreactors (R1 and R2) after M2P was added to the influent. However, isopropanol was not observed because at this phase of the study, the biomass was already well adapted after more than 100 days of degrading E2P; thus, the formed isopropanol should be undetectable by the equipment. Therefore, the hypothetical mechanism schematized in Figure 4.9 would explain the presence of the detected organics in the effluent of the reactors. In this regard, the degradation of acetone to methane and carbon dioxide, acetate was the only intermediate trasnferred between a fermenting bacterium and a methanogen, as first reported Platen and Shink (1989). The authors suggested that acetone is first carboxylated to acetoacetate by condensation with carbon dioxide, from which acetate is formed and then transferred to *Methanosatea* sp. (formerly *Methanothrix* sp.). Methanol can be directly utilized as a carbon and energy source by several species of methanogens and acetogens.

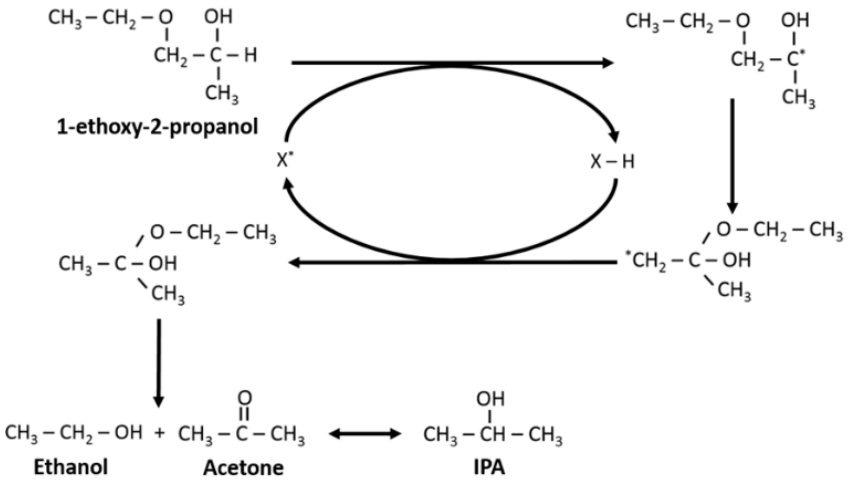

Figure 4.9. Proposed mechanism for the anaerobic degradation of 1-ethoxy-2-propanol, including isopropanol (IPA) formation from acetone.

4.4.3 Biomass retention capacity of AHR and EGSB reactor

Granular and non-granular accumulated solids of each stage collected from the effluent of reactors R1 (EGSB) and R2 (AHR) can be found in Table 4.3, and the evolution of total accumulated solids of both reactors are plotted in Figure 4.10,

which shows that during stage S-I, the solids in the effluent of R1 were considerably higher than in R2, reaching 73.2 g and 29.0 g of accumulated solids, respectively. In the beginning of S-II, the solids collected from both effluents were similar, but from day 133 to day 161, a granular sludge flotation occurred in R1. Therefore, the accumulated solids in the effluent increased considerably with respect to R2, where no granular sludge flotation was registered. Thus, during this stage, 255.0 g of solids were collected from R1 and only 109.0 g from R2. During stage S-III, the solids in the effluent were also slightly higher in the EGSB reactor (28.3 g) than in the AHR (20.1 g). Anaerobic granular sludge flotation was observed in the systems (stage S-IV), reaching 45.9 g and 36.5 g of accumulated solids for R1 and R2, respectively. It is important to clarify that the sludge flotation observed has not been a total breakdown that had led to a complete wash out as those described extensively in literature (Macarie et al., 2017; Yoda and Nishimura, 1997; Lafita et al., 2015). In fact, if the leakage of solids is quantified in reference to the effluent flow, the average outlet concentration of reactor R1 in stage S-II (stage and reactor most unfavorable in terms of leakage of solids) is 228 g TSS m^{-3} which can be considered as a normal value for this kind of anaerobic systems.

Table 4.3. Accumulated suspended solids (SS) in the effluent from each reactor in the different stages. EGSB (R1) and AHR (R2)

	Stage	**S-I**	**S-II**	**S-III**	**S-IV**	**S-VI**	**S-VII**
Non granular solids (g)	EGSB	73.1	101.2	17.3	6.7	15.0	15.4
	AHR	29.0	81.5	16.6	7.1	12.2	27.5
Granular solids (g)	EGSB	< 0.1	153.8	11.0	39.2	39.1	91.3
	AHR	< 0.1	27.5	3.5	29.4	36.5	22.9
Ratio SS_{EGSB}/SS_{AHR}	Non-granular	2.52	1.24	1.04	0.94	1.24	0.56
	Granular	-	5.59	3.14	1.34	1.07	3.98
	Total	2.52	2.34	1.40	1.26	1.11	2.12

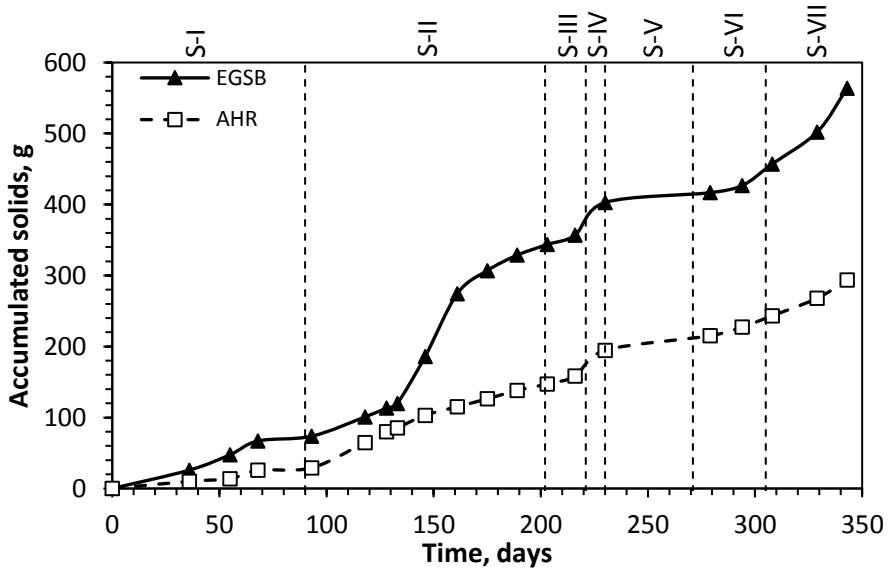

Figure 4.10. Accumulated suspended solids in the effluent of EGSB reactor (R1) and AHR (R2).

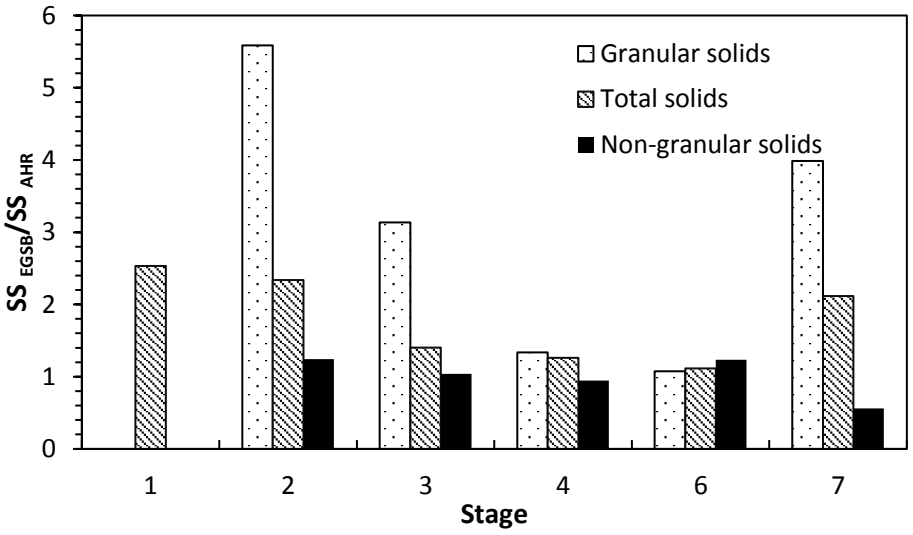

Figure 4.11. Ratio of suspended solids in the efluent of EGSB and AHR differenciating between granular, non-granular and total solids.

After the shut-down period (S-V), when feeding was resumed, 20.0 g of solids in total were collected from R2, while only 13.8 g were washed out from R1 during the first week of stage S-VI. This unusually larger amount of biomass washed out from R2 could be explained by the lysis that occurred during the starvation period; as more biomass was retained by R2 than by R1, more granules were proportionally broken in R2, yielding greater biomass washout. The last two stages also showed greater washout from R1, in accordance with the global experimental trend. At the end of the experiment, the accumulated solids collected in the effluent were 563.2 g and 293.7 g for R1 and R2, respectively. This difference shows the AHR's (R2) higher capacity to retain biomass, supporting the study of Borja et al. (1995), who reported that the packing of a hybrid anaerobic reactor significantly enhanced the retention of active biomass treating slaughterhouse wastewater. Furthermore, Guiot and van den Berg (1985) demonstrated that the AHR's biomass retention capacity was between 1.5 and 2.8 times higher than those of other bioreactor configurations such as the UASB or the anaerobic baffled reactor.

The significant improvement in the biomass retention capacity of the hybrid system (R2) was not reflected in its performance since no significant differences in the REs of the different compounds were observed over the experimental period. Only slightly more stable and lower concentrations of VFA were observed in the effluent of R2 (Figure 4.3).

In previous research, episodes of biomass flotation in the AHR had been attributed to lipid adsorption in granules (Belançon et al., 2010), but as no lipids were present in the feeding of the present study, the biomass flotation produced during stages S-II and S-IV had to be caused by other factors. Furthermore, in the AHR, no biomass flotation was observed during stage S-II, probably due to the higher capacity to retain biomass. However, the episode of anaerobic granular sludge flotation observed in the AHR at stage S-IV indicated that the filter was highly clogged and close to its maximum retention capacity. This fact can also be observed in Table 4.3 and in Figure 4.11 where, in addition to the absolute values of the leakage of solids from each reactor, the ratios of the solids in the effluent between control EGSB (R1) and AHR (R2) have been included (granular, non-granular, and total solids). The ratio of the total solids (R1/R2) decreased over the experimental period from 2.5 at stage S-I to 1.1 at stage S-VI, and then, at stage S-VII, it increased again to 2.11 when the packing material was replaced by new and clean polyethylene rings. The decrease in the ratio at every stage was even higher if only granular solids were considered—from 5.6 at stage S-II to 1.1 at stage S-VI, and then, after the replacement of the packing material, the ratio increased again to 4.0 at

stage S-VII. In contrast, the non-granular solids (suspended solids) collected from both systems were not significantly different at each of the stages studied (the ratio varied from 1.2 to 0.6). These results indicated that the packing material selectively retained the granular biomass versus the solids in suspension. Furthermore, the recovered capacity to retain the solids after the replacement of the packing material suggested that the filter was clogged; thus, to maintain proper performance, it should be periodically cleaned or replaced. It seems that the leakage of granules would be related with regular discharge of surplus biomass in normal operation as reported by Pereboom et al. (1994). Likewise, Hulsshoff Pol et al. (2014) affirmed that light sludge is separated from mature granules using the rapid upward velocity of EGSB reactor. This selective wash-out, resulting in an increased growth of retained (heavier) sludge agglomerates would be crucial for the granulation process (Lim and Kim, 2004). Thus, it is interesting to highlight that the observed leakage throughout the experimental series did not have significant influence with the performance of the EGSB reactor. Even more interesting is that no difference has been observed between the performance of both systems (control EGSB and AHR) for any stage, which would indicate that the activity of the excess sludge (that was retained in the case of the AHR system) was practically negligible and no comparable to the activity of retained granules.

In the next chapter of this work, the study of the dynamic evolution of the microbial community and their interelation with the bioreactor performance and operation can be found.

4.5 CONCLUSIONS

Granular anaerobic treatment was shown to be a feasible technology for the biodegradation of ethanol-glycol ether mixtures such as E2P and M2P after a short period of acclimatization. Of the two systems tested, the AHR showed a higher solid retention capacity than the EGSB but did not result in the AHR's improved performance. Both systems could completely remove M2P (M2P OLR of 8.3 kg COD m^{-3} d^{-1}) and 70% of the E2P (E2P OLR of 10.6 kg COD m^{-3} d^{-1}). The detected byproducts allowed clarifying the degradation pathways of the studied glycol ethers.

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5 ANAEROBIC DEGRADATION OF GLYCOL ETHER-ETHANOL MIXTURES USING EGSB AND HYBRID REACTORS: MICROBIAL COMMUNITY STUDY

5.1 ABSTRACT

The temporal dynamics of the microbial community within an anaerobic hybrid reactor (AHR) and an expanded granular sludge bed reactor (EGSB) treating mixtures of ethanol and glycol ethers, such as 1-ethoxy-2-propanol (E2P) and 1-methoxy-2-propanol (M2P), was investigated. After an acclimation period, this study revealed the adaptation capacity of a complex microbial community to consume glycol ethers as substrates. A similar evolution was observed in both reactors during the whole experiment and the microbial community exhibited a longitudinal homogeneity within both bioreactors. The introduction of E2P in the polluted feed led to a decrease of microbial population densities, but this drop was not observed when M2P was introduced into the gas. Furthermore, a shift in Proteobacteria, which was the most predominant phylum in the first stages, and Firmicutes was observed during the experiment, indicating an adaptation of the bacterial community to new operating conditions.

5.2 INTRODUCTION

Flexographic industries produce air emissions characterised by a content of volatile organic compounds (VOCs) due to the use of solvents in the manufacturing processes. Ethanol, ethyl acetate, 1-methoxy-2-propanol (M2P) and/or 1-ethoxy-2-propanol (E2P) are among the main VOCs present in these air emissions (Sempere et al., 2012). Different environmental regulations limiting these emissions can be found (Council Directive 2010/75/EU, USEPA, 2006) and frequently an end-of-pipe treatment is needed. In this sense, a new biotechnology based on an anaerobic bioscrubber has been proved to be a feasible treatment to meet regulatory requirements (Bravo et al., 2017). This new technology transfers VOCs from the gas phase to the liquid phase and then VOCs are biotransformed into biogas in an expanded granular sludge bed (EGSB) reactor (Waalkens et al., 2015).

Several recent studies have demonstrated the feasibility of the treatment of these contaminants in high rate anaerobic reactors. An EGSB reactor from a pilot-scale anaerobic bioscrubber treating ethanol, ethyl acetate and E2P as the main pollutants achieved an average removal efficiency (RE) of 93% (Bravo et al., 2017). Torres et al. (2018) demonstrated the almost complete biodegradation of ethanol and ethyl acetate and a maximum RE for E2P of 78% in chitosan-assisted UASB reactors fed with a mixture of ethanol:ethyl acetate:E2P in a mass ratio of 7:2:1. Lafita et al. (2015) demonstrated that more than 85% of M2P could be degraded anaerobically in an EGSB reactor, but a long adaptation period - more than 40 days - was required to achieve this performance.

The anaerobic degradation of these solvents seems to strongly rely on the developed microbial population in the anaerobic granules, so the microbial dynamics of these reactors is especially important for a better understanding of the system. In addition, this better understanding can provide control, diagnostic and prediction tools for process monitoring (Cabrol and Malhautier, 2011). To improve biomass retention, some authors used an anaerobic hybrid reactor (AHR) to treat industrial wastewaters (Belançon et al., 2010; Shivayogimath and Ramanujam, 1999), where an up-flow granular reactor was modified by adding an anaerobic filter in the upper zone to replace the gas-liquid-solid (GLS) separator. Furthermore, the links between microbial and functional components need better understanding in order to improve bioreactor performance. In this sense, different molecular tools are available in order to investigate the microbial component in different bioreactors. In this regard, denaturing gradient gel electrophoresis (DGGE), quantitative polymerase chain reaction (qPCR) and high throughput sequencing

technologies are broadly appreciated. In the last decade, DGGE has been frequently used to monitor the bacterial and archaeal populations in bioreactors; Cabrol et al. (2012) revealed a highly dynamic total bacterial community structure and a high and stable performance in gas biofilters and Zhang et al. (2011) showed a succession in the dominance of the archaeal and bacterial populations in an anaerobic baffled reactor treating acetone-butanol-ethanol fermentation wastewater. qPCR is widely used to quantify and monitor microbial populations, such as methanogenic populations, at order or family taxonomic level (Yu et al., 2005). In this sense, Siggins et al. (2011) studied the temporal dynamics of the microbial community in anaerobic digestion of a wastewater containing trichloroethylene using DGGE and qPCR. It has been highlighted that the microbial communities are affected by environmental conditions and it seems that temperature has a stronger influence on the bacterial community structure than the concentration of trichloroethyelene. High throughput sequencing technologies are now currently used as the "next generation" sequencing methods by allowing targeted DNA of an entire community to be sequenced in parallel, providing a very deep phylogenetic fingerprint of complex communities in environmental samples, such as from anaerobic reactors. The resulting data may help the operators to improve/maintain reactor performances. By using these sequencing methods, Antwi et al. (2017) and Ambuchi et al. (2016) observed a microbial stratification along the bed height in an up-flow anaerobic sludge bed (UASB) reactor and an EGSB reactor, respectively. This interesting result could be used to improve performances by designing an adapted compartmentalised anaerobic reactor.

Other recent studies have reported the link between the microbial diversity of an EGSB reactor and operational conditions, such as the hydraulic retention time (HRT) and the organic loading rate (OLR). Researchers observed that *Proteobacteria* was enhanced with higher OLR and lower HRT (Yang et al., 2018, 2017). A shift in the methanogenic population was also observed when the OLR was increased drastically, from an acetoclastic to a hydrogenotrophic community (Wang et al., 2015). Another study also identified a succession in methanogenic populations during conditions of stress, where the hydrogenotrophic community changed from *Methanospirillum* to *Methanoculleus* (Li et al., 2016). In this context, several studies have reported contradictory results. Krakat et al. (2011) indicated that the phylum *Acidobacteria* was related to high OLR, while Song et al. (2017) observed that this phylum could be negatively affected by the increase of the OLR. This discrepancy could be explained by the substrates used, which highlights the importance that the

substrates have over the dynamics of the microbial community structure in anaerobic reactors.

In the previous chapter it has been highlighted the potential of anaerobic bioreactors (AHR and EGSB bioreactors) for the treatment of typical solvents containing glycol ethers, such as E2P and M2P, emitted by the flexographic industry. Nevertheless, at this time, no information about the links between the microbial community structure and the bioreactor performance is available. Therefore, the purpose of this study is to: i) evaluate the microbial stratification in high rate anaerobic reactors with high up-flow velocities (≈10 m h^{-1}); ii) determine the impact of glycol ethers, such as E2P and M2P, on the microbial community structure within the reactor; and iii) examine the temporal dynamics of the microbial populations of an AHR and an EGSB reactor treating wastewater containing typical solvents from a flexographic industry.

5.3 MATERIALS AND METHODS

5.3.1 Bioreactors: Source of biomass and performance evolution

Biomass samples were taken at the bottom (0.15 m) and at the top (0.90 m) of the sludge bed during all the experiment carried out in the previous chapter (Chapter 4) through installed sampling ports. The experiment was performed over a period of almost one year in two anaerobic EGSB and AHR bioreactors with identical dimensions and configuration (Figure 4.1), except that the EGSB did not contain the packing material in the upper part of the bioreactor (Figure 4.1, part 4). Both reactors were seeded with anaerobic granular sludge from a brewery wastewater treatment plant. Each anaerobic reactor was equipped with two peristaltic pumps that provided constant feeding and recirculation flows of 10 L d^{-1} and 32 L h^{-1}, respectively, to operate with an up-flow velocity of 10 m h^{-1}. Reactor temperatures were maintained at 25 °C with an external jacket. The operating conditions and performance of reactors are summarised in Table 5.1.

Table 5.1. Conditions and performance of EGSB (R1) and AHR (R2).

Stage	**S-I**	**S-II**	**S-III**	**S-IV**	**S-V**	**S-VI**	**S-VII**
Time, d	0-90	91-202	203-221	222-230	231-271	272-305	306-335
OLR, kg COD m^{-3} d^{-1}	9.1 to 47.0	47.4 ± 7.4	54.1 ± 9.5	48.8 ± 5.1	-	15.9 to 40.7	46.2 ± 4.8
EGSB Total RE, %	99.0 ± 0.7	85.3 to 97.6	93.7 ± 1.4	92.5 ± 0.6	-	94.8 ± 3.4	95.4 ± 1.1
AHR Total RE, %	99.0 ± 1.1	85.3 to 97.7	94.2 ± 0.7	94.7 ± 2.6	-	94.3 ± 3.2	94.8 ± 2.6
E2P OLR, kg COD m^{-3} d^{-1}	0	5.1 ± 0.8	10.6 ± 0.7	6.1 ± 2.3	-	1.7 to 5.3	4.8 ± 0.6
EGSB E2P RE, %	-	21.4 to 86.0	75.4 ± 6.4	68.0 ± 7.7	-	77.3 ± 2.7	75.5 ± 2.6
AHR M2P RE, %	-	16.3 to 90.5	73.7 ± 4.2	72.1 ± 0.3	-	76.4 ± 3.8	69.3 ± 10.2
M2P OLR, kg COD m^{-3} d^{-1}	0	0	0	4.8 ± 0.2	-	1.5 to 4.7	8.3 ± 1.1
EGSB M2P RE, %	-	-	-	92.1 ± 6.9	-	97.8 ± 4.3	99.7 ± 0.5
AHR M2P RE, %	-	-	-	93.6 ± 7.7	-	99.7 ± 0.7	99.2 ± 1.4

During S-I (from day 0 to 90) the start-up of the reactors was carried out by increasing the OLR with a very readily biodegrable substrate, such as ethanol, until achieving 47.0 kg chemical oxygen demand (COD) $m^{-3}d^{-1}$. At this stage, the global RE of both reactors was 99%. Then, in S-II (from day 91 to 202) the synthetic feed was modified by introducing E2P with a mass ratio ethanol:E2P of 9:1 and the OLR was maintained in 47.4 kg COD m^{-3} d^{-1}. During the first days of this period, the global RE decreased to 85% in both reactors as a consequence of the low degradation of E2P (21% and 16% in the EGSB reactor and in the AHR respectively). Then, after an acclimation period of 42 days, global RE increased to 97% in both reactors and the removal efficiency of E2P reached high levels in both reactors (86% for EGSB reactor and 91% for AHR). In S-III (from day 203 to 221), the OLR was increased to 54 kg COD m^{-3} d^{-1} (ethanol:E2P mass ratio of 8:2) and the global and E2P RE decreased to 94% and 74%, respectively, in both reactors. In S-IV (from day 222 to 230), the composition of the synthetic feed was modified by introducing M2P with an ethanol:E2P:M2P mass ratio of 8:1:1. The OLR was 49 kg COD m^{-3} d^{-1}. The global and E2P RE were similar to the previous stage (approximately 94% and 70%, respectively) in both reactors. Besides, M2P reached very high RE values (>90%) in both reactors from the beginning of the period. During S-V (from day 231 to 271), no feeding and recirculation flow was applied (shut down). In S-VI (from day 272 to 305), the re-startup was carried out by increasing the total OLR until achieving 41 kg COD m^{-3} d^{-1} with the same solvent mixture used in S-IV, and the global, E2P and M2P RE were very similar to those obtained at previous stage (S-IV). Finally, in the stage S-VII (from day 306 to 335), the average OLR was 46 kg COD m^{-3} d^{-1} (ethanol:E2P:M2P mass ratio of 7:1:2). During this stage, the performance of both reactors remained constant as in the previous stages. In addition, methane yield production was constant with an average value of 0.324 ± 0.051 m^3 CH_4 kg^{-1} $COD_{degraded}$ and 0.318 ± 0.049 m^3 CH_4 kg^{-1} $COD_{degraded}$ for the EGSB and the AHR, respectively, whatever the considered period. A detailed discussion of the performance results can be found in the Chapter 4.

5.3.2 DNA extraction

Genomic DNA of each sample was extracted by using the PowerSoil DNA isolation kit following the supplier's protocol. Extracted DNA was then quantified by using NanoDrop® and then DNA samples were preserved at -20 °C for further analysis. The experimental design is presented in Table 5.2.

Table 5.2. Sampling days at each stage and molecular techniques applied at samples from top port.

Stage	S-I	S-II	S-III	S-IV	S-V	S-VI	S-VII
DGGE	0[a], 89[b]	93[b], 108[a], 132[b]	-	230[a]	-	-	336[a]
qPCR	0[a], 89[a]	103[a], 132[a], 160[a], 189[a]	217[a]	230[a]	-	296[a]	336[a]
Illumina	0*[a], 89*[a]	132*[a]	-	230[a]	-	-	336[a]

[a] Biomass sampled at the top of the reactor; [b] Biomass sampled at both top and bottom of the reactor; *Analysis of AHR samples only.

5.3.3 Denaturing gradient gel electrophoresis (DGGE)

Two primers sets were used to amplify the V3 region of 16S rDNA as follows: F357-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and R518 (5'-ATT ACC GCG GCT GG-3') for bacterial 16S (Toffin et al., 2004) with an amplicon size of 162 bp , and F787-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAT TAG ATA CCC SBG TAG TTC-3') and R1059 (5'-GCC ATG CAC CWC CTC T-3') for archaeal 16S (Hwang et al., 2008) with an amplicon size of 273 bp. PCR amplification was carried out under the following conditions: firstly 20 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 0.5 min, and then 10 or 15 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 0.5 min for *Bacteria* and *Archaea*, respectively, and a final extension of 72 °C for 10 min. Then, the amplicons were electrophoresed into a 2% (w/v) agarose gel to check the extent of the amplification. PCR products from each sample were then separated on an 8% acrylamide gel with a linear denaturing gradient, using the KuroGel Verti 2020 DGGE system (VWR International Eurolab, Spain). DGGE was conducted using 20 µL of PCR product in 1× Tris-acetate-EDTA buffer at 60 °C and 100 V for 840 min. DGGE gels were visualised using the MiniBIS Pro System (DNR Bio-Imaging System Ltd., Spain). Predominant bands were excised and resuspended in 30 µL of sterilised Mili-Q water at 4 °C overnight, allowing the migration of DNA from the gel to the water. This DNA was reamplified by PCR using the previously reported conditions and was then purified with the mi-PCR Purification Kit (Metabion, Germany). The obtained PCR products were sequenced using an automated DNA analyser (3730 KL DNA Analyzer, Applied Biosystems, Spain). Sequences were analysed with MEGA 5.0 and then compared with those available from the NCBI GenBank using BLAST software.

5.3.4 Quantitative Polymerase Chain Reaction (qPCR)

Genomic DNA of each sample was quantified by PicoGreen (ThermoFischer Scientific, USA). The abundances of *Bacteria*, *Archaea*, *Methanosarcinales*, *Methanomicrobiales* and *Methanobacteriales* were quantified by quantitative PCR by targeting a sequence of 16S rDNA. Specific primer and probe sets are summarised in Table 5.3. Amplification protocol for each population had been previously adjusted. Triplicate samples were run on a RotorGene 6000 thermocycler (Qiagen, Hilden, Germany) as follows: 4 min at 95 °C and then 45 cycles of 10 s at 95 °C and 30 s at 60 °C for *Bacteria* and *Archaea*; 4 min at 95 °C and 45 cycles of 10 s at 95 °C and 50 s at 60 °C for *Methanobacteriales* and *Methanosarcinales*; 4 min at 95 °C and 45 cycles of 10 s at 95 °C and 30 s at 63 °C for *Methanomicrobiales*.

Table 5.3. Primer and probe sequence and concentration for each target population used in qPCR.

Target population	Primer and probe	Conc. (nM)	Sequence
Archaea	ARC787F	200	ATTAG ATACC CSBGT AGTCC
	ARC915S	200	GCCAT GCACC WCCTC T
	ARC1059R	200	AGGAA TTGGC GGGGG AGCAC
Bacteria	BAC338F	200	ACTCC TACGG GAGGC AG
	BAC516S	200	GACTA CCAGG GTATC TAATC C
	BAC805R	200	TGCCA GCAGC CGCGG TAATA C
Methanomicrobiales	MMB282F	200	ATCGR TACGG GTTGT GGG
	MMB749S	200	CACCT AACGC RCATH GTTTA C
	MMB832R	200	TYCGA CAGTG AGGRA CGAAA GCTG
Methanobacteriales	MBT857F	400	CGWAG GGAAG CTGTT AAGT
	MBT929S	200	TACCG TCGTC CACTC CTT
	MBT1196R	400	AGCAC CACAA CGCGT GGA
Methanosarcinales	MSL812F	350	GTAAA CGATR YTCGC TAGGT
	MSL860S	200	GGTCC CCACA GWGTA CC
	MSL1159R	350	AGGGA AGCCG TGAAG CGARC C

The standard curves for each qPCR 16S rRNA gene sequence were obtained from a strain of each population that was amplified and cloned into pGEM-T Easy

vectors. For each plasmid, dilutions from 10^7 to 10^1 copies mL^{-1} were generated. The amplification was carried out in a reaction volume of 20 µL, containing 18 µL mix and 2 µL of DNA. Results were normalised to copy numbers g^{-1} volatile suspended solids (VSS).

5.3.5 High throughput sequencing

Selected DNA samples from the top port of AHR (0, 89, 132, 230 and 336 days) were analysed by high throughput sequencing. The high throughput sequencing was carried out by amplifying the V4 hyper variable region of 16S RNA gene of the extracted DNA from samples of AHR with the universal primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3'). Sequencing was performed using a MiSeq System (Illumina, San Diego, CA, USA). Raw 16S rRNA gene sequences obtained were screened and trimmed by using the Quantitative Insights Into Microbial Ecology (QIIME) software, with a sequence length of 200 nt and mean quality score cut-off of 25 nt.

5.4 RESULTS AND DISCUSSION

5.4.1 Impact of E2P and M2P addition on microbial community structure

5.4.1.1 *DGGE bands dynamics*

Archaeal and bacterial DGGEs from samples obtained during the previous experiment (Chapter 4) can be observed in Figures 5.1 and 5.2, respectively. While archaeal and bacterial DGGEs from samples taken at the bottom port (BP) and top port (TP) of EGSB and AHR before and after the introduction of E2P can be observed in Figures 5.3 and 5.4, respectively.

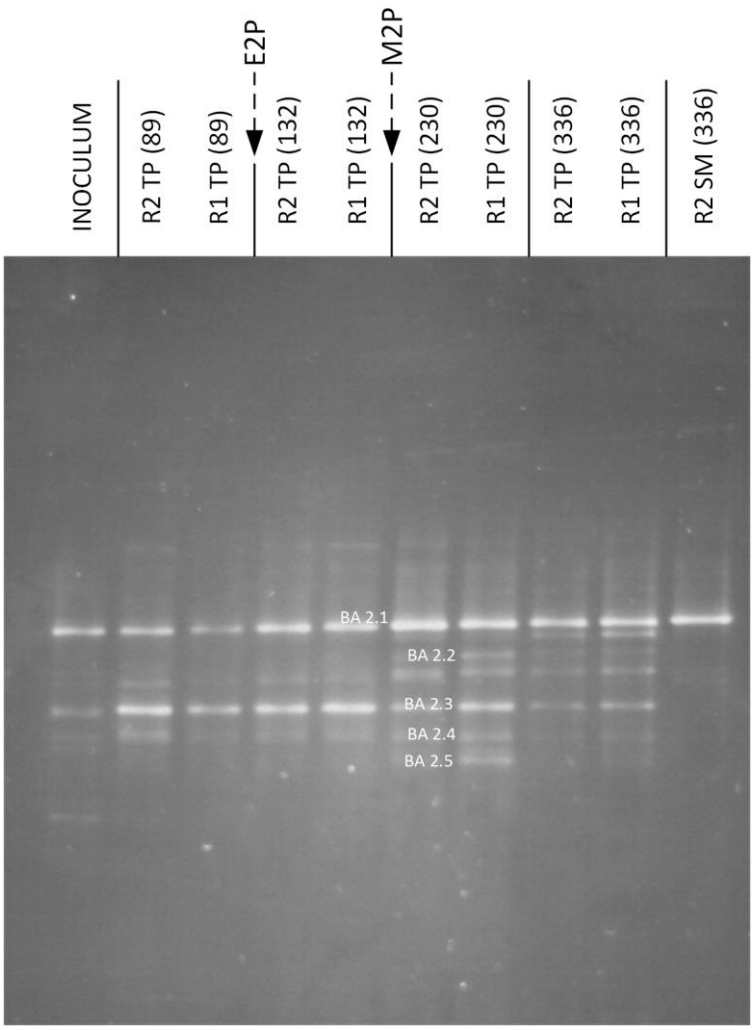

Figure 5.1. Archaeal DGGE profiles of biomass samples from the top port (TP) of both reactors and the support material (SM) of AHR during all experimental period. Day of experiment into brackets. R1: EGSB, R2: AHR. E2P introduced in day 91 and M2P introduced in day 221 (Table 5.1).

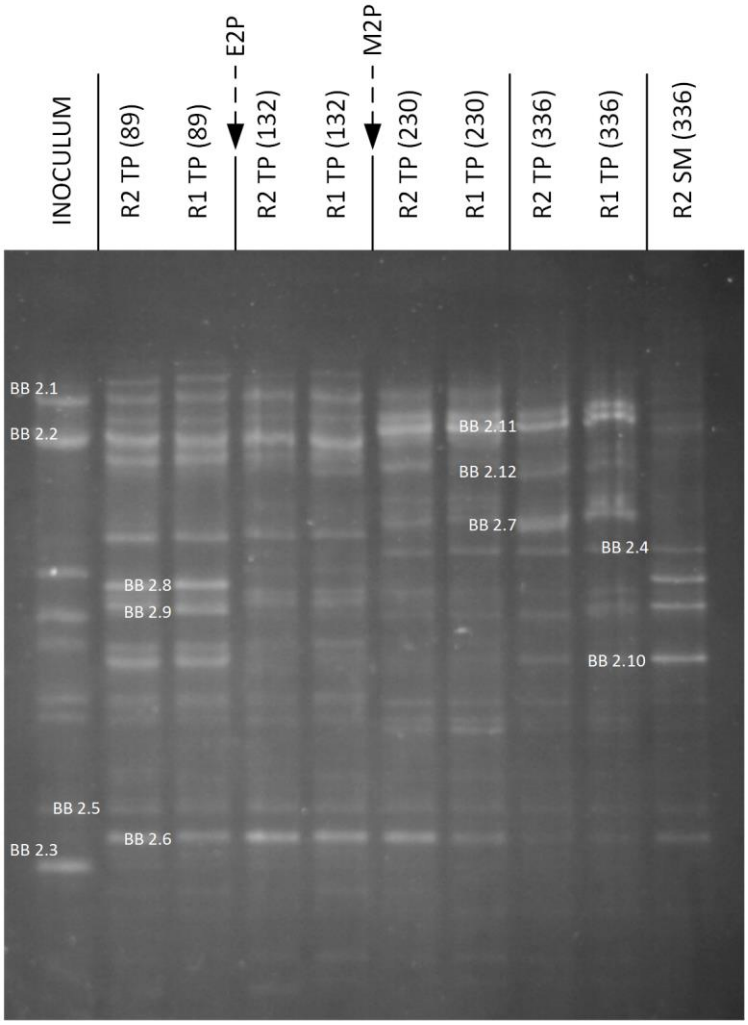

Figure 5.2. Bacterial DGGE profiles of biomass samples from the top port (TP) of both reactors and the support material (SM) of AHR during all experimental period. Day of experiment into brackets. R1: EGSB, R2: AHR. E2P introduced in day 91 and M2P introduced in day 221 (Table 5.1).

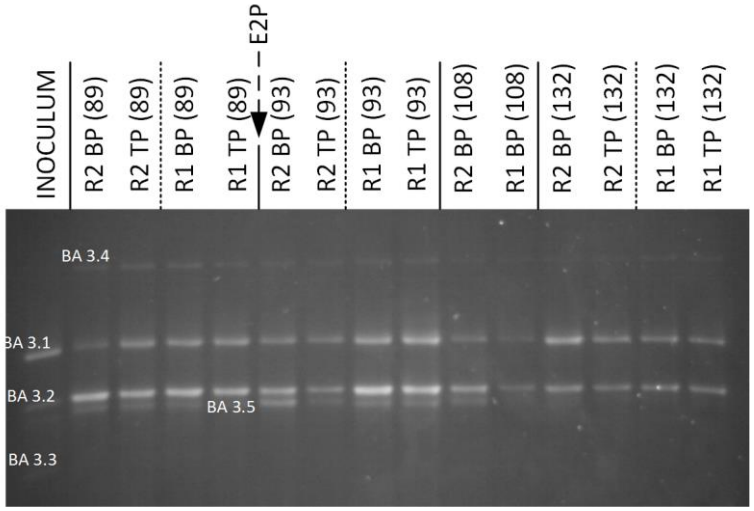

Figure 5.3. Archaeal DGGE profiles of biomass samples from bottom port (BP) and the top port (TP) from each reactor after the introduction of E2P. Day of experiments into brackets. R1: EGSB, R2: AHR. E2P introduced in day 91 (Table 5.1).

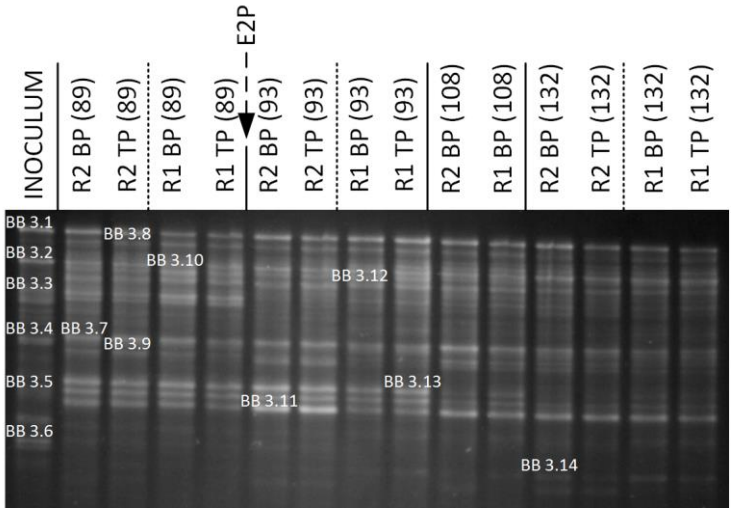

Figure 5.4. Bacterial DGGE profiles of biomass samples from bottom port (BP) and the top port (TP) from each reactor after the introduction of E2P. Day of experiments into brackets. R1: EGSB, R2: AHR. E2P introduced in day 91 (Table 5.1).

Both reactors developed a very similar and stable archaeal community with few predominant bands during the whole the experiment (BA 2.1, BA 2.3 and BA 2.4) (Figure 5.1). Nevertheless, when M2P was introduced into the synthetic feed, new bands appeared (BA 2.2 and BA 2.5). As these populations were not detected in the sludge source (inoculum), it is probable that these were involved in M2P biodegradation. With regard to *Bacteria* (Figure 5.2), a marked shift of the bacterial community structure from the beginning to the end of the trial can be observed. The intensity of some initial predominant bands (BB 2.1, BB 2.6, BB 2.8 and BB 2.9) decreased with time, while other bands (BB 2.4, BB 2.7, BB 2.11 and BB 2.12) became predominant. It is also noted that both EGSB and AHR bacterial communities stayed in relative proximity to each other. The addition of E2P and then M2P led to temporally synchronised microbial structure shifts and then new structures, still similar between both bioreactors. These temporal dynamics seem to explain the similar performance levels achieved by both reactors throughout the experimental period. The shift in the bacterial community due to the introduction of E2P can also be observed in Figure 5.4, leading to a progressive decrease in biodiversity. After E2P was introduced into the synthetic feed, the intensity of some predominant bands (BB 3.5, BB 3.7, BB 3.9 or BB 3.13) decreased, while the intensity of other bands increased (such as BB 3.10, BB 3.11 or BB 3.12). The addition of a new substrate (E2P) seemed to induce population losses and the emergence of more adapted bacterial groups. On the other hand, a stable diversity was observed for *Archaea* (Figure 5.3), regardless of the composition of the feed (addition of E2P); the emerging bands remained constant during the first 40 days after both reactors were supplied with E2P. In addition, the qualitative analysis of DGGE gels seemed to reveal a similar temporal dynamics of bacterial and archaeal community structure for both bioreactors fed with E2P.

5.4.1.2 *Identification of bands from DGGE gels*

Labelled bands (Figures 5.1, 5.2, 5.3 and 5.4) were sequenced and identified. Tables 5.4 and 5.5 summarise DGGE band designation of Figures 5.1 and 5.2, and Figures 5.3 and 5.4, respectively, and the level of similarity to related Genbank sequences and the phylogenetic group of each strain.

Table 5.4. DGGE band designation, accession numbers in GenBank and levels of similarity to related organisms according to Figures 5.1 and 5.2.

DGGE Band	Closest microorganism in GenBank (accession number)	Similarity (%)	Phylogenetic group
BA 2.1	*Methanosaeta concilii* (NR_102903)	98	*Methanosarcinales* [a]
BA 2.2	*Methanospirillum hungatei* (NR_118366)	97	*Methanomicrobiales* [a]
BA 2.3	*Methanobacterium formicicum* (NR_115168)	99	*Methanobacteriales* [a]
BA 2.4	*Methanobacterium aggregans* (NR_135896)	86	*Methanobacteriales* [a]
BA 2.5	*Methanobrevibacter arboriphilus* (NR_042783)	98	*Methanobacteriales* [a]
BB 2.1	*Anaerosporomusa subterranea* (NR_152052)	76	*Firmicutes* [b]
BB 2.2	*Syntrophothermus lipocalidus* (NR_102767)	74	*Firmicutes* [b]
BB 2.3	*Thermomarinilinea lacunifontana* (NR_132293)	84	*Chloroflexi* [b]
BB 2.4	*Mariniphaga anaerophila* (NR_134076)	79	*Bacteroidetes* [b]
BB 2.5	*Lentimicrobium saccharophilum* (NR_149765)	86	*Bacteroidetes* [b]
BB 2.6	*Desulfotomaculum tongense* (NR_133738)	90	*Firmicutes* [b]
BB 2.7	*Clostridium cellulolyticum* (NG_041947)	80	*Firmicutes* [b]
BB 2.8	*Pelobacter propionicus* (NR_074975)	94	*Proteobacteria* [b]
BB 2.9	*Geobacter psychrophilus* (NR_043075)	97	*Proteobacteria* [b]
BB 2.10	*Smithella propionica* (NR_024989)	97	*Proteobacteria* [b]
BB 2.11	*Clostridium sufflavum* (NR_041497)	79	*Firmicutes* [b]
BB 2.12	*Cellulosilyticum lentocellum* (NR_119067)	88	*Firmicutes* [b]

[a] Order; [b] Phylum.

Bands BA 3.1 and BA 2.1 were identified as a *Methanosaeta concilii* (*Methanosarcinales* order). This specie is anaerobic and mesophilic and is known as an acetoclastic methanogen. *Methanosaeta* species are usually found in anaerobic digesters, especially where volatile fatty acid (VFA) concentrations are low, due to its higher affinity to acetate than other acetoclastic methanogens, such as *Methanosarcina* (Jetten et al., 1990). This population is then consistent with the known ecophysiology of this bacteria as low levels of VFA were measured within both bioreactors (average values of 32.9 mg L^{-1} and 20.6 mg L^{-1} in the EGSB and AHR, respectively) during the whole experimental period, as it can be observed in the previous chapter. Bands BA 3.2, BA 1.5, BA 2.3 and BA2.4 were identified as *Methanobacterium*, known as hydrogenotrophic methanogens, and most species of this genus have been isolated from mesophilic neutral systems, such as anaerobic digesters (Mori and Harayama, 2011). Band BA 3.4 was identified as *Methanocorpusculum*, a strictly anaerobic archaea with an optimum growth temperature of 37 °C. These archaea can produce methane from H_2/CO_2, formate and 2-propanol/CO_2 and have been identified in anaerobic reactors. Luo et al. (2016) found that an external recirculation combined with a HRT of less than two days induced the dominance of *Methanobacterium* and *Methanocorpusculum*. This finding is in accordance with results of our study as both reactors worked with similar external recirculation and HRT. Band BA 2.2 was identified as *Methanospirillum hungatei*, a known hydrogenotrophic methanogen which has been identified in anaerobic reactors, as Fu et al. (2011) found *Methanospirillum hungatei* to be predominant in an EGSB reactor. Besides, this archaea appeared when both bioreactors were fed with M2P. It is possible that this population is involved in the removal of this contaminant. Nevertheless, syntrophic partners are required to remove M2P as *Methanospirillum hungatei* uses only H_2 and CO_2 for its development.

Table 5.5. DGGE band designation, accession numbers in GenBank and levels of similarity to related organisms according to Figures 5.3 and 5.4.

DGGE Band	Closest microorganism in GenBank (accesion number)	Similarity	Phylogenetic group
BA 3.1	*Methanosaeta concilii* (NR_102903)	99	*Methanosarcinales* [a]
BA 3.2	*Methanobacterium formicicum* (NR_115168)	99	*Methanobacteriales* [a]
BA 3.3	*Methanolinea tarda* (NR_028163)	99	*Methanomicrobiales* [a]
BA 3.4	*Methanocorpusculum aggregans* (NR_117749)	99	*Methanomicrobiales* [a]
BA 3.5	*Methanobacterium petrolearium* (NR_113044)	99	*Methanobacterium* [a]
BB 3.1	*Anaesporomusa subterranea* (NR_152052)	76	*Firmicutes* [b]
BB 3.2	*Paludibaculum fermentans* (NR_134120)	88	*Acidobacteria* [b]
BB 3.4	*Desulfomicrobium aestuarii* (NR_133694)	89	*Proteobacteria* [b]
BB 3.5	*Lentimicrobium saccharophilum* (NR_149795)	89	*Bacteroidetes* [b]
BB 3.6	*Thermomarinilinea lacunifontana* (NR_ 132293)	90	*Chloroflexi* [b]
BB 3.8	*Bacteroides propionicifaciens* (NR_112941)	86	*Bacteroidetes* [b]
BB 3.9	*Geobacter daltonii* (NR_074916)	95	*Proteobacteria* [b]
BB 3.10	*Desulfonispora thiosulfatigenes* (NR_026497)	78	*Firmicutes* [b]
BB 3.11	*Geobacter uraniireducens* (NR_074940)	93	*Proteobacteria* [b]
BB 3.12	*Syntrophorhabdus aromaticivorans* (NR_041306)	81	*Proteobacteria* [b]
BB 3.14	*Desulfoglaeba alkanexedens* (NR_043705)	93	*Proteobacteria* [b]

[a] Order; [b] Phylum.

Regarding the identification of bands from bacterial DGGEs, it is remarkable that during the start-up (day 89), dominant bacterial populations (bands BB 3.4, BB 3.9, BB 3.11, BB 3.12, BB 3.13, BB 2.8, BB 2.9 and BB 2.10) belong to the *Proteobacteria* phylum and, more specifically, to the *Geobacter* genus (BB 3.9, BB 3.11 and BB 2.9). Some species from this genus can couple the reduction of Fe(III) with the oxidation of substrates, such as ethanol, acetate, formate, butanol benzoate and toluene (Nevin et al., 2005; Prakash et al., 2010; Shelobolina et al., 2008). Furthermore, it has been demonstrated that these bacteria can be associated syntrophically with methanogens, such as *Methanosaeta* species, by direct interspecific electron transference (Rotaru et al., 2014). It is probable that, in our study, such metabolic interactions between community members occur within the biofilm. However, at the end of the trial, the predominant bacterial groups belong to *Firmicutes* phylum (bands BB 2.1, BB 2.2, BB 2.6, BB 2.7, BB 2.11 and BB 2.12) and to *Clostridia* genus (BB 2.7 and BB 2.11). These microorganisms became dominant when the reactors were fed with M2P. So the enrichment of these populations favoured by specific operating conditions (addition of M2P) is probably due to their competitive advantages in terms of growth and substrate affinity. The development of *Clostridium* is probably due to the presence of methanol as an intermediate compound of M2P degradation, as this population has the ability to metabolise methanol.

5.4.1.3 *qPCR study*

qPCR results for EGSB reactor and AHR can be observed in the Figures 5.5 and 5.6, respectively. The concentration of *Bacteria* varied from $6.1 \cdot 10^9$ to $3.9 \cdot 10^{10}$ copies g^{-1} VSS and from $6.1 \cdot 10^9$ to $7.0 \cdot 10^{10}$ 16S rRNA gene copies g^{-1} VSS within bioreactors EGSB and AHR (Figures 5.5 and 5.6), respectively. During the experimental period, the densities of total *Archaea* fluctuated from $2.3 \cdot 10^8$ to $2.4 \cdot 10^{10}$ 16S rRNA gene copies g^{-1} VSS and from $2.3 \cdot 10^8$ to $1.1 \cdot 10^{10}$ 16S rRNA gene copies g^{-1} VSS within EGSB and AHR bioreactors respectively (Figures 5.5 and 5.6). Other studies with anaerobic granular sludge reported similar values (Siggins et al., 2011). Furthermore, *Methanobacteriales*, *Methanosarcinales* and *Methanomicrobiales* orders were quantified for all the samples. It is possible that the selective pressure was not high enough to induce shifts in community composition, leading to the dominance of a specific order. These results are in agreement with the archaeal DGGE study which reveals a stable methanogenic population in which different populations belonging to the three orders above

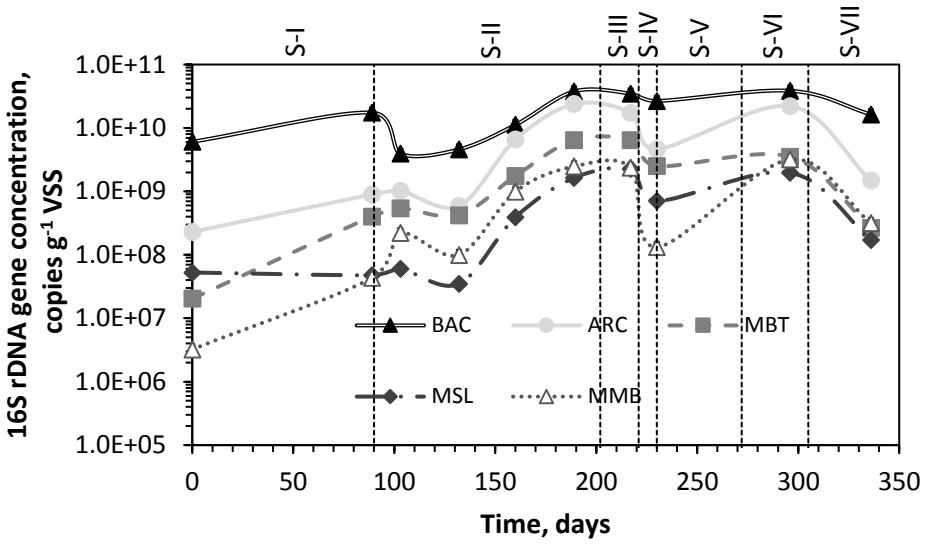

Figure 5.5. Evolution of 16S rDNA gene concentration in the EGSB reactor for *Bacteria* (BAC), *Archaea* (ARC), *Methanobacteriales* (MBT), *Methanosarcinales* (MSL) and *Methanomicrobiales* (MMB).

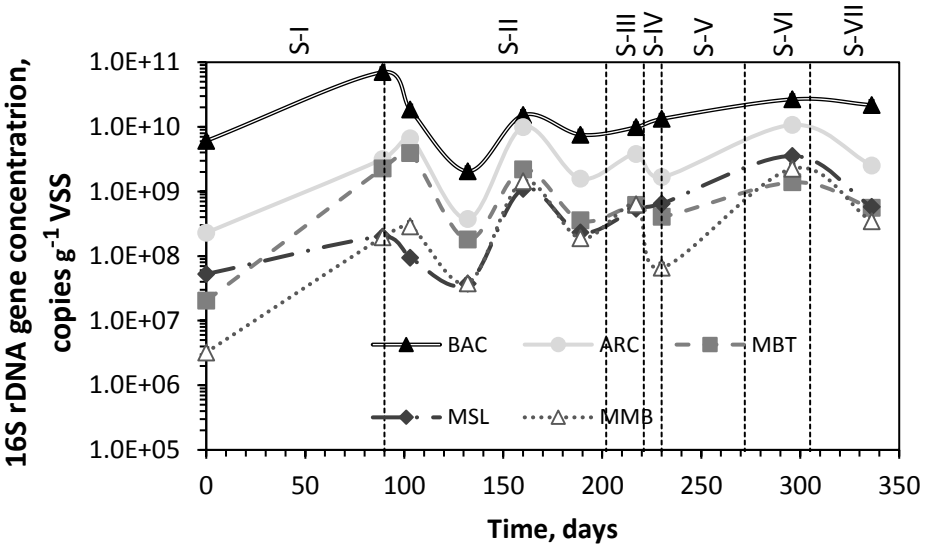

Figure 5.6. Evolution of 16S rDNA gene concentration in the AHR reactor for *Bacteria* (BAC), *Archaea* (ARC), *Methanobacteriales* (MBT), *Methanosarcinales* (MSL) and *Methanomicrobiales* (MMB).

are detected. Thus, acetoclastic and hydrogenotrophic methanogenesis seem to occur in both reactors. This finding is in concordance with the work of Connelly et al. (2017). These authors reported that acetoclastic and hydrogrenotrophic methanogenesis mainly occurs in stable laboratory-scale operated bioreactors, while the hydrogenotrophic pathway is dominant within full-scale bioreactors.

Although the concentration of the three orders varied throughout the experimental period and *Methanosaeta* species (member of *Methanosarcinales* order) are known to dominate EGSB reactors with low VFA concentrations (Connaughton et al., 2006), *Methanobacteriales* was the population with the higher number of copies g^{-1} VSS in all samples, except in the inoculum, which could suggest that hydrogenotrophic methanogenesis was an important pathway for producing methane in these reactors. The same finding was reported by Song et al. (2010) in a pilot-scale UASB plant treating swine wastewater at low HRT (3.5 days). They also concluded that *Methanosaetaceae* had a role in the granule sustainability and promoted a stable operation despite of the lower concentration of *Methanosaetaceae*. In any case, consumption of generated VFA by *Methanosaeta* can have an important role in the granule sustainability, as was postulated by Song et al. (2010).

During the start-up period (S-I), densities of bacterial and archaeal groups increased (Figures 5.5 and 5.6). This development could be linked to the increase of the OLR in both reactors (Table 5.1). In S-II, E2P was introduced with an average OLR of 5.1 kg COD m^{-3} d^{-1}; the total OLR remaining unchanged (Table 5.1). From the beginning of S-II to day 132, the RE of E2P was relatively low (<50 %) (Chapter 4). As can be observed in Figures 5.5 and 5.6, an important drop in the bacterial and archaeal densities was observed. This decrease demonstrates, not only an incapacity of the biological system to use E2P as a substrate, but a toxic effect when it reached a concentration of 1800 mg COD L^{-1} (OLR of 4.5 kg COD m^{-3} d^{-1}), causing a decay of a significant fraction of the quantified active biomass. From day 132, the RE gradually improved through an acclimatisation period that could be related to the development of ether-cleavage enzymes to degrade E2P, as it has been suggested in the previous chapter (Section 4.4.2). Thus, the removal of E2P causes a gradual increase in densities for each population until values were similar to those prior to E2P introduction in the synthetic feed. During S-III, the OLR of E2P was increased to 10.6 kg COD m^{-3} d^{-1}, but in this period the populations seem to be not affected negatively as the density values are stable or slightly increase; this could be due to the previous adaptation of the microorganisms (S-II) and the selection of the fittest species. M2P was then introduced as new substrate (Table 5.1) with an OLR

of 4.8 kg COD m^{-3} d^{-1} (S-IV). In this stage (Figures 5.5 and 5.6), the concentration of *Bacteria* remained practically unchanged within both reactors. Thus, it seems that this substrate does not have a negative impact on bacterial densities. This result supports the hypothesis formulated in the previous chapter that this substrate would be biodegraded by using the same metabolic pathway as E2P. Additionally, this can explain the difference between this no adaptation period to degrade M2P and the long adaptation period (40 days) reported by Lafita et al. (2015), where M2P was the first glycol ether added. In contrast to *Bacteria*, a slight decrease of *Archaea*, *Methanomicrobiales* and *Methanobacteriales* densities was observed for both reactors. This behaviour could be attributed to the production of intermediate compounds during biodegradation of M2P. In this sense, in the previous chapter it was showed that E2P and M2P degradation generate production and accumulation of acetone and isopropanol, as well as ethanol (in case of E2P) or methanol (in case of M2P). Besides, Cerrillo et al. (2016) reported a decrease of the relative abundance of both *Methanobacteriaceae* and *Methanoregulaceae*, members of *Methanobacteriales* and *Methanomicrobiales* orders, respectively, in an UASB reactor fed with methanol. This could support the view that the densities of these archaeal populations were mainly affected by the production of intermediate products (methanol) rather than the primary substrate (M2P). In the period S-VI, a start-up was carried out after a long starvation period using a mixture of ethanol, E2P and M2P. During this period, bacterial densities remained stable and all archaeal populations recovered previous density levels (S-III). Finally, when the OLR of M2P was increased to 8.3 kg COD m^{-3} d^{-1} (S-VII), RE (around 98-99%) and biogas production values were unchanged while abundances of all archaeal populations decreased more drastically. As previously discussed, this decrease could be due to the toxic effect of intermediate compounds on some methanogens leading to population losses.

5.4.1.4 *High throughput sequencing study*

The analysis of total bacterial diversity within the AHR bioreactor is presented in Figures 5.7 and 5.8. *Proteobacteria*, *Firmicutes* and *Bacteroidetes* phyla were dominant along the experiment. Yang et al. (2018) observed a similar distribution within sludge from a digester implemented into a municipal wastewater treatment plant. They reported that most *Proteobacteria* were facultative bacteria and the dominance of this phylum increased with shorter HRT.

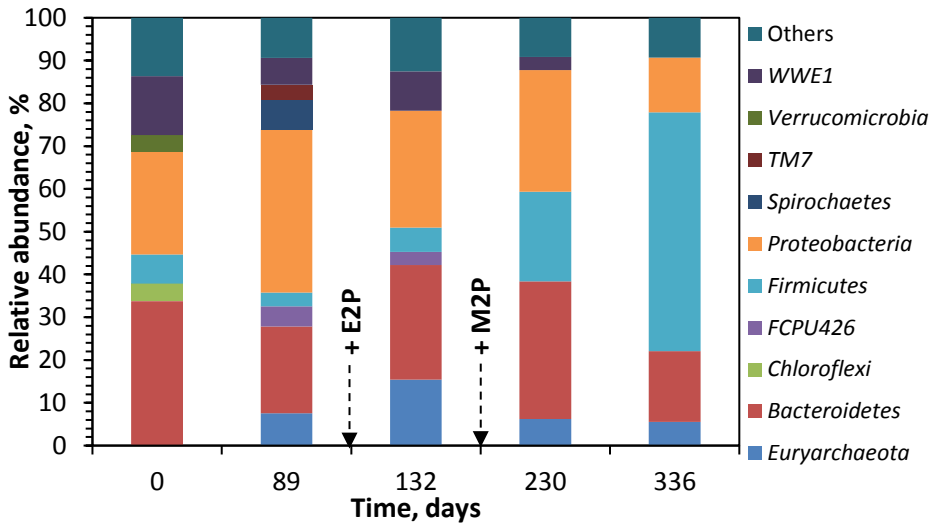

Figure 5.7. Taxonomic classification of the microbial communities at phylum level in the samples of AHR taken in the top port at different times. Arrow show glycol ether introductions.

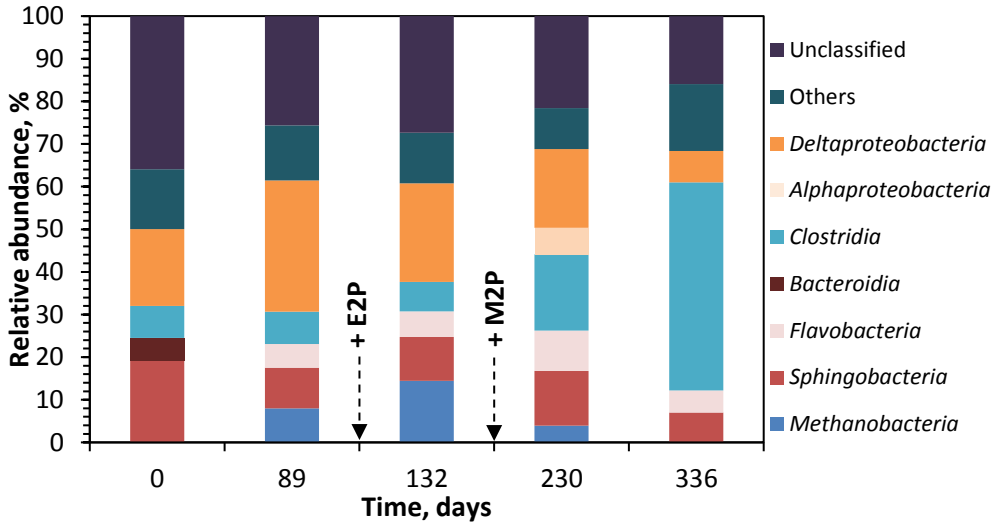

Figure 5.8. Taxonomic classification of the microbial communities at class level in the samples of AHR taken in the top port at different times. Arrow show glycol ether introductions.

Microorganisms belonging to *Firmicutes* phylum are facultative microorganisms and are known to produce various organic acids (Mohan et al., 2011). Microorganisms from the phylum *Bacteroidetes* are known to be involved in hydrolysis and acidogenesis steps (Dias et al., 2016).

In the inoculum, the phylum *Bacteroidetes* was the dominant phylum with a relative abundance of 34%, but at the end of the start-up of the reactors the relative abundance of this phylum decreased to 20%. This drop could be associated with the composition of the synthetic feed, since during the start-up period, only ethanol was used as substrate, therefore no hydrolysis step was required. These operating conditions could explain the population loss of *Bacteroidetes* during this stage as *Bacteroidetes* is involved in such a metabolic pathway. Then, when a more complex substrate, such as E2P and M2P, was introduced into the synthetic feed, *Bacteroidetes* recovered its relative abundance up to 32% in the stage S-IV (day 230). During the last stages, when M2P was added to the feeding tank of reactors, the *Bacteroidetes* population decreased to 17% (S-VII) and this could be due to the competition with more adapted microorganisms.

Proteobacteria was the second most represented phylum in the inoculum (24%). The relative abundance of this phylum increased notably up to 38% during the start-up period as these microorganisms are able to grow using only ethanol as a carbon source. Furthermore, most of sequences of this phylum belong to *Deltaproteobacteria* class (Figure 5.8) and *Geobacter* genus and it has been reported that *Geobacter* species are dominant in anaerobic reactors operating with ethanol (Xing et al., 2017). Then, the relative abundance of *Proteobacteria* phylum decreased from 38 to 13% as E2P and M2P were added in the synthetic feeding from 38 to 13%. This important loss could be explained by competition exclusion and/or toxic effect of by-products linked to the metabolical pathways of E2P and M2P.

The *Firmicutes* phylum was poorly represented in the inoculum during the first 132 days of operation (maximum relative abundance of this phylum reached 7%). Nevertheless, this trend changed drastically with the introduction of M2P in the polluted feed, as the relative abundance increased from 6% at day 132 to 21% at day 230 and to 56% at the end of the trial. This result indicates that *Firmicutes* has some competitive advantages in terms of M2P affinity or intermediate biodegradation compounds (methanol and acetone). As acetone is another intermediate compound of the ether cleavage of E2P, as it has been showed in the previous chapter, and no significant increase of the relative abundance of *Firmicutes* was observed after the introduction of E2P (day 89), the competitive advantage

could be preferentially linked to the anaerobic degradation of methanol. Furthermore, almost all the sequences of *Firmicutes* phylum belong to the *Clostridia* class (Figure 5.8). Some microorganisms of this class, such as *Clostridium*, are acetogens, and some species can ferment methanol to acetate (Widdel, 1998). Moreover, it has been assumed that *Clostridium fervidus* could establish syntrophic relationships with methanogens to grow on methanol (Roest et al., 2005). Therefore, the increase in the relative abundance of *Firmicutes* during the last steps of the run could be associated with the ability of some species of *Clostridia* class to metabolise methanol.

Euryarchaeota phylum, which includes the methanogenic archaea, was not detected in the inoculum, but with time the relative abundance of this phylum changed during the experimental period within the range of 6 to 16%. The maximum relative abundance (16%) of this phylum was achieved after the introduction of E2P (day 132). Besides, the major sequences represented in this phylum corresponded to *Methanobacteria* class which was the most abundant methanogenic population throughout the experimental period, as revealed qPCR analysis.

WWE1 phylum was the third most represented phylum in the inoculum (14%). Nevertheless, the relative abundance diminished with time and this finally became a minority group. Practically all the sequences identified in this phylum belong to *Cloacamonae* class (Figure 5.8) and some bacteria from this class, such as *Candidatus cloacamonas acidaminovorans*, are probably syntrophic bacteria that are present in many anaerobic digesters. This microorganism have been also identified in the anaerobic granular sludge from a brewery water treatment plant used as inoculum in the prototype reactor, as it will be discussed in Chapter 7

5.4.2 Stratification of microbial community from DGGE analysis

Almost identical biodiversity was highlighted for all sludge samples collected at the top and the bottom of both bioreactors (Figures 5.3 and 5.4). No microbial stratification was detected, which could be due to the fluid flow behaviour of the granular sludge bed of both reactors being closer to a mixed flow model than to a plug flow model, and in contrast to that reported by Ambuchi et al. (2016). The low value of Peclet number (<1) can suggest the behavior of a perfectly mixed reactor.

Furthermore, the structure of the bacterial community retained in the packing material at the top of the AHR was considerably different from the bacterial

community within the sludge bed. Moreover, the structure of the bacterial community within the packing material, as can be observed in Figure 5.2, seemed to be close to the bacterial structure observed at the first stages of the trial (S-I, day 89); some populations (BB 2.8, BB 2.9 and BB 2.10) observed at the beginning of the experiment (S-1) within the sludge bed were still dominant at the end of the trial (day 336) within the packing material. In addition, the diversity of the archaeal community of the packing material (Figure 5.1) seems to be largely reduced as the population corresponding to the band BA 2.1 was predominant. This could be explained by a specialization of the archaeal population in this part of the reactor. As almost identical performance of both systems was observed for both reactors, the populations within the packing material seem not to be directly involved in the contaminant removal. This band (BA 2.1), identified as *Methanosaeta concilii*, could indicate that the methanogenic population developed and supported on the packing separator material contains, notablym acetoclastic species, that are able to develop more favorably in an environment with a low acetate concentration.

Concerning the bacterial composition within the packing material, at the end of the period the predominant bands (BB 2.8, BB 2.9 and BB 2.10) belonged to the *Proteobacteria* phylum. As the replacement of colonised packing material with new material induces a slight increase of VFA concentration in the effluent of AHR, as it has been described in the previous chapter (Chapter 4), it is possible that these populations are propionate, formate and/or acetate consumers (Liu et al., 1999; Nevin et al., 2005; Schink et al., 1987).

5.5 CONCLUSIONS

The microbial evolution of sludge within both AHR and EGSB bioreactors used for the treatment of ethanol-glycol ether mixtures were similar, except in the packing material located into the upper part of the AHR wherein a VFA degrading population was identified. No stratification was observed along the sludge bed in either reactor, probably due to the high mixture effect inside the reactors. A toxic effect of E2P on both bacterial and archaeal densities was observed, while the addition of M2P only temporarily affected the archaeal population. High throughput sequencing highlights a succession from Proteobacteria to Firmicutes when anaerobic bioreactors were fed with M2P.

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6 BEHAVIOUR, STABILITY, AND MICROBIAL COMMUNITY ANALYSIS OF EGSB REACTOR AT HIGH CONTENT OF GLYCOL-ETHER SOLVENTS IN MIXTURES WITH ETHANOL

6.1 ABSTRACT

Performance and microbial community analysis of an expanded granular sludge bed reactor (EGSB) treating a mixture of ethanol and relatively high content of glycol ethers, such as 1-ethoxy-2-propanol (E2P) and 1-methoxy-2-propanol (M2P), was evaluated. Results showed good EGSB performance in terms of global Removal Efficiency (RE > 95%) when only ethanol was fed. When glycol ethers were added, RE was intially 80%, but further increased to 90%, indicating a period of adaption. When the proportion of glycol ethers was increased and a binary mixture of E2P and M2P was fed, RE dropped to 80%. RE of individual glycol ethers was significantly different after the acclimatation period, while M2P degradation was complete (100%), RE of E2P reached only 65%. In the last stage, when the EGSB reactor was fed only E2P and M2P, a partial degranulation of the sludge bed occurred, revealing the importance of ethanol as a substrate for maintaining the granular structure of the sludge. Microbial community analysis showed a relation between the substrate utilized in the reactors and the microbial populations that developed, such as *Methanosaeta*, an acetate consumer, and *Methanomethylovorans*, a methanol consumer, which were the predominant microorganisms in the archaeal population; the predominant bacterium found was Geobacter, an ethanol consumer. Extracellular Polymer Substances (EPS) analysis revealed a lower content of proteins and polysaccharides in the EPS of degranulated biomass, and the ratio of protein to polysaccharides decrased from 6.3 to 5.4 in the degranulated biomass, suggesting that this parameter could be used as an indicator of the granule health.

6.2 INTRODUCTION

Glycol ethers have many uses as solvents due to their amphiphilic structure; one end binds to water—the hydrophilic part—and one end binds to oils—the lypophilic part. These compounds have been extensively used as solvents for resins, lacquers, paints, varnishes, gum, perfume, dyes, inks, and as a constituent of paints and pastes, cleaning compounds, liquid soaps, cosmetics, and hydraulic fluids. Although some of these compounds such as 2-methoxyethanol, 2-ethoxyethanol and 2-butoxyethanol have been classified as hazardous compounds because both short and long term exposure may be toxic in humans (EPA 2000), and specific precautions and handling procedures are required (Ketttenis 2005). Other ether glycols, such as 1-methoxy-2-propanol (M2P) and 1-ethoxy-2-propanol (E2P), have negligible human toxicity and has increasinged in its use. These compounds are usually mixed with other main solvents, such as ethanol in printing or the flexographic industry, and later are evaporated, promoting emissions of volatile organic compounds (VOCs) (Passant, 2002). These air emissions have been regulated in many countries (USEPA 2006, Council Directive 2010/75/EU) and recent studies have reported that these source, among other similar sources, now constitute half of fossil fuel VOC emissions in industrialized areas (Mc Donald et al. 2018), and therefore, end-of-pipe treatment still seems necessary. In this regard, a new process for treatment of atmospheric emissions of VOCs based on an anaerobic treatment has recently been described (Waalkens et al. 2015); VOCs in the air are first scrubbed with water and then degraded anaerobically in an EGSB reactor, thus recycling dilute organic waste gases into bioenergy.

The anaerobic degradation of both M2P and E2P has been scarcely reported, except in an early study by the European Chemicals Bureau (2006) where M2P anaerobic degration has been described using an inoculum of a municipal digester. The rest of the research has been carried out recently by our research group. Lafita et al. (2015) demonstrated that in an EGSB reactor supplied with a binary mixture of M2P and ethanol at a 4:1 mass ratio, anaerobic degradation is feasible at mesophilic (18°C) and psychrophilic (25°C) temperatures. At first, an adaptation of the biomass was carried out with ethanol, obtaining efficiencies that were close to 100% for psychrophilic conditions and introducing loads of 35 kg COD m^{-3} d^{-1}. Subsequently the composition was changed by introducing M2P. Finally, efficiencies of 94% at 18°C and 97% at 25°C were achieved with organic M2P charges of 6.1 and 9.0 kg COD m^{-3} d^{-1}. The anaerobic degradation of the M2P and E2P together with ethanol has also been studied (Vermorel, 2017) in a stirred tank reactor, showing that the degradation of ethanol is not inhibited by both the presence of M2P or E2P. In the same study, the organic load rates and acclimation times were also determined on a pilot scale. As would be expected, the degradation of ethanol was faster than the rest of the solvents. The anaerobic bioscrubber technology has

proved to be an effective solution for the treatment of industrial and real emissions of VOCs coming from the flexographic sector using an EGSB reactor (Bravo et al., 2017). In this study, the residual current was composed of ethanol as the main contaminant and in smaller quantities other contaminants could be found, such as 1-propanol, 2-propanol, M2P and 3-ethoxy-1-propanol, obtaining efficiencies of 99.6%.

In this regard, EGSB is one of the best-selling high-rate anaerobic reactors based on granular sludge technology for wastewater treatment (van Lier, 2008). The success of this configuration is due to the greater contact between biomass and wastewater, enabling treatment of poorly biodegradable compounds (Zouttberg, 1997; Puyol, 2009) and higher loading rates (Seguezzo, 1998), compared to wastewater treated with an upflow anaerobic sludge blanket (UASB) reactor. One of the potential drawbacks of an EGSB reactor is the eventual loss of biomass. Although several strategies have been studied to enhance the suitable retention of biomass granules, including the addition of granulation promoter compounds (Lafita et al., 2018) or biomass retaining elements, as has been studied in Chapter 4, further research is still needed to give a deeper understanding of the aspects involved. In this previous chapter it has been shown that EGSB treatment is a feasible technology for the biodegradation of ethanol-glycol ether mixtures like E2P and M2P after a short period of acclimatization. The system could completely remove ethanol and M2P (M2P organic loading rates (OLR) of 8.3 kg COD m^{-3} d^{-1}), but only removed 70% of the E2P (E2P OLR of 10.6 kg COD m^{-3} d^{-1}). In that study, (Chapter 4 and 5) only ternary mixtures of ethanol:E2P:M2P with a relatively low proportion of glycol ethers (< 30% in weight) were used. The analyses of the microbial communities, performed through denaturing gradient gel electrophoresis and high-throughput sequencing, revealed that E2P showed an initial toxic effect on bacterial and archaeal populations, while M2P only affected the archaeal population; however, both populations recovered after the aclimatation period. In addition, the same study noted that the introduction of glycol-ethers shifted the microbial community analysis, especial for M2P, showing that different populations can be predominant, depending on the type of the solvent.

The work presented here represents the continuation of previous studies. Thus, the main goal was to determine the influence of diminishing the ethanol content, until its disappearance, in the treated mixture and the effect on granules and microbial communities.

6.3 MATERIALS AND METHODS

6.3.1 Reactor design and operation

As in previous chapters, the experiment was carried out in an EGSB reactor with an effective volume of 4 L and a total volume of 19 L. Anaerobic granular sludge from a brewery wastewater treatment plant (Font Salem SL, El Puig) was used to seed the reactor. A gas seal containing NaOH was used to retain CO_2 and H_2S from biogas. A recirculation flow of 30 L h^{-1} was provided with a peristaltic pump reaching an upflow velocity close to 10 m h^{-1}. Feed was buffered with bicarbonate (5 g L^{-1}) and Ca^{+2} and Mg^{+2} were introduced to the feed with a constant concentration of 40 mg L^{-1}. The rest of the macro and micronutrients were dosed in relation with the COD concentration in the same way that it was carried out in previous chapters as can be found in Table 6.1. Flow was kept constant at 10 L d^{-1}, so the increases in OLR were achieved by increasing the COD concentration in the feed. A schematic of the EGSB reactor can be found in Figure 4.1 in Chapter 4.

Table 6.1. Macronutrient and micronutrient supplementation influent.

Compound	Dose, mg g^{-1} COD	Compound	Dose, mg g^{-1} COD
NH_4Cl	15.7	H_3BO_3	0.1143
Yeast extract	7.5	EDTANa	0.100
KCl	6.1	$(NH_4)_2Mo_7O_{24}{\cdot}4H_2O$	0.0625
$(NH_4)_2HPO_4$	5.6	Al_2O_3	0.0595
$FeCl_3{\cdot}6H_2O$	0.4208	$NiSO_4{\cdot}6H_2O$	0.0447
$CoCl_2{\cdot}6H_2O$	0.1615	$CuCl_2{\cdot}2H_2O$	0.0134
$MnCl_2{\cdot}4H_2O$	0.1441	$ZnSO_4{\cdot}7H_2O$	0.132

Operational conditions can be observed in Table 6.2. In a manner similar to previous studies, the start-up of the reactor was carried out during the first three stages by progressively increasing the OLR with a very readily biodegradable substrate, such as ethanol, until an average OLR was achieved in Stage III of 40.05 kg COD m^{-3} d^{-1}. After that, ethanol was replaced gradually by glycol ethers (M2P and E2P), maintaining a constant OLR around 45 kg COD m^{-3} d^{-1} in the feed (Table 6.2).

Table 6.2. Operational Conditions of the EGSB reactor at the different stages.

Stages	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
Duration, d	4	7	7	32	17	7	11	3
EtOH: M2P: E2P*	10:00:00	10:00:00	10:00:00	8:01:01	7:1.5:1.5	5:2.5:2.5	2:04:04	0:05:05
OLR, kg COD m^{-3} d^{-1}	15	32.83	40.05	41.01	45.01	39	47.16	44.05
C_S, g COD L^{-1}	6	13.13	16.02	16.4	18	15.6	18.86	17.62

* Mass ratio
C_s concentration

In stage IV the total average OLR was 41.01 kg COD m^{-3} d^{-1} with a mass ratio ethanol:M2P:E2P of 8:1:1; in stage V the total average OLR was 45.01 kg COD m^{-3} d^{-1} with an ethanol:M2P:E2P mass ratio of 7:1.5:1.5. In stage VI the total average OLR was 39.00 kg COD m^{-3} d^{-1} with an ethanol:M2P:E2P mass ratio of 5:2.5:2.5; in stage VII the total average OLR was 47.16 kg COD m^{-3} d^{-1} with a ethanol:M2P:E2P mass ratio of 2:4:4, and finally in the last stage (VIII) the total OLR average was 44.05 kg COD m^{-3} d^{-1} in the absence of ethanol and with a mass ratio M2P:E2P of 5:5.

6.3.2 Effluent and biogas analysis

Inlet and outlet COD, volatile fatty acids (VFA), total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods (American Public Health Association, 1999). Total removal eficiencies were determined from inlet and outlet COD values. Solvent concentration was analysed using a gas chromatograph equipped with a flame ionization detector, where solvents were separated in a Restek Rtx-VMS (USA) column (30 m long x 0.25 mm i.d. x 1.4 µm film thickness), and helium was used as the carrier gas. Compounds RE was obtained from these values. Methane flow rate was obtained using a gas meter (MGC-10PMMA, Ritter, Germany) after removing CO_2 and H_2S in the gas seal. Biogas composition was measured with a portable biogas analyzer (COMBIMASS® GA-m, Binder, Germany).

Additionally, to monitor the biomass substrate yield (Y_{XS}), solid concentrations and content of total solids inside the reactor were measured. For that, the recirculation and feeding systems were stopped, volume of the sludge bed was measured, and then samples of biomass were collected from a sampling port installed in the sludge bed at 0.75 m of reactor height.

6.3.3 Specific methanogenic activity (SMA)

Samples of biomass used in this assay were the inoculum (initial biomass) and the final biomass (day 88), which was differentiated in granulated and degranulated biomass (obtained after a degranulation episode). The tests were conducted in triplicate at 25°C. Five hundred mL bottles were filled with medium that contained 4.8 g of COD and macro and micronutrients in proportion to COD, as shown in Table 6.1. A 3 ratio of inoculum/substrate (I/S) was used in the experiment. Bottles were placed in an Automatic Methane Pontential Test System (AMPTS II, Bioprocess Control, Sweden), where the stirring system worked in discontinuous way (on 60 s/off 60 s). CO_2 and H_2S produced in the biogas were removed by passing the biogas through a solution containing NaOH, measuring only CH_4 flow.

6.3.4 Extraction and characterization of extracellular polymeric substances (EPS)

Extraction of EPS was carried out according to D'Abzac et al. (2010) by the use of a cationic exchange resin (Dowex 20–50 mesh, Sigma-Aldrich, Spain) with a proportion of 70 mg g^{-1} VSS. Polysaccharide and protein content of EPS were measured by colorimetric methods, as indicated by Dubois et al. (1956) and Lowry et al. (1951), respectively.

6.3.5 Microbial community analysis

Microbial community analyses were carried out for initial (day 0) and final (day 88) biomasses, and for granular biomass and degranulated. Initially, DNA from each sample was extracted following the instructions supplied by the manufacturer (DNeasy PowerSoil kit, Qiagen, Germany). The correct amount of DNA extracted was then confirmed by measuring the DNA concentration via Nanodrop (Nanodrop 2000, ThermoFisher Scientific, USA). Amplification of the V3 region of the 16S rRNA gene in bacterial and archaeal population was performed by PCR using the 357F-GC and 538R primers for Bacteria (Toffin et al., 2004) and 787F-GC and 1059R for Archaea (Hwang et al., 2008). PCR conditions can be found in Chapter 5. Correct amplification was checked using electrophoresis in an agarose gel (2%). After that, denaturing gradient gel electrophoresis (DGGE) was conducted in a gel with a denaturing gradient from 35% to 55%, and the PCR products were electrophoresed for 14 h at 100 V. DGGE results were visualised with an image acquisition system (MiniBIS pro, DNR, Israel) after staining the gel. Predominant bands were excised, resuspended in water and crushed to promote the migration of DNA from the gel to the water. Then, the DNA extracted from each band were reamplified with the same conditions that were used in the previous PCR. PCR products were purified with the mi-PCR purification kit (Metabion, Germany), following the manufacturer's protocol. Purified PCR products from each band were sequenced in an automated DNA analyser (3730 KL DNA Analyser, Applied Biosystems, Spain). Sequences were compared with those available in the NCBI database, using the BLASTn tool.

In the DGGE two markers, one for *Bacteria* and other one for *Archaea*, were used for a better analysis of the gel. These markers were contructed from DNA of pure strains of different microorganisms (*Methanocorpusculum bavaricum*, *Methanosarcina mazei* and *Methanobrevibacter smithii* for the archaeal marker and *Pseudomonas aeruginosa*, *Escherichia coli*, *Thiobacillus thiooxydans* and *Xantobacter autotrophicus* for the bacterial marker). DNA of these microorganisms were amplified separately through PCR with the same conditions and primers that were used previously. Then, amplicons were purified with the mi-PCR purification

kit (Metabion, Germany). After the purification, they were quantified by using the Nanodrop (Nanodrop 2000, ThermoFisher Scientific, USA), and finally the amplicons of each microorganism were mixed to get a final concentration of each one of 5 ng μL^{-1}.

6.4 RESULTS

6.4.1 Reactor performance

Total RE in terms of COD removal is shown in Figure 6.1. As can be observed, a high RE (> 97%) was achieved during the start-up (from Stage I to Stage III; feeding only ethanol) of the reactors, despite the rapid increase in the OLR (from 15 to 45 kg COD m^{-3} d^{-1} in less than 3 weeks). This behavior can be explained due to the origin of the initial sludge, taken from a brewery wastewater treatment plant, and the substrate used during this period, which was a readily biodegradable substrate, such as ethanol. The VFA evolution during start-up (Figure 6.2) showed a relatively high increase, with values around 300–500 mg CH_3COOH L^{-1}, which could be attributed to a rapid increase in OLR in a place where the methanogenic population was not developed enough and/or adapted to the increasing loads. Once OLR was stable (after 20 days; Stage IV to Stage VIII) the VFA concentrations were always relatively low (< 250 mg CH_3COOH L^{-1}), indicating no significant kinetic decoupling between the acidogenic and the methanogenic communities. pH values were always stable around 7.3 ± 0.2, even in the initial periods, showing that solution was properly buffered by the addition of bicarbonate in the feed (with stable alkalinity values > 2200 mg $CaCO_3$ L^{-1} in the reactor effluent). Conductivity values were also very stable, beginning at Stage III (OLR > 40 kg COD m^{-3} d^{-1}), with values of 5.73 ± 0.08 mS cm^{-1}. These results are similar to those obtained previously (Chapter 4).

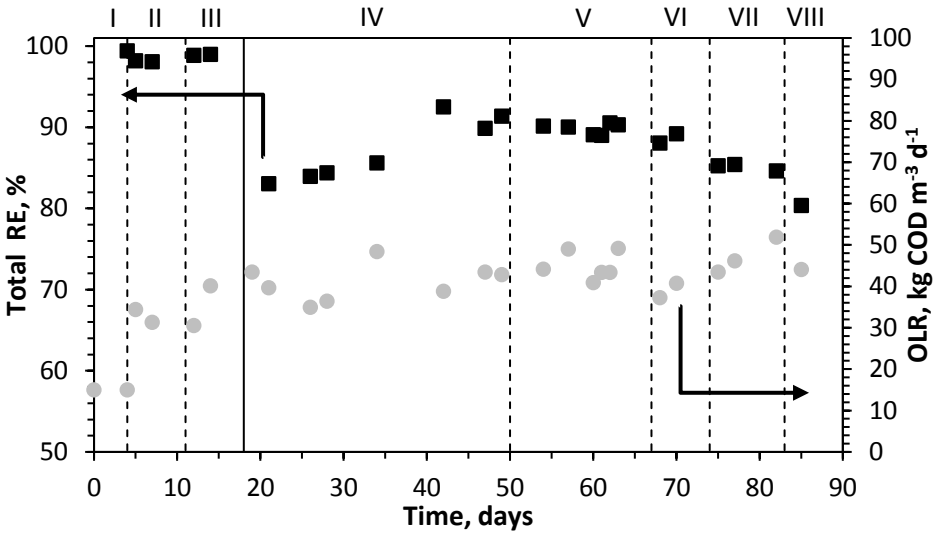

Figure 6.1. Evolution of total organic loadind rate (OLR) and total removal efficiency (RE) of EGSB reactor. Roman numerals show the different stages.

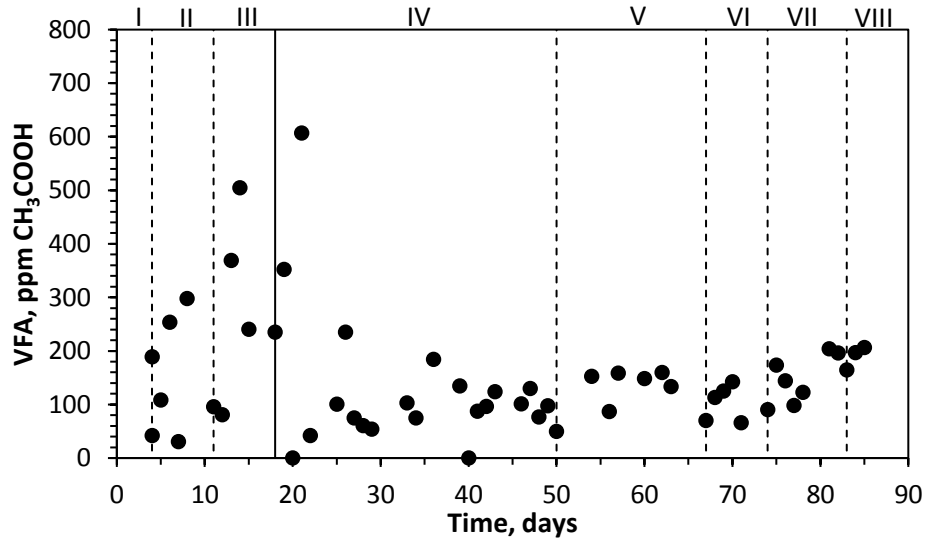

Figure 6.2. Evolution of Volatile Fatty Acids (VFA) concentration throughout the experiment. Roman numerals show the different stages.

When glycol-ethers were introduced in stage IV on day 18 (Table 6.2), total RE decreased suddenly from 99% to 83%, and further, the RE increased progressively until a stable value of about 90% was achieved at the end of stage IV (Figure 6.1). This behavior is similar to that observed in our previous experiments, when M2P or E2P was introduced into the feed (Chapter 4), confirming that biomass needs an acclimatisation period to metabolize these glycol-ethers.

Figures 6.3 and 6.4 illustrate the evolution of the load and the RE of E2P and M2P, respectively. As can be seen, the RE was very low at the beginning (20%) and increased progressively for both glycol-ethers. In the case of M2P, after just 10 days an almost complete elimination was obtained and maintained, even when OLR was increased. For E2P a maximum RE value of around 65% was obtained after 22 days, and when OLR was increased in the next stages (V–VII) a decrease in RE was usually observed, especially at the beginning of the stages and when OLR achieved the highest value.

This behavior could indicate that, although both glycol ethers were degraded through the same mechanism, the biomass adapted better to changes in M2P loads than in E2P, which usually needs two days to recover values close to the average value of RE when OLR was increased. However, this could also be indicative of a preference of microorganisms to degrade M2P against E2P.

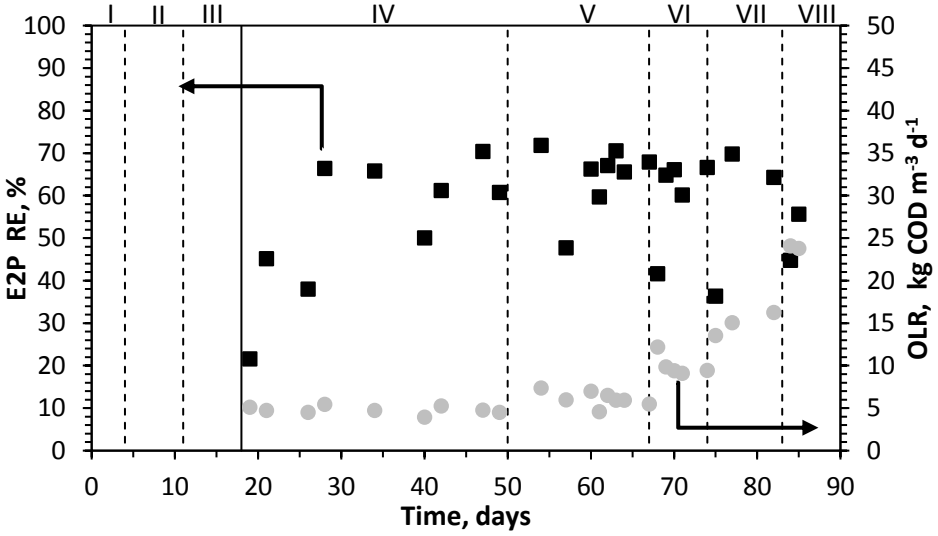

Figure 6.3. Evolution of the OLR and removal efficiency (RE) of E2P. Roman numerals show the different stages.

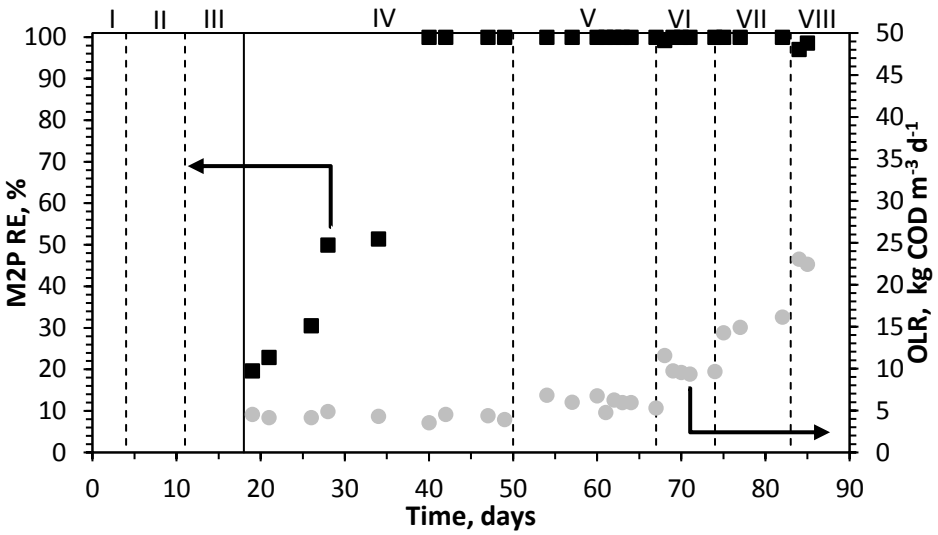

Figure 6.4. Evolution of the OLR and removal efficiency (RE) of M2P. Roman numerals show the different stages.

This performance could be explained by taking into account that in the anaerobic degradation of glycols-ethers, hydrolysis initially occurs to obtain more simple compounds. The enzymes that carry out this hydrolysis contain an active center, which is responsible for interacting with the substrate. For this reason, as has been explained in Chapter 4, the enzymes responsible for the anaerobic degradation of glycol-ethers needs to translocate a methoxy or ethoxy group, and as the methoxy group of M2P is smaller than the ethoxy group of E2P, the translocation could be done easier in the case of M2P, due to the shorter chain of its functional group, which could be more accesible for the active center of the enzyme complex.

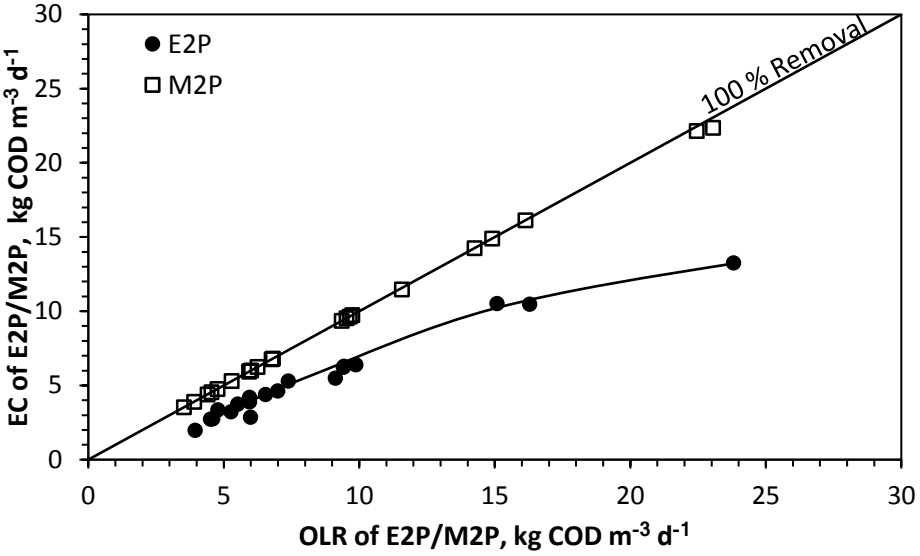

Figure 6.5. Elimination Capacity (EC) versus Organic Load Rate (OLR) for both glycol-ethers.

Figure 6.5 shows that the elimination capacity of M2P is practically complete for the tested inlet loads, whereas a kinetic limitation is observed in the case of E2P. Quantitatively, the behavior of E2P degradation can be adjusted to a Monod model with a half-velocity constant of 8.1 kg COD m^{-3}. Obviously, the value of this parameter would be much lower for the case of M2P, since no kinetic limitation has been observed under experimental tested conditions. Thus, it is clear that the affinity for the substrate of the biological system is much greater for M2P than for E2P. This fact could be a key factor for choosing the most suitable solvents for an industrial application. However, Vermorel (2017) found in batch assays that when using only E2P as a carbon source, the compound could be completely degraded at higher concentrations than were used in our experiment, without kinetic limitations. This discrepancy could be explained by the use of a mixture of both glycol-ethers in our experiment, as opposed to only using E2P, which promoted a competition for accessibility to the active center of the enzyme. Thus, this could suggest that the low half-velocity constant found in this experiment for E2P degradation could be caused by a competitive enzymatic inhibition mechanism, as both glycol-ethers (E2P and M2P) are degraded by the same enzymes, the shorter functional group of M2P could make it preferable to degradation. In contrast, in our

previous experiments (Chapter 4), complete removal of E2P was not obtained even without the presence of M2P, which could indicate that other factors could also be influencing the degradation behaviour of E2P in our EGSB reactor.

During the first 15 days of stage IV, when glycol-ethers were introduced, some intermediate compounds, such as acetone (0.30–0.45 g DOQ L^{-1}), isopropanol (0.05-0.25 g DOQ L^{-1}) and, in minor amounts, methanol (< 0.05 g DOQ L^{-1}) were observed, as described in previous chapters. The appearance of these compounds, which were not detected when the only substrate to be degraded was ethanol, corroborates the previously proposed mechanism for the ether cleavage. The anaerobic degradation of M2P could be producing intermediate products, such as acetone or another compound that could be propionaldehyde or methanol, which would justify the appearance of these compounds in the effluent. Regarding the appearance of isopropanol, it could be related to the presence of acetone, since the anaerobic degradation of isopropanol is carried out by means of a dehydrogenation; acetone production (Widdel, 1986), and in further studies the reversible process in the presence of hydrogen, has been demonstrated (Tonouchi, 2004; Zellner and Winter, 1987).

The period in which intermediate compounds were detected corresponds to the period in which E2P and M2P RE had not reached its maximum (days 18–34 in Figures 6.3 and 6.4), which seems to indicate that accumulation of intermediate compounds could be especially related with the difficulty of the anaerobic degradation of M2P and E2P.

Flow rate and composition of the biogas produced in the reactor were also monitored. The generated biogas had an average composition of 76.4 ± 4.8 % (v/v) CH_4, 23.6 ± 2.1% (v/v) CO_2 and H_2S concentration varied from 121.5 ± 27.6 ppmv during the first month of operation to 59.0 ± 8.9 ppmv at the end of the experiment. Thus, this could indicate that the sludge used as inoculum contained a high content of sulphur. Methane content was close to the estimated stoichiometric content for anaerobic degradation of these compounds (around 75%), although some research carried out in an UASB reactor with methanol and sacharose as degrading compounds also reported similar results (Zhan et al., 2017), methane content was higher than usual for most anaerobic digestors (Rasi et al., 2007). The relatively high methane content could be linked to the fact that in these studies the residual water was buffered with bicarbonate, although using different concentrations (1500 mg L^{-1} for the study carried out by Zhen et al. (2017) and 3000 mg L^{-1} for the present

work). This buffer maintains a balance with the CO_2, thus producing a biogas enriched in methane.

This high methane content in biogas has usually been associated with the pH, bicarbonate alkalinity and VFA concentrations in the system (Grady et al., 2011). In previous studies (Henares et al., 2018), methane contents even higher than 88% were reported, and these values were well correlated with these three parameters.

Methane production is a key factor in anaerobic degradation. It has been established that 1 kg of COD of degraded substrate could provide stoichiometrically 0.35 Nm^3 of CH_4 (Metcalf and Eddy, 2003). As indicated in materials and methods, flowrate of CH_4 has been monitored, so methane yield (m^3 methane per kg of COD of substrate consumed) was estimated. The results expressed as an average of the methane yield throughout the experiment are shown in Figure 6.6.

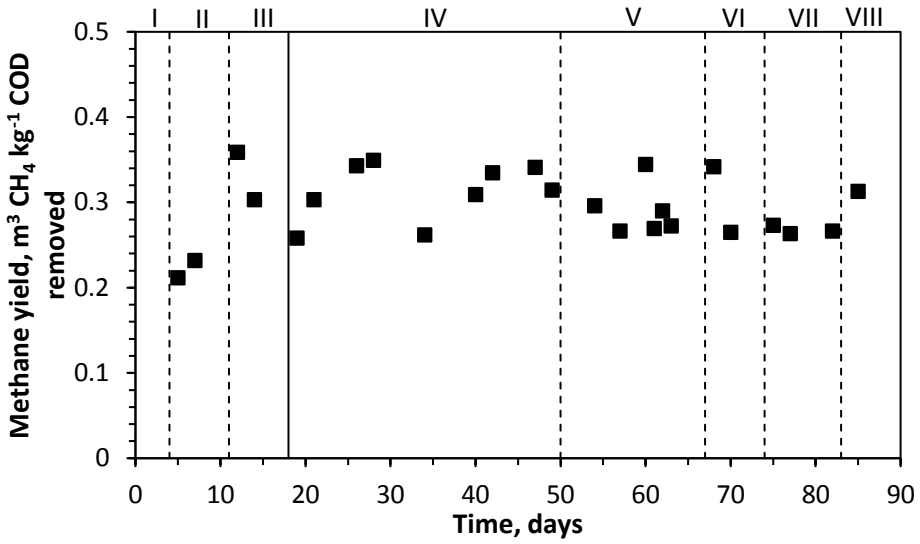

Figure 6.6. Methane yield variation during the experiment.

Methane yield values were always kept below the stoichiometric value, except for day 12 (0.359 m^3 CH_4 kg^{-1} $COD_{degraded}$), which was slightly higher. Throughout the experiment, relatively high value dispersion could be observed, obtaining an average yield of 0.295 ± 0.039 m^3 CH_4 kg^{-1} $COD_{degraded}$, obtaining the lowest values during the first week of the experiment. This fact could be explained by the high content of sulphur in the inoculum, and more sulphate reduction could have occurred, effectively lowering the methane yield, as the biogas composition

revealed. In other similar studies, such as that carried out by Enright et al. (2005), methane yields between 0.11 and 0.35 m^3 CH_4 kg^{-1} $COD_{degraded}$ were obtained using ethanol, acetone, propanol and methanol as substrates. Yields ranging from 0.292 to 0.335 m^3 CH_4 kg^{-1} $COD_{degraded}$ were also obtained using mixtures of ethanol, ethyl acetate and E2P, with a mass ratio of 7:2:1 (Torres et al., 2018). Methane yield values higher than the stoichiometric one have also been observed in a study carried out by Vermorel et al. (2017), in which a maximum value of 0.45 m^3 CH_4 kg^{-1} $COD_{degraded}$ was obtained, although in this case a peak in the value of volatile fatty acids acting as COD accumulators could explain this unusually high value. In Chapter 4, similar methane yields (≈ 0.3 m^3 CH_4 kg^{-1} $COD_{degraded}$) were reported.

In stage VIII of the experiment (days 80–90), the reactor was fed with only glycol-ethers, without ethanol. During the first few days, a decrease in the total removal was observed (Figure 6.1), due soley to a decrease in the removal of E2P (from 65% to 40–50%) (Figure 6.3), since removal of M2P remained at nearly 100%. After day 88 of the test, a partial degranulation of the bed occurred, causing great problems with operation, among which we can highlight the effluent discharge of a large part of the degranulated biomass and the obstruction of the recirculation pump and the effluent. For these reasons, the test could not be continued and had to be finished at a subsequent time, which will be discussed in more detail later.

Enough amount of biomass must be assured inside of the reactor for its suitable performance, so an excess of biomass purge through the effluent should be avoided. In this regard, the amount of suspended solids in the effluent and inside the reactor was regularly monitored to control this factor. Result showed that almost all the suspended solids present in the effluent (> 95%) were of a volatile nature, and concentrations of suspended solids in the effluent remained quite stable, at values close to 50 mg L^{-1}. Finally, the total accumulated amount of suspended solids was 53.7 g after day 84 of the operation, which was before the degranulation episode; this was noticeably lower than in the EGSB experiment of the previous chapter that had more than 90 g of accumulated suspended solids after 90 days of operation, showing that the evolution of this parameter was not critical in this experiment. Regarding the amount variation of solids inside the reactor, an average increase of more than 1.2 g d^{-1} was estimated in stages VI–VII when the maximum OLR was used. The biomass produced in the reactor was estimated from the variation of total solids inside the reactor and from the values of the solids that escaped through the effluent; that is, the non-granular suspended solids.

The proper methanogenesis of anaerobic degradation can be carried out in two ways (acetoclastic and reducing), as has been pointed out above. Both have a very low substrate biomass yield, thus producing a gaseous product and not significantly contributing to the formation of new biomass. The corresponding yield with acetoclastic methanogenesis varies between 0.03 and 0.07 g of COD of biomass produced per g of COD consumed, and the reductive methanogenesis varied from 0.01 to 0.06 g of COD of biomass produced per g of COD consumed (Metcalf and Eddy, 2003). The average value obtained in this experiment was of 0.02 g of COD of biomass produced per g of COD consumed. This value ranges within the established margins, but is closer to the lower value. Authors Handel and Lettinga (1994) reported values of 0.03 g of COD of biomass produced per g of consumed COD, which is quite higher than that obtained in our experiment.

6.4.2 Specific methanogenic activity (SMA) test

Specific methanogenic activity (SMA) determines the methane-producing capability of the sludge for a specific substrate. The methanogenic activity test can be used to delineate the operating conditions for anaerobic systems and as a parameter to assess the system performance by giving a better perceptive of the system and its stability. At the beginning of the start-up period of a new digester, the SMA is of great importance in the determination of proper initial OLRs. In different phases, a regular determination of SMA also ascertains the developmental stages of the sludge. Also, a change in SMA indicates an inhibition or an accumulation of slow degradable, or even non-biodegradable, organic matter from the influents. For this purpose, four different SMA tests were carried out, combining two different solvents to be degraded (pure ethanol or a mixture of solvents, as in stage VII) and biomass source (inoculated biomass or granulated final biomass). Every test was repeated three times. The evolution of methane production in these assays is shown in Figure 6.7, and a summary of the methanogenic activity results can be found in Table 6.3.

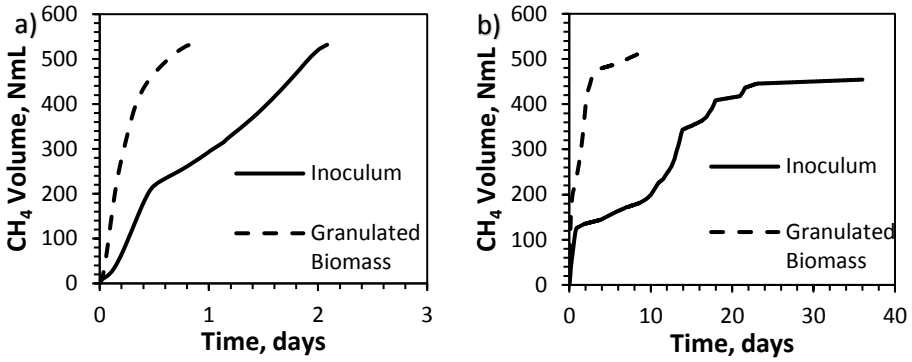

Figure 6.7. Evolution over time of the methane production using the initial (inoculum) and the final granulated sludge with different substrates. a) ethanol and b) the mixture of solvents used in stage VII (ethanol:M2P:E2P in a mass ratio 2:4:4).

As can be observed, the methane production presented a diauxic beharviour when the mixture of solvents and the initial biomass was used, where two or more slopes can be seen. This behaviour can be explained due to the preference for ethanol that the active biomass has compared to other solvents utilized (glycol-ethers). The first slope is associated with the degradation of ethanol, and the others slopes are associated with the degradation of glycol-ethers and their intermediate compounds. This is confirmed in Figure 6.7b, where it can be observed that the first slope achieved 120 mL of CH_4 when the initial sludge was used, which agrees with the stimated volume of CH_4 associated with ethanol degradation, and there are other slopes associated to the glycol-ether degradation. In contrast, when the final granulated sludge was used, the first slope reached 200 mL of CH_4, which indicates that glycol-ethers had been degraded since the beginning of the experiment.

The test duration with inoculated biomass and a mixture of solvents, included glycol-ethers, was much longer (> 36 days) than that for the rest of the experiments (average < 4 days), which could be related to the need for an acclimation period for the biomass to adjust to the glycol-ethers, as confirmed by the lag phase (14 ± 5 d) of the degradation of glycol-ethers. In the assay using the final granulated biomass and a mixture of solvents, a lag phase was not observed, due to the previous adaptation of biomass to the degradable compounds.

Table 6.3. Specific methanogenic activity experiments. Mixture as in Stage VII (ethanol:M2P:E2P in 2:4:4 mass ratio). Average biomethane potential (BMP) and SMA of with standard deviation values.

Substrate	Biomass	BMP (L CH_4 g^{-1} COD)	Ethanol	Glycol ethers	
			SMA (mL CH_4 g VSS^{-1} d^{-1})	SMA (mL CH_4 g VSS^{-1} d^{-1})	LagPhase (d)
Ethanol	Inoculum	0.26±0.02	139±15	-	-
Ethanol	Granulated	0.25±0.05	295±66	-	-
Mixture	Inoculum	0.27±0.03	45±2	9±5	14±5
Mixture	Granulated	0.38±0.09	144±4	32±1	0

The biomethane potential (BMP) with ethanol and/or innoculum was a similar value, close to 0.26 L CH_4 g^{-1} $COD_{degraded}$, recovering 74% of the methane expected from the stoichiometric value (0.35 L CH_4 g^{-1} $COD_{degraded}$). Nevertheless, in tests containing a mixture of solvents and granulated final biomass, the BMP value was 0.38 L CH_4 g^{-1} $COD_{degraded}$, which is a 8% higher value than what was expected and can be attribuited to the biases of the measurement system.

Regarding the results of the SMA for ethanol degradation (Table 6.3), the highest value was achieved when the final granulated biomass was used, with ethanol as a substrate (295 NmL CH_4 g VSS^{-1} d^{-1}), and the lowest value was obtained with the inoculum and the mixture combination (45 NmL CH_4 g VSS^{-1} d^{-1}). When the different types of sludge were compared, the highest values usually corresponded to the final granulated biomass. The difference in behaviour between the final biomass and the initial biomass (inoculum) may be due to a lack of adaptation, both to the substrate and to the introduced organic load. Finally, SMA results in tests where ethanol was used as a substrate were higher than when the mixture was used, which could be attributed to the fact that the degradation of glycol-ethers needs a hydrolysis step, which delays the process, while the ethanol degrades by an easier and faster mechanism. Thus, it seems that the hydrolysis stage of the glycol-ethers is the limiting stage of its degradation process.

Torres et al. (2017) used an EGSB reactor biomass that was aquired during 219 days of operation and obtained an ethanol SMA value of 461.6 NmL CH_4 g VSS^{-1} d^{-1}, which is higher than the value obtained in our study. In that study authors also reported SMA values obtained for E2P, which was of 76.6 NmL CH_4 g VSS^{-1} d^{-1} , also higher values than SMA values obtained using our mixture test (about 32 NmL CH_4 g VSS^{-1} d^{-1} in Table 6.3). On the other hand, Lafita et al., (2015) carried out a study

of the methanogenic activity of ethanol and M2P separately, using the biomass of an EGSB reactor operated during 120 days with a maximum organic load of these compounds of 33.7 kg of COD m^{-3} d^{-1}, they obtained 214.5 NmL CH_4 g VSS^{-1} d^{-1} for ethanol and 24.3 NmL CH_4 g VSS^{-1} d^{-1} for M2P, respectively. These values are lower but silimar to that obtained here (295 NmL CH_4 g VSS^{-1} d^{-1} for ethanol and 32 NmL CH_4 g VSS^{-1} d^{-1} for glycol-ethers). The higher values obtained in this study could be related to the higher OLR and the higher glycol-ether concentration that was treated comparing with the study of Lafita et al. (2015).

6.4.3 Degranulation episode

As previously mentioned, after day 88 of the test, degranulation of the EGSB biomass occurred and the experiment had to be stopped. This episode occurred after just 8 days of operation in tests where only glycol-ethers (E2P and M2P) without ethanol (stage VII, Table 6.2) were fed into the reactor.

Similar problems of degranulation in anaerobic reactors have been previously described. In this regard, more than 250 days into the experiment, Lafita et al. (2015) observed degranulation of the sludge bed of an EGSB reactor that was treating wastewater containing a mixture of ethanol andv M2P. The degranulation occurred when supplying the feeding intermitently, and the authors attributed the degranulation to the discontinuous operational conditions. Nishio et al., (1993) carried out a study in a UASB reactor fed with methanol and acetate, in which granulation was not observed with methanol-only medium, but granulation occurred in mixed substrates medium containing methanol and acetate. This fact could suggest that acetate is necessary to maintain the granular structure, and this compound would not be generated in the anaerobic degradation of methanol, which could be degraded anaerobically by three different routes, depending on the specific environmental conditions and the evolution of the consortium (Paulo et al., 2003). The first route of anaerobic degradation is by direct methylotrophic methanogenesis, the second acetoclastically obtaining acetic acid as an intermediate product and the third is by reductive methanogenesis (Zhen et al., 2017); the first route is the most predominant (Gonzalez-Gil and Kleerebezem, 1999) and avoids the presence of acetic acid. Additionally, the negative effect of methanol on the methanogenic fermentation of propionate and the growth of granular methanogenic sludge in a methanol-propionate mixture has been also described in the performance of an UASB reactor (Fukuzaki and Nishio 1997). The

results were presented, while taking into account that the observed methanol inhibition was due to the toxicity of methanol to propionate-degrading bacteria.

This fact has been also shown in other researches; for example, in the work done by Kobayashi et al. (2011), the authors investigated the effect of starch in the treatment of methanolic waters in a UASB reactor. In this case, two different experiments were carried out, one with methanol as the only substrate and another with a mixture of methanol and starch. In the first experiment, the bed degranulated; in the second, the granular structure was maintained. This result exposed the need of starch for the correct development of the process due to the appearance of acetate, which was produced by the anaerobic degradation of starch. It was assumed that acetate stimulated the archaea growth of the Methanosaeta genus, which was responsible for the maintenance of the granular structure (Ahn et al., 2004). In our experiment, we assumed that ethanol would act as starch, producing the acetic acid necessary to maintain the granular structure, because acetogenesis is one of the main routes of ethanol degradation. Therefore, degranulation in stage VIII could be explained by taking into account the absence of ethanol in the feed. Nevertheless, the influence of other factors, such as, the high concentration of glycol ethers which could act as solvent or extractors of EPS, should not be completely discarded

Furthermore, SMA of the degranulated biomass was also evaluated, as it has been previously described for other kinds of sludges (Section 6.4.2). SMA of degranulated biomass with only ethanol was 82 ± 7 NmL CH_4 g VSS^{-1} d^{-1}, while the value for the glycol-ethers in an assay with the mixture was 32 ± 1 NmL CH_4 g VSS^{-1} d^{-1}. When comparing the results for degranulated versus granulated sludges (Table 6.3), it seems that granulated biomass had a greater capacity for degradation of ethanol, perhaps due to the fact that the granules were set up with a closer relationship to microorganisms, promoting a better mass transference between granules and microorganisms. It has to be pointed out that when the final degranulated biomass was used, the obtained SMA only with ethanol was even lower than for the initial biomass (82 vs 139 NmL CH_4 g VSS^{-1} d^{-1}); however, for the glycol ethers when a mixture of solvents was used, the inverse results were found (32 versus 9 NmL CH_4 g VSS^{-1} d^{-1}). In contrast, SMA of granulated and degranulated biomass for glycol-ethers was identical, suggesting that in this case the limiting step for methane production was not the mass transference of the intermediate compounds, but the ether cleavage. These results could be explained by taking into account that final degranulated biomass was less active, but was better adapted to the glycol-ethers than the initial biomass.

To identify the nature and causes of this degranulation, the content of an extracellular polymeric substance was compared in granulated and degranulated biomass.

Extracellular polymeric substances (EPS) are natural polymers of high molecular weight that are secreted by microorganisms into their environment (Morgan et al., 1990). EPSs establish the functional and structural integrity of biofilms and granules and are considered the fundamental component that determines the physiochemical properties of a microorganism's attachment structures. EPSs are mostly composed of polysaccharides (exopolysaccharides) and proteins, but include other macro-molecules, such as DNA, lipids and humic substances. EPSs are the construction material of bacterial settlements and either remain attached to the cell's outer surface, or are secreted into its growth medium. These compounds are important in biofilm formation and cell-attachment to surfaces. EPSs constitute 50–90% of a biofilm's total organic matter (Fleming et al. 2000). Consequently, these substances play an important role in maintaining the integrity and mechanical strength of the granules and the long-term stability of the reactor (Li et al., 2012).

In the present work, because partial degranulation of the bed was observed in the last stage, samples of granulated and degranulated biomass were taken, to later perform the EPS extraction.

Table 6.4. Polysaccharide and protein content in the EPS of each biomass.

Biomass	Polysaccharides (mg g^{-1} VSS)	Proteins (mg g^{-1} VSS)	Ratio PN/PS
Degranulated	13.71 ± 0.88	73.90 ± 14.35	5.4
Granulated	23.71 ± 0.86	150.21 ± 1.44	6.3

The results obtained from the concentration of polysaccharides and proteins can be seen in Table 6.4, which shows that in granules there was a higher concentration of proteins than polysaccharides. When comparing biomass types, the concentrations of polysaccharides and proteins were 58% and 55% higher in the granulated biomass, respectively. The ratio of proteins / polysaccharides (PN / PS) in the case of the degranulated biomass was 5.4, and for the granulated biomass it was 6.3.

The reduction of EPS can cause negative effects with respect to the stability of the granules, since they can be converted into more porous and dispersed granules, thus causing them to easily rupture (Lu et al., 2015). The difference

between proteins and polysaccharides could be due to the fact that a greater amount of proteins has been generated during the experiment; this fact has been proven by Zhang et al. (2007), where they observed that during granulation the protein concentration had increased from 49.4 mg g^{-1} VSS in the seed sludge to 148 mg g^{-1} VSS in the granules on day 45, while the extracellular polysaccharides remained almost unchanged. Therefore, the ratio of proteins to polysaccharide within the sludge EPS increased with the granulation, from 2.3 to 4.9. Comparing these values with that obtained in our experiment, protein content in granulated sample was very similar, around 150 mg g^{-1} VSS in both cases, but the PN/PS ratio was higher in our samples. These authors (Zhang et al. 2007) suggested that the proteins were linked to the granulation, since a higher concentration of these molecules corresponded to a more hydrophobic and less negatively charged surface; that is, electrostatic repulsion was reduced. In addition, they observed that when the organic load increased, there was a decrease in the concentration of proteins and an increase in the polysaccharides, justifying that an increase in excessive organic load could cause degranulation of the biomass. The PN / PS ratio has also been a proposed indicator of biomass stability because when this ratio was relatively high, the biomass values were more prone to float (Franco et al., 2006). In addition, Lu et al. (2015) explained that low PN / PS ratio values (4.50, which is even lower than those obtained in this work) were associated with the disintegration and breakage of granules. This finding corroborate those obtained in our study where the PN / PS ratio of degranulated biomass decreased from 6.3 to 5.4, confirming that the PN / PS ratio is a good indicator of the health and stability of the granules. In this regard, Torres et al. (2017) carried out a study on granulation, observing that an increase in organic load also produced an increase in the concentration of polysaccharides, and that as the granulation improved, the concentration of proteins increased. The values obtained in that study were 127.9 mg g^{-1} VSS of proteins and 18.8 mg g^{-1} VSS of polysaccharides, which are very similar to the values obtained in our granulated biomass samples.

6.4.4 Microbial community analysis

Results of archaeal DGGE can be found in Figure 6.8a, where predominant bands have been marked, excised and sequenced. Results of identification of marked bands through the BLASTn analysis can be observed in Table 6.5, where the most closest microorganism in the database, the accession number and the level of similarity are indicated.

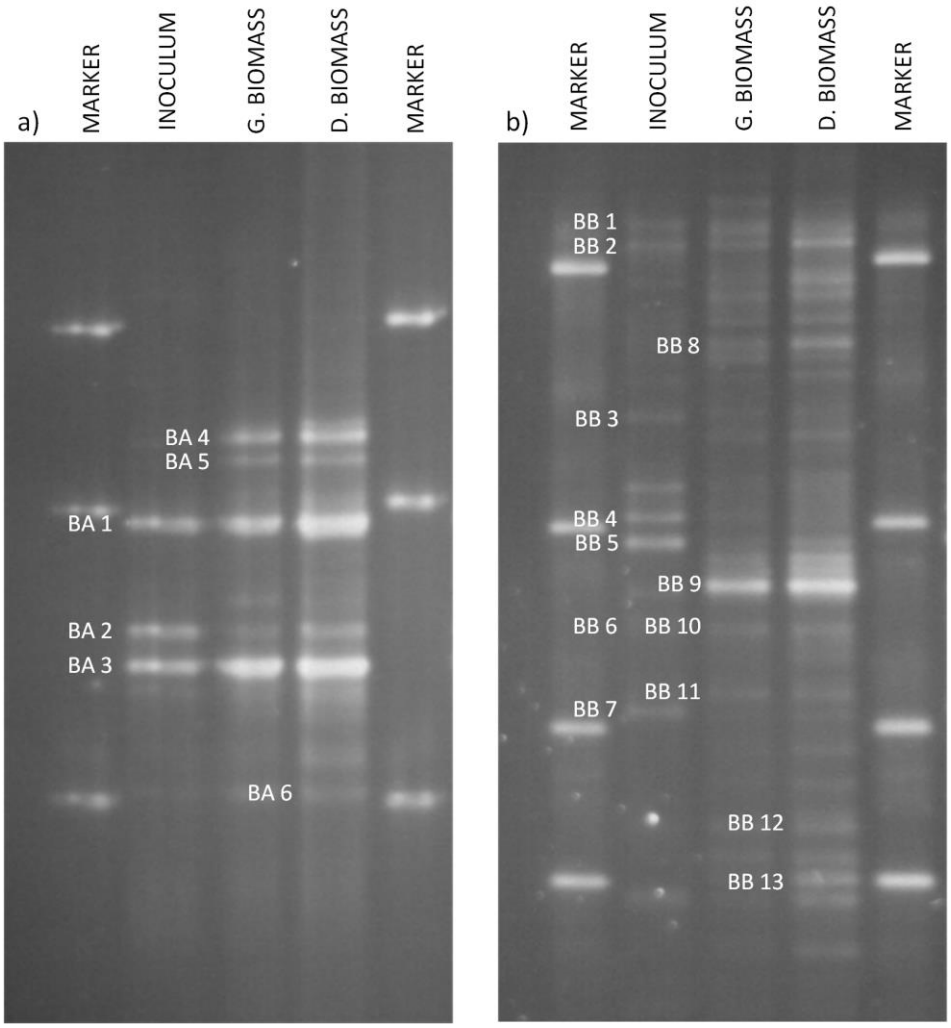

Figure 6.8. DGGE profiles of biomass samples from the inoculum and both final biomass samples granulated (G) and degranulated (D). a) Archaeal profile. b) Bacterial profile.

Table 6.5. DGGE band designation, accession numbers in GenBank and levels of similarity to related organisms according to Figure 6.8a.

Band	Closest microorganism in GenBank (accession number)	Similarity (%)	Phylogenetic group
BA 1	*Methanosaeta concilii* (NR_102903)	98	*Methanosarcinales*[a]
BA 2	*Methanobacterium ferruginis* (NR_113045)	99	*Methanobacteriales*[a]
BA 3	Methanomethylovorans uponensis (NR_133781)	99	*Methanosarcinales*[a]
BA 4	*Methanolinea tarda* (NR_028163)	88	*Methanomicrobiales*[a]
BA 5	*Methanospirillum hungatei* (NR_118366)	85	*Methanomicrobiales*[a]
BA 6	*Methanoculleus receptaculi* (NR_043961)	95	*Methanomicrobiales*[a]

[a]Order

As can be observed in Figure 6.8a, only three bands (BA 1–BA 3) were predominant in the inoculum, while in both final sludges (granulated and degranulated) at least six significant bands could be found (BA 1–BA 6), revealing an evolution in the archaeal population. Furthermore, there are no significative differences between the final sludges, as the same bands were detected in both of these communities. In the final sludges, two bands (BA 1 and BA 3) had a higher intensity, revealing a greater predominance of the associated microorganisms in the archaeal community. This fact could be related to a competitive advantage in connection with the substrate of these microorganisms. As can be observed in Table 6.5, BA 1 was identified as *Methanosaeta concilii*, an acetoclastic species of archaea that uses acetate to produce methane. This species was found in different reactors using granular sludges (Patel and Sprott, 1990) and was proposed as a key microorganism in maintaining the integrity of the granular structure (Xing et al., 2009). BA 3 has been identified as *Methanomethylovorans uponensis*, a microorganism that is able to use methanol, methylated amines, dimethyl sulfide and methanethiol as catabolic and methanogenic substrates; however, this microorganisms cannot use other methanogenic susbtrates, such as H_2/CO_2, formate, 2-propanol and acetate. It can grow in temperatures ranging 25–40˚C and in a neutral pH (5.5–7.5), and its growth rate increases in the presence of H_2 (Cha et al., 2013). The development of this species was enhanced by the introduction of M2P, due to the fact that the hydrolysis of this compound produces methanol, which is utilized by *Methanomethylovorans uponensis* to grow and produce methane.

Other bands identified, but with less intentisity than BA 1 and BA 3, were BA 2 as *Methanobacterium ferruginis*, BA 4 as *Methanolinea tarda*, BA 5 as *Methanospirillum hungatei* and BA 6 as *Methanoculleus receptaculi*; all of these microorganisms are hydrogenotrophic archaeas (Cheng et al., 2008; Gunsalus et al., 2016; Imachi et al., 2008; Mori and Harayama, 2011). This brought attention to the fact that the methane production in the reactor is carried out by three different pathways: i) from acetate, revealed by the presence of *Methanosaeta concilii*, ii) from hydrogen linked to different hydrogenotrophic methanogens as the presence of *Methanobacterium ferruginis*, *Methanolinea tarda*, *Methanospirillum hungatei* and *Methanoculleus receptaculi* revealed and iii) from methanol as a methylotrophic species, as shown by the presence of *Methanomethylovorans uponensis*. In addition, the high intensity of bands associated with *Methanosaeta* and *Methanomethylovorans* could indicate that the predominant pathways of methane production in the reactors were the acetoclastic and the methylotrophic pathways.

The bacterial DGGE results can be found in Figure 6.8b, where predominant bands have been marked, excised and sequenced. The microorganism identification of each band is shown in Table 6.6, where the closest microorganism in the database, the accession number, the level of similarity and the phylogenetic group are indicated.

As shown in Figure 6.8b, a shift in the bacterial population occured from the beginning to the end of the experimental period, where the predominant bands in the inoculum decreased in intensity, or even disappeared in both final sludges. In contrast, some new bands that were not present in the inoculum sludge were predominant in the sludges at the end of the experiment. This fact could indicate the adaptation of the biomass to the new substrates used in the experiment, to the new operational conditions of reactors such as OLR or HRT, or an adaptation to a synthetic wastewater containing only solvents and very limited amounts of complex substrates. On the other hand, when comparing the final sludges (granulated and degranulated), an almost identical fingerprint can be observed with the same predominant bands, and every band that is present in the final granulated biomass was also found in the final degranulated sludge.

Table 6.6. DGGE band designation, accession numbers in GenBank and levels of similarity to related organisms according to Figure 6.8b.

Band	Closest microorganism in GenBank (accession number)	Similarity (%)	Phylogenetic group
BB 1	*Anaerosporomusa subterranea* (NR_152052)	78	*Firmicutes*[b]
BB 2	*Anaerosporomusa subterranea* (NR_152052)	77	*Firmicutes*[b]
BB 3	*Desulfobulbus rhabdoformis* (NR_029176)	96	*Proteobacteria*[b]
BB 4	*Sulfuricurvum kujiense* (NR_074398)	98	*Proteobacteria*[b]
BB 5	*Sulfuricurvum kujiense* (NR_112144)	95	*Proteobacteria*[b]
BB 6	*Desulfomicrobium orale* (NR_113205)	84	*Proteobacteria*[b]
BB 7	*Desulfomicrobium aestuarii* (NR_132594)	93	*Proteobacteria*[b]
BB 8	*Desulfovibrio vietnamensis* (NR_026303)	83	*Proteobacteria*[b]
BB 9	*Geobacter psychrophilus* (NR_043075)	90	*Proteobacteria*[b]
BB 10	*Halanaerobaculum tunisiense* (NR_044464)	74	*Firmicutes*[b]
BB 11	*Clostridium methoxybenzovorans* (NR_024917)	95	*Firmicutes*[b]
BB 12	*Rectinema cohabitans* (NR_156915)	87	*Spirochaetes*[b]
BB 13	*Lentimicrobium saccharophilum* (NR_149795)	83	*Bacteroidetes*[b]

[b] Phylum

Regarding the identification of the bacterial bands , BB 1 and BB 2 were identified as *Anaerosporomusa subterranean*, an anaerobic, mesophilic and netrophilic bacteria that grows fermenting short chains of carboxylic acids (Choi et al., 2016). Band BB 3 was identified as *Desulfobulbus rhabdoformis*, a strictly anaerobic, mesophilic and neutrophilic bacterium that utilizes sulfate as an electron acceptor, as well as electron donnors like ethanol, propanol, lactate and pyruvate (Lien et al., 2018). Bands BB 4 and BB 5 were identified as *Sulfuricurvum kujiense*, a facultatively anaerobic, chemolithoautotrophic and sulfur-oxidiing bacterium. It uses sulfide, elemental sulfur thiosulfate and hydrogen as electron donors and

nitrate as electron acceptor (Ismail et al., 2016). Band BB 6 was identified as *Desulfomicrobium orale*, an anaerobic and mesophilic bacterium that is able to use lactate, pyruvate, hydrogen, ethanol and formate as electron donors substrates and sulfate as an electron acceptor. Furthermore, it produces acetate from the incomplete oxidation of lactate and pyruvate (Langendijk et al., 2001). Band BB 7 was identified as *Desulfomicrobium aestuarii*, an anaerobic and mesophilic bacterium which uses sulfate, thiosulfate and nitrate as electron acceptors and lactate, formate, malate and H_2 as electron donors (Dias et al., 2008). Band BB 8 was identified as *Desulfovibrio vietnamensis*, which is an anaerobic, mesophilic and neutrophilic bacterium. It is able to use lactate, pyruvate, malate, formate, fumarate, ethanol and glycerol as electron donors in presence of sulfate, which is an electron acceptor (Nga et al., 1996). Band BB 9 was identified as *Geobacter psychrophilus*, an anaerobic bacterium that is able to grow at temperatures between 4 and 30˚C. It can couple the reduction of Fe(III) to the oxidation of different substrates, such as acetate, butanol, ethanol or formate (Nevin et al., 2005). Band BB 10 was identified as *Halanaerobaculum tunisiense*, an anaerobic, mesophilic and neutrophilic bacterium that can ferment such substrates as glucose, sucrose or starch, and the final products of the fermentarion are acetate, butyrate, lactate, H_2 and CO_2 (Hedi et al., 2009). Band BB 11 was identified as *Clostridium methoxybenzovorans*, an anaerobic bacterium that grows on some carbohydrates, organic compounds, methanol and methoxylated aromatic compounds as the sole source of carbon and energy. Fermentation products of carbohydrates are formate, acetate and ethanol, while methoxylated aromatic compounds are fermented to form acetate and butyrate (Mechichi et al., 1999). Band BB 12 was identified as *Rectinema cohabitans*, an anaerobic bacterium that grows through fermentation of carbohydrates (Koelschbach et al., 2017). Band BB 13 was identified as *Lentimicrobium saccharophilum*, a strictly anaerobic, mesophilic and neutrophilic bacterium that use carbohydrates like glucose, ribose or starch to grow (Sun et al., 2016).

Identification of bands revealed that most of bands in the inoculum (BB 3, 4, 5, 6 and 7) were closely related to sulfur metabolism and that these bands decreased their intensity or disappeared at the end of the experiment, confirming that the reactor of which was taken the sludge could have a higher concentration of sulfate of sulfur compounds than our EGSB reactor , as indicated by the higher H_2S concentration in biogas during the first weeks of operation. In addition, the predominant bacterium at the end of the experimental period in both sludges was *Geobacter psycrophylus* (band BB 9), possibly due to a competitive advantage for substrates like ethanol, since this bacterium is able to use ethanol as electron donor (Nevin et al., 2005). Ethanol was used as a substrate during most of the experiment, and it is also an intermediate product of the E2P degradation. Further, the

prevalence of this species could also explain the predominance of *Methanosaeta concilii* in the archaeal population, as both microorganisms are syntrophic partners for ethanol degradation (Morita et al., 2011). Another microorganism that seems to have a competitve advantage for our substrate composition is *Clostridium methoxybenzovorans*, which can ferment methoxylated aromatic compounds and could possibly ferment methoxylated compounds like M2P. Moreover, it is able to use methanol, which is one of the intermediate products of M2P degradation. Therefore, the appearance of these microorganisms could be related to the substrate utilized along the experimental period. As expected, the fast changes in the physico-chemical properties of the granular sludge did not directly cause any sudden changes in the microbial communities, since the fingerprints of both granulated and degranulated biomass from *Archaea* and *Bacteria* were almost identical.

Comparing these results with those obtained in Chapter 5, in the archaeal communities *Methanosaeta* was one of the predominant methanogens in both reactors. However, in this study the high content in M2P promotes the apparition of methanogens able to directly use methanol for methane formation as *Methanomethylovorans uponensis* which was not detected when relatively low concentration of M2P (< 20% in weight) was in the feed. In the bacterial communities, some species were identfied in both studies as *Geobacter* and *Clostridium*, which could revealed that these species are important microorganisms for the anaerobic degradation of solvents from the flexographic sector.

6.5 CONCLUSIONS

Anaerobic treatment of ethanol and glycol-ethers mixtures has been shown as a feasible technology, with a high RE (≈ 90%). However, ethanol has been demonstrated as an important substrate for the maintenance of the granular structure of sludge in anaerobic degradation, since partial degranulation occurred when ethanol was not introduced in the feed. M2P had a higher RE than E2P, probably due to enzymatic competitive inhibition. Microbial community analyses revealed an evolution of the inoculum to the final sludge and adaptation to the substrate. This is because the predominant microorganisms identified at the end of the experiment had a close relationship with the substrates used during the trial. Meanwhile, EPS analyses showed that protein and carbohydrate content in the EPS of degranulated biomass were lower than in granulated biomass. The PN/PS ratio also decreased in the degranulated biomass, which suggests that this parameter could be a good indicator of the health and stability of the granules.

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7 MICROBIAL COMMUNITY ANALYSIS OF AN ON SITE PILOT ANAEROBIC BIOSCRUBBER TREATING VOCS FROM PRINTING PRESS AIR EMISSION

7.1 ABSTRACT

Microbial community analysis of an anaerobic reactor from an on-site anaerobic bioscrubber prototype installed in a flexographic facility was carried out. The anaerobic reactor treated solvents used in the printing industry, mainly ethanol, ethyl acetate and 1-ethoxy-2-propanol (E2P). Despite the fluctuations and variations in the organic load applied to the reactor, high removal efficiencies (average of 93%) were obtained and volatile fatty acids accumulation was only detected when the organic load was higher to 3 kg h^{-1}. The microbial community analysis revealed a decrease in biodiversity during the first months of operation in the domains *Archaea* and *Bacteria*. This shift was associated to the limitation of the carbon source to only a few organic solvents. Archaeal populations were more affected by these changes in the carbon source, resulting in a drop of the Shannon index from 1.07 to 0.41 in the first 123 days of experimentation. In this domain, *Methanosaeta* was the dominant microorganism and its dominance persists along all the experimental period. In *Bacteria*, species from *Geobacter* and *Pelobacter* genera (microorganisms specialized in ethanol degradation), were the predominant microorganisms. Besides, when the behavior of the anaerobic bioscrubber prototype scale reactor was compared with the laboratory scale reactors, similar performance was observed in terms of removal efficiency and adaptation period needed to degrade efficiently E2P. The only difference between both scale reactors was that an organic load operational limit, associated to volatile fatty acid accumulation, was observed in the proptotype scale but not at laboratory scale. Dynamics of microbial community structure has been compared with that obtained in laboratory scale reactors.

7.2 INTRODUCTION

Flexographic sector represents the 17% and it contributed around 1,7% of the total turnover in 2003 of the European printing sector (Ernst & Young, 2007). The consumed solvents are mainly oxygenated compounds such as ethanol, ethyl acetate, isopropanol, n-propanol, 1-methoxy-2-propanol, n-propyl acetate, 1-methoxy-2-propyl acetate, acetone and 1-butanol (Granström et al., 2002). Flexographic air emissions are characterized by high flow rates and low volatile organic compound (VOC) concentrations (Sempere et al., 2012), with temperatures ranging from 40 to 70°C and relative humidity varying from 5 to 15% (Rothenbuhler et al., 1995). These air emissions must be controlled according to the European Directive on industrial emissions (Council Directive 2010/75/EC).

Biotreatments represent well-developed air pollution control techniques for removing VOCs in these conditions (Deshusses, 1997). Among biotreatments, bioscrubbers can handle higher gas loads than biotrickling filters and biofilters, up to 3000 - 4000 $m^3\ m^{-2}\ h^{-1}$ (Kennes et al., 2009). However, there are scarce available studies on aerobic bioscrubbers, Le Cloriec et al. (2001) reported removal efficiencies (RE) between 90.1 - 100% in a lab-scale bioscrubber, with liquid to air ratios ranged between $0.6{\cdot}10^{-3}$ and $2{\cdot}10^{-3}$, and with ethanol concentration in waste gas from 18.8 to 291.7 mg-C m^{-3}. Granström et al. (2002) investigated an onsite pilot-scale for the treatment of a waste gas from printing processes. In this study, the major VOC of the waste air was ethanol, with smaller amounts of ethyl acetate, 1-propanol, 2-propanol, 1 methoxy-2-propanol and 3-ethoxy-1-propanol. The flow of the waste gases varied from 1.68 to 3.73 $m^3\ h^{-1}$ with 99.6% of VOC RE excluding evaporation losses. Nevertheless, aerobic bioscrubber is not still widespread within the biotreatment market due to the high-energy consumption of the aerobic bioreactor. In contrast, anaerobic bioscrubber could be an alternative to recycle waste gases into bioenergy, resulting in a positive net energy balance.

To the best of our knowledge, except the studies carried out in our research group, no previous literature exists about anaerobic bioscrubber treating VOC waste gases. Nevertheless, the anaerobic degradation of solvents such alcohols (Eichler and Schink, 1985; Widdel, 1986; Zellner and Winter, 1987) or esters (Oktem et al., 2008; Yanti et al., 2014) is well documented. Recently, a research carried out in Gi^2AM group (Lafita et al., 2015) demonstrated that anaerobic degradation of glycol ethers is feasible by reporting the treatment of a synthetic packaging wastewater, which contains a mixture of ethanol and 1-methoxy-2-propanol in a mass ratio of 4:1. In this study, carried out in an expanded granular sludge bed (EGSB) reactor, a

RE up to 94% at 18 ˚C and 97% at 25 ˚C were obtained, with organic loading rates (OLR) of methoxy-2-propanol of 6.4 and 9.3 kg COD m^{-3} d^{-1}, respectively.

The anaerobic degradation of organic solvents in granular sludge reactors relies on the microbial population developed in the anaerobic granules, which in turn should maintain their physical integrity. Leclerc et al. (2004) studied the microbial population of 44 anaerobic digesters treating effluents from several sectors. These authors indicated that the occurrence and significance of the different species are influenced by the running and environmental conditions. Anaerobic granulated sludge coming from breweries is a common source of biomass for other industrial sectors. In this sense, the study of the evolution of the microbial population is an interesting tool to investigate the impact that the change of the substrate composition could have on the feasibility and robustness of the anaerobic degradation of solvents.

The characterization of microbial population can be done by molecular biology tools such as Denaturing Gradient Gel Electrophoresis (DGGE), which is based on the electrophoretic separation of Polymerase Chain Reaction (PCR) products with the same length, but with different sequences, on a linear denaturing gradient polyacrylamide gel (Muyzer and Ramsing, 1995). DGGE has been applied to evaluate the microbial diversity of anaerobic reactors such as an upflow anaerobic sludge blanket (UASB) reactor treating wastewater of a brewery, showing that the dominant archaeal bands were closely related with *Methanosaeta* and *Methanobacterium* (Chan et al., 2001). The DGGE technique has also shown that the microbial population of an UASB treating wastewater from an unbleached pulp plant persisted throughout the experimental period (Buzzini et al., 2006). DGGE studies also can demonstrate the importance that the environmental conditions have in the diversity of microbial population; for example LaPara et al. (2000) indicated that a thermophilic reactor showed less biodiversity than a mesophilic one by treating wastewater from a pharmaceutical facility.

The purpose of this study was: (i) to evaluate the dynamics of the microbial community structure of an anaerobic reactor of an anaerobic bioscrubber prototype installed in a flexographic facility; (ii) to link the operational conditions and carbon sources to the microbial populations identified; (iii) to compare the performance of the anaerobic pilot scale reactor with the performance of the laboratory scale reactors described in Chapters 4 and 6 ; and (iv) to compare the structure and the dynamics of the microbial communities present in both scale anaerobic reactors.

7.3 MATERIALS AND METHODS

7.3.1 Characterization of air emissions

A proptoype pilot scale unit was operated in an industrial flexographic facility (Altacel Transparant Verpakkingsind, Weesp, the Netherlands), where high variable emissions associated with the number of printing press in operation were occurred. The flexographic site operates on two-shift (16 h) basis from Monday to Friday and on one-shift (8 h) basis on Saturday. Airflow ranged between 184 and 1253 m^3 h^{-1}, average VOC concentration was 1126 mg-C Nm^{-3} with standard deviation of 470 mg-C Nm^{-3}. The main detected compounds (% in weight) were ethanol (60-65%), ethyl acetate (20-25%) and 1-ethoxy-2-propanol (E2P) (10-15%); other minor compounds were isopropanol (1-0.5%), isopropyl acetate (0.5-0%), n-propyl acetate (3-0%), 4-hydroxy-4-methyl-2-pentanone (5-0.2%) and n-propanol (2-0%). In this study M2P was not present in the emission to be treated because the facility where the prototype was installed did not used this compound.

7.3.2 Anaerobic bioscrubber prototype

Samples of biomass were collected from an expanded granular sludge bed (EGSB) reactor from an anaerobic bioscrubber prototype, which was provided by Pure Air Solutions B.V. (Heerenveen, the Netherlands) and installed in the flexographic site. A diagram of the system is shown in Figure 7.1. It comprised two connected parts: i) a scrubber of 3.06 m in total height (2.0 m in packing material height) and 0.5 m in diameter, which was assembled onto a bottom tank of 2 m^3 in volume, and ii) an anaerobic reactor of 5.08 m of total height and 1.59 m of diameter, resulting in a volume of 8.7 m^3. Scrubber was needed in prototype unit to transfer the soluble organic solvents from air emissions to water, which was subsequently fed to the anaerobic bioreactor. Water and air flow rates, temperatures, inlet and outlet gas and liquid composition, water pH and biogas production and composition, and so on, were monitored and/or controlled. A more thorough overview of the prototype unit can be found elsewhere (Waalkens et al., 2015 and Bravo, 2017).

The anaerobic bioscrubber was operated for 484 days. During the experimental period two packing materials were tested in the scrubber: a cross-flow structured packing material (KFP 319/619, ENEXIO, Germany) used from day 0 to day 95 and from day 266 to day 484, and a vertical flow structured packing material

(KVP, 323/623, ENEXIO, Germany) used from day 96 to day 130 and from day 181 to day 265. A spray tower configuration of the scrubber was used from day 131 to day 180. The water stream from the scrubber, which contained the solvents (mainly ethanol, ethyl acetate and E2P), was supplemented with macro and micronutrients and with sodium carbonate to keep controlled the pH and it was fed to the EGSB reactor that was inoculated with 3 m^3 of granular sludge from a brewey wastewater treatment (Heineken, the Netherlands) and therefore, different from the sludge used as inoculum in previous chapters. The EGSB reactor operated at 3 h of hydraulic residence time and a constant upflow velocity of 3 m h^{-1}. Respect to the operational temperature of the reactor, the experiment can be divided in two periods. During the first period (from day 0 to day 334) the temperature of the reactor depends only on the temperature of the industrial air emissions because there was any temperature control, and during the second period (from day 335 to day 484) an air temperature control system was installed, so the temperature was more stable during this period without big differences between the minimum and the maximum temperature as can be observed in Table 7.1. Organic loading (OL), shown in Figure 7.2, ranged function of the production of the facility from low OL as 0.37 kg h^{-1} to high OL as 6.96 kg h^{-1}. A more detailed description of the operation and discussion of the performance of the prototype unit can be found elsewhere (Bravo, 2017).

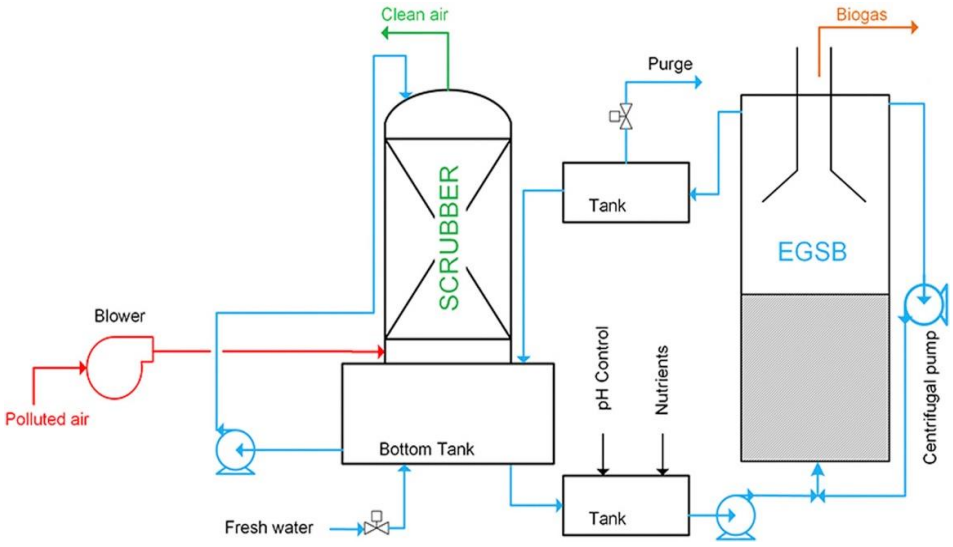

Figure 7.1. Scheme of the anaerobic bioscrubber prototype.

7.3.3 Microbial community analysis

7.3.3.1 *DGGE study*

Samples of biomass were taken along all the experimental period (days 0, 123, 238, 334, 413 and 430) to investigate the microbial dynamics occurred in the reactor. Samples were taken at the facility, and then they were frozen and sent to the Universitat de Valencia to continue with the procedure. DNA from each sample was extracted with a Power Soil Isolation Kit (Mo Bio Laboratories, USA), using supplier's protocol. DNA concentration and purity were measured using NanoDrop® (Thermo Scientific, USA). Extracted DNA was stored at -20 °C for the analysis. To amplify 16S rDNA, two universal primer sets were used: F357-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and R518 (5'-ATT ACC GCG GCT GG-3') for bacterial 16S and F787-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAT TAG ATA CCC SBG TAG TTC-3') and R1059 (5'-GCC ATG CAC CWC CTC T-3') for archaeal 16S. The PCR amplification was conducted according to the following protocol: 20 cycles of 94 °C for 1 min; 65 °C for 1 min; 72 °C for 0.5 min; 10 cycles of 94 °C for 1 min; 55 °C for 1 min; 72 °C for 0.5 min and final extension at 72 °C for 10 min. After amplification, the PCR products were electrophoresed in 0.5% (w/v) agarose gel to evaluate the extent of amplification. PCR product generated from each sample was separated on an 8% acrylamide gel with a linear denaturant gradient increasing from 20% to 35% using the KuroGel Verti 2020 DGGE System (VWR International Eurolab, Spain). DGGE was performed using 20 µl of PCR product in 1× TAE buffer at 60 °C with a sequence of 50 V for 5 min, 150 V for 120 min and 200 V for 60 min. The DGGE gels were visualized in the MiniBIS Pro system (DNR Bio-Imaging System Ltd., Spain). Predominant bands were excised and resuspended in 30 µL of sterilized Mili-Q water, and then bands were stored at 4°C allowing DNA to migrate to the liquid. The eluted DNA was reamplified by PCR with the same conditions as the previous PCR to the DGGE. The PCR product was purified with High Pure PCR Product Purification Kit. Successfully reamplified and purified PCR products were sequenced by using an automated DNA analyzer (3730 KL DNA analyzer, Applied Biosystems, Spain). Sequences were analyzed with MEGA 5.0 and then compared with those available from the NCBI GenBank using BLAST software.

7.3.3.2 *Shannon index*

DGGE images were analyzed in order to identify each band. In this study, bacterial and archaeal biodiversity was estimated using Shannon Index (H'), which increases as both the richness and the evenness of the community increase. For every sample, relative intensity of each band was determined through the free software ImageJ, and the H' index was calculated as follows:

$$H' = -\sum (n_i/N)\, log(n_i/N)$$

Where n_i was the intensity of the band i, and N the sum of the intensities of the bands of each sample.

7.3.3.3 *Granule size distribution*

In order to investigate the potential growth or desintegration of the granules, a study of the evolution of particle size distribution of biomass samples was carried out. The granule size was measured by a Malvern Mastersizer 2000 instrument (Worcestershire, UK) with a detection range of 0.02-2000 µm. Samples of days 0, 238 and 430 (BS-0, BS-3 and BS-6, Table 7.1) were analyzed.

7.4 RESULTS AND DISCUSSION

7.4.1 The EGSB reactor performance

The performance of the prototype EGSB reactor has been reported by Bravo (2017). A summary of the results has been included in this section (7.4.1) in order to facilitate the understanding of this chapter.

The OL (daily average) applied to the EGSB is shown in Figure 7.2 along with the weekly RE of COD (RE_{COD}). The daily average organic load to EGSB was quite fluctuating due to modifications on the facility's production and the performance of the scrubber, values ranging from 0.37 (day 169) to 6.96 kg h^{-1} (day 47). Despite the OL fluctuations, RE of the reactor was kept in very high values for the whole experimental period, with average and standard deviation values of 93 ± 5%, proving the feasibility of the anaerobic biodegradation of a mixture of solvents containing mainly ethanol, 1-ethyl acetate and E2P. Methane content in the biogas was stable at 94 ± 3% (n = 18). The average methane yield was 0.32 $Nm^3\ CH_4\ kg^{-1}$

$COD_{removed}$, close to the stoichiometric value (0.35 m^3 CH_4 kg^{-1} $COD_{removed}$, Grady et al., 1998) and very similar to the obtained in the experiments of previous chapters of this work. The growth yield coefficient derived from the methane yield was 0.06 mg VSS mg^{-1} COD.

Table 7.1 summarizes the VFA concentration, temperature and pH of the water effluent of the anaerobic reactor grouped between biomass sampling events. The average temperature for all intervals was usually in the mesophilic range during the first 330 days, except some eventual days when temperature was of around 19 °C. Variations were associated to industrial air emission temperature. So with the aim to prevent any day to be below 20 ˚C, a temperature control system was installed on day 334.

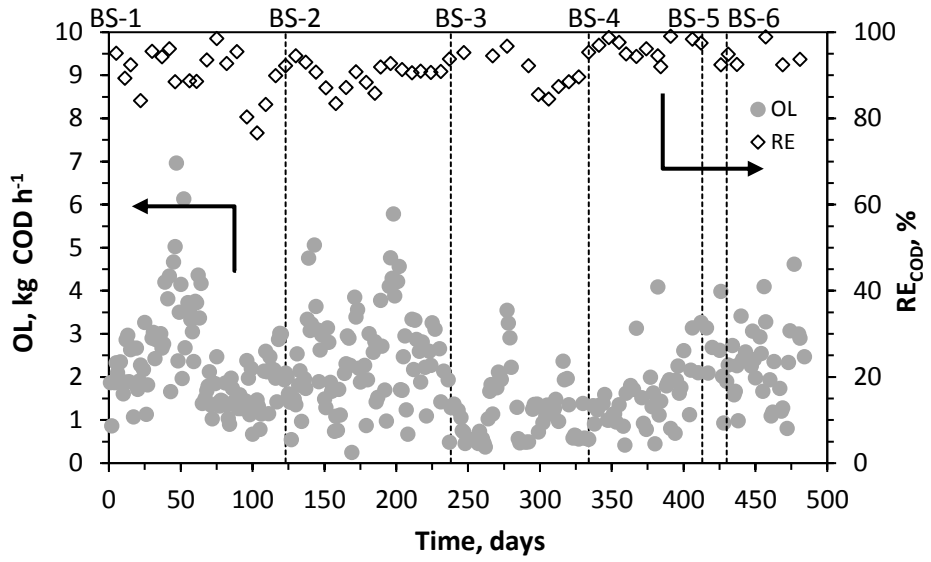

Figure 7.2. Daily average of the organic load (OL) and weekly removal efficiency of COD (RE_{COD})in the EGSB reactor. Broken lines indicate biomass sampling events. Adapted from Bravo (2017)

VFA concentration was normally kept in values lower than 361 mg CH_3COOH L^{-1}, indicating a good balance between acidogenesis and methanogenesis, although some VFA accumulation (>1000 mg CH_3COOH L^{-1}) occurred at high OLR. The pH was chemically controlled by adding sodium carbonate, keeping the pH above the minimum value for optimal growth of methanogens (6.8) (Grady et al.,

1998). The minimum pH values were reached on days when OLR were high and VFA accumulated in water; for example a daily average pH of 6.78 was measured on day 200 after 3 days running with OLR higher than 4.5 kg COD h^{-1} and resulting in a maximum VFA concentration of 1143 mg CH_3COOH L^{-1}.

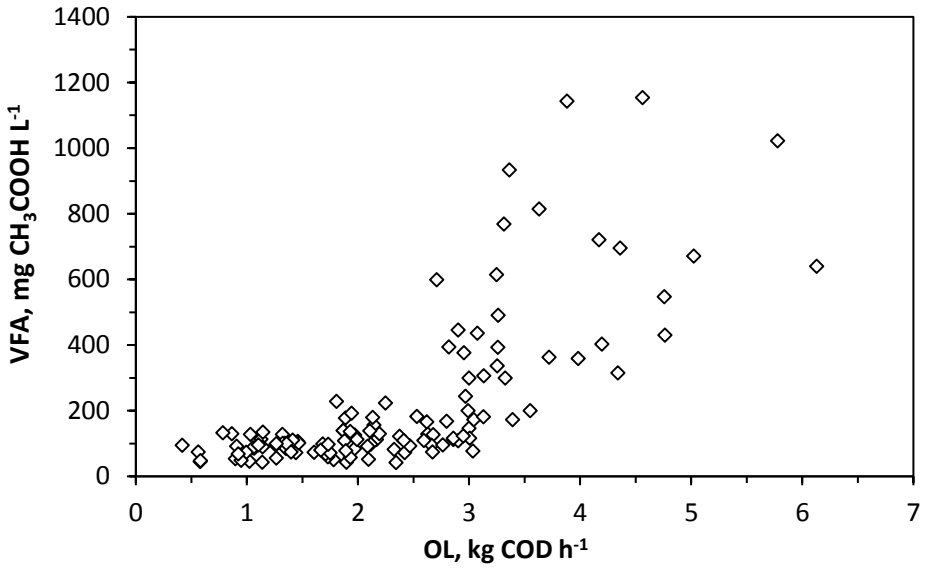

Figure 7.3. Effect of the organic load (OL) on the water effluent VFA concentration of the EGSB reactor. Adapted from Bravo (2017)

Figure 7.3 shows the variation of VFA concentration with OL. Accumulation of VFA in water for average daily loads higher than 3.0 kg COD h^{-1} can be observed. The accumulation of VFA indicates that the slowly growing methanogens cannot sufficiently and rapidly metabolize the intermediate products from VFA producers (acidogens and acetogens population). This imbalanced situation could derived, if keep over time, in the desestabilization of the reactor. Assuming that biomass volume of the reactor was around 3 m^3, the design organic load should be less than 24 kg COD m^{-3} d^{-1} for ensuring stable RE over 94% (VFA < 400 mg CH_3COOH L^{-1} in Figure 7.3).

Table 7.1. Daily average parameters of the water effluent of the EGSB reactor. Partially adapted from Bravo, 2017.

Biomass sampling events	Days of operation	VFA concentration, mg CH_3COOH L^{-1}			Temperature, °C			pH		
		Average	Min	Max	Average	Min	Max	Average	Min	Max
BS-1 (0)	0 - 122	141 ± 130	42.6	696	22.8 ± 2.0	19.4	26.4	7.55 ± 0.33	7.04	8.41
BS-2 (123)	123 – 237	361 ± 285	58.1	1143	24.7 ± 2.9	18.2	30.6	7.37 ± 0.22	6.78	7.96
BS-3 (238)	238 – 333	178 ± 172	43.5	615	23.2 ± 1.6	18.8	26.7	7.39 ± 0.39	6.82	8.75
BS-4 (334)	334 – 412	137 ± 106	49.2	393	27.2 ± 1.7	23.8	30.8	7.48 ± 0.42	6.88	8.63
BS-5 (413)	413 – 429	183 ± 152	90.5	359	29.7 ± 0.8	28.3	30.9	7.26 ± 0.18	7.05	7.74
BS-6 (430)	430 – 484	141 ± 96	80.4	359	28.1 ± 1.4	25.5	30.9	7.35 ± 0.29	6.85	8.45

7.4.2 Granule size distribution

Figure 7.4 shows the granule size distribution of the samples taken during the trial (BS-1, BS-3 and BS-6) based on volume. A narrow range of size distribution was observed in all samples, showing large mean diameters (0.88 mm for BS-1, 0.95 mm for BS-3, and 1.03 mm for BS-6). The results demonstrate that the shift of the substrate from ethanol (inoculum - BS-1) to a mixture of ethanol, ethyl acetate and E2P in which ethanol was the major component had not a marked difference in the granular size, and a relatively small increase in particle size was observed.

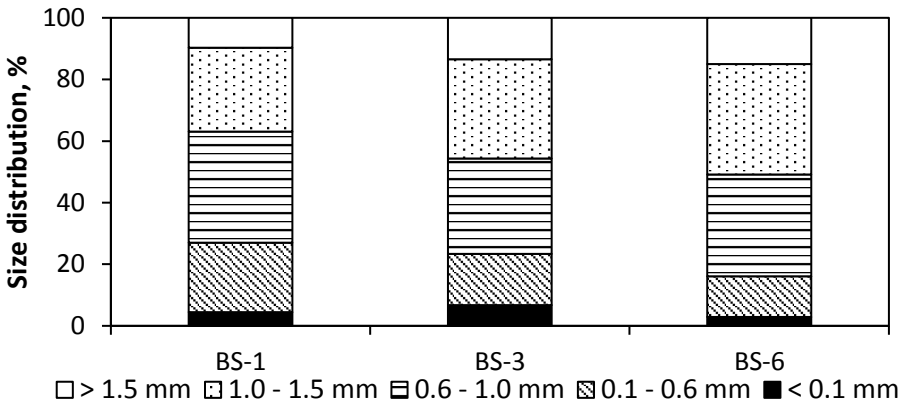

Figure 7.4. Variation of the granule size distribution of biomass over time.

This result contrasts with that previously reported by Lafita et al. (2015). These authors indicated a progressively deterioration in methane production and granule disintegration by working at OLR of 35 kg COD $m^{-3}d^{-1}$ of a mixture of ethanol and 1-methoxy-2-propanol (M2P) (4:1 in mass) intermittently applied (16 hours per day, 5 days a week) to a 4-L reactor. Although the carbon source, the type of sludge and the feeding operation (intermittently) were similar for both studies, the granule disintegration could be due to the different glycol ether used (M2P in the case of Lafita et al. (2015) and E2P in this study) because as it was detailed in the section 4.4.2 one of the intermediate compounds of M2P degradation is methanol and some problems to keep the granule integrity (Kobayashi et al., 2011) or to achieve the granule formation in presence of methanol (Nishio et al., 1993) have previously been described.

7.4.3 Archaeal DGGE

The result of the DGGE for archaeal population is shown in Figure 7.5 for all biomass samples collected during the trial. The predominant bands of the samples, which have been labelled in Figure 7.5, were excised and sequenced. In addition, Shannon's index (H') for each sample was indicated. Table 7.2 summarizes the DGGE band designation, the level of similarity to related GenBank sequences and the phylogenetic group of each strain.

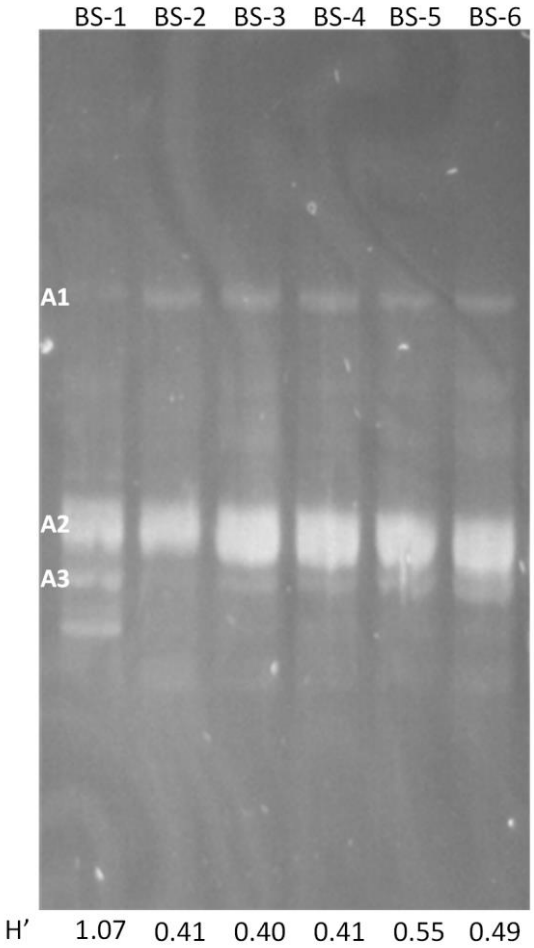

Figure 7.5. Archaeal DGGE profiles of biomass samples from the EGSB reactor including their Shannon Index.

Archaeal DGGE (Figure 7.5) shows a shift of population during the first 123 days, where the biodiversity decreased, as Shannon index (H') decreased from 1.07 (BS-1) to 0.41 (BS-2). After this initial shift in the archaeal population, H' remained stable with no high variations for more than a year (BS-2 to BS-6). The developed archaeal population after the shift in the EGSB reactor presented low archaeal biodiversity, being A2 the predominant band, which has been identified as *Methanosaeta concilii* (Table 7.2). It is a well-known acetotrophic archaea, and the most abundant microorganism in anaerobic granular processes like EGSB and UASB (Díaz et al., 2006).

Table 7.2. DGGE band designation, accession numbers in GenBank and levels of similarity to related organisms according to Figure 7.5.

DGGE band	Closest organism in the GenBank (accession number)	Similarity (%)	Phylogenetic group
A1	*Methanospirillum lacunae* (NR_112981.1)	99	Methanospirillaceae [a]
A2	*Methanosaeta concilii* (NR_102903.1)	100	Methanosaetaceae [a]
A3	*Methanobacterium formicicum* (NR_115168.1)	99	Methanobacteriaceae [a]

[a]Family

Methanospirillum species, band A1, are hydrogenotrophic archaea. Tsushima et al. (2010) found that *Methanospirillum* species were able to proliferate under psychrophilic conditions, in line with that *Methanospirillum* were found in some anaerobic reactors working at low temperatures (Xing et al., 2009). Initially the intensity of this band was lower than in the rest of the samples, the development of *Methanospirillum* in the reactor can be attributed to the operational temperature of the reactor, especially from days 0 to 334. During the first year of experiment, temperature evolves spontaneously associated to the temperature of the air emissions in the facility, daily average temperature reached values as low as 18.5 ˚C (Table 7.1), with 8 days lower than 20 ˚C; favoring the development of *Methanospirillum*. Anycase, the smooth variation of temperature during the whole trial (average of 25.1 ± 3.2 ˚C) did not seem to impact on the removal efficiency of the process, showing that microbial functionality was not adversely influenced. *Methanobacterium* species, band A3, are hydrogenotrophic archaeas. Wang et al. (2015) found that *Methanobacterium* species became predominant in the reactor when a drastic increased in OL was applied. In our study, the intensity of this band was high in the inocucum, a brewery granular sludge that

was taken from a reactor working at high OL; and then, the intensity of *Methanobacterium* increased again in BS-3 (day 238). This could be because working at average daily OLs higher than 3.5 kg COD h^{-1} since day 195 to day 202 seems to be an advantage to this specie.

No *Methanosarcina* was found in the reactor, this could be explained because of the competition with *Methanosaeta* for acetate. *Methanosaeta* has a higher affinity to acetate than *Methanosarcina* (Jetten et al., 1990), hence *Methanosaeta* can be predominant against *Methanosarcina* in stables reactors with low acetate concentrations (McMahon et al., 2001). In our study, the EGSB reactor showed quite stable performance and the acetate concentrations used to be lower than 200 mg L^{-1}. Furthermore, the archaeal population that has been found in this study is very similar to the population found by Xing et al. (2009), who run an EGSB treating a synthetic brewery wastewater at 15 °C where *Methanosaeta*, *Methanobacterium* and *Methanospirillum* reached the 95 % of the archaeal population, being *Methanosaeta* the most predominant archaea. Besides, *Methanosaeta* has been described as an important microorganism in anaerobic granulation processes and also has a key role in the granule integrity (Xing et al., 2009). Therefore, its dominance facilitated the maintenance of the granule integrity along the trial as Figure 7.5 shows. As conclusion, methane in this anaerobic reactor was produced by hydrogentrophic and acetotrophic pathways as the presence in the reactor of hydrogenoclastics and acetoclastics species has been revealed.

7.4.4 Bacterial DGGE

The result of the DGGE for bacterial population is shown in Figure 7.6 for all biomass samples collected during the trial. The predominant bands of the samples, which have been labelled in Figure 7.6, were excised and sequenced. In addition, Shannon's index (H') for each sample was indicated. Table 7.3 summarizes the DGGE band designation, the level of similarity to related GenBank sequences and the phylogenetic group of each strain.

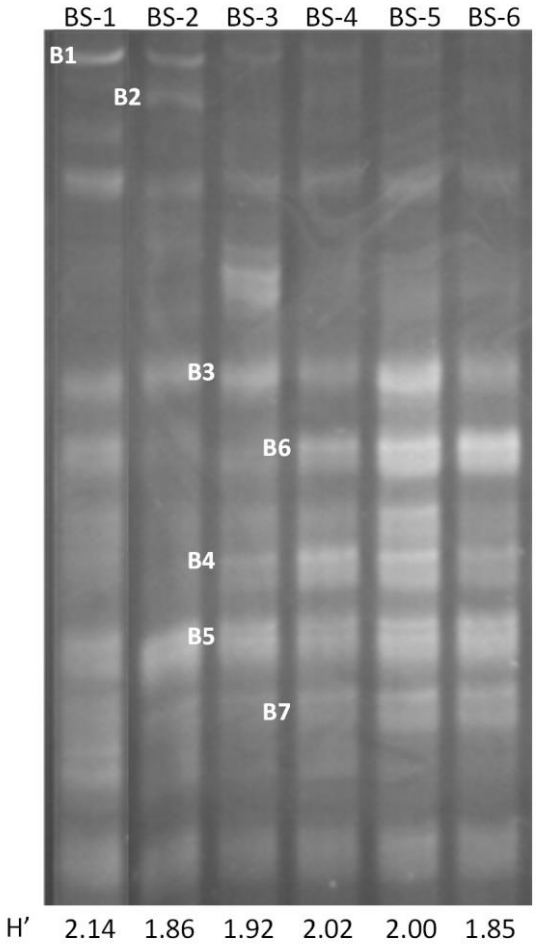

Figure 7.6. Bacterial DGGE profiles of biomass samples from the EGSB reactor including their Shannon Index.

Bacterial DGGE (Figure 7.6) also shows a smooth shift in the bacterial diversity at the beginning of the trial, where the Shannon index revealed a drop, from 2.14 (BS-1) to 1.86 (BS-2), in the diversity of the bacterial population. This decrease in the biodiversity from the initial sludge seems to be due to the differences in the operational and environmental conditions of the reactor. As the granular sludge comes from a reactor treating a complex brewery wastewater, the microorganisms have to adapt to a defined wastewater containing only organic solvents as carbon source, with few major compounds (ethanol, ethyl acetate and

E2P). The increase in the Shannon index from 1.86 (BS-2) to 2.00 (BS-5) from day 123 to day 413, indicates that the biodiversity increased due to new microorganisms were slowly becoming abundant, such as bands B4, B6 and B7. At the end of the experiment, the Shannon index slightly decreased to 1.85 (BS-6) because bands that initially were predominant, B1 and B2, decreased in intensity progressively and finally, in this sample, disappeared. As conclusion, the use of granular sludge from brewery wastewater treatment plant seemed to be a good choice for treating oxygenated solvents coming from VOC emissions of flexographic industry. The predominant bands (A2, B5, B6, and B7) initially came with the sludge, and the change of the carbon source to pure solvents resulted in a population with less biodiversity in which some microorganisms prevailed.

Table 7.3. DGGE band designation, accession numbers in GenBank and levels of similarity to related organisms according to Figure 7.6.

DGGE band	Closest organism in the GenBank (accession number)	Simil (%)	Phylogenetic group
B1	*Candidatus Cloacamonas acidaminovorans* (NR_102986.1)	89%	*Cloacimonetes* [b]
B2	*Candidatus Cloacamonas acidaminovorans* (NR_102986.1)	87%	*Cloacimonetes* [b]
B3	*Sulfurovum sp* (NR_074503.1)	97%	*Epsilonproteobacteria* [c]
B4	*Pelobacter propionicus* (NR_074975.1)	100%	*Pelobacteraceae* [a]
B5	*Geobacter argillaceus* (043575.1)	92%	*Geobacteraceae* [a]
B6	*Geobacter psychrophilus* (043075.1)	97%	*Geobacteraceae* [a]
B7	*Geobacter toluenoxydans* (NR_116428.1)	83%	*Geobacteraceae* [a]

[a] Family; [b] Phylum; [c] Class.

Respecting the bacterial community, B1 and B2 were identified as *Candidatus Cloacamonas acidaminovorans*. Previous studies suggest that these bacteria are probably syntrophic bacteria (Pelletier et al., 2008). *Sulfurovum agreggans*, band B3, is a strictly chemolithoautotrophic bacteria (Mino et al., 2014). This bacterium was previously found in an EGSB working with high sulfate concentration (Chen et al., 2008). In our reactor the sulfate average was low (values < 10 mg L^{-1}), so probably this bacterium came within the brewery sludge and was able to survive under low sulfate concentrations. *Pelobacter propionicus*, band B4, produce acetate and propionate from ethanol with sulfate presence (Schink et al.,

1987), so involved in the VFA production. It was found in anaerobic reactors treating winey wastewater (Cresson et al., 2009). Furthermore, it is remarkable that this bacterium can be associated with *Methanospirillum* species, to which transferring H_2, to degrade primary alcohols and diols (Eichler and Schink, 1985). Yanti et al. (2014) proposed that the mechanism of ethyl ester degradation is the same that the mechanism for methyl ester degradation, so ethyl acetate probably is transformed into acetate and ethanol. In the case of E2P, it has been detected intermediate compounds of its degradation that indicate the mechanisms of the anaerobic degradation of glycol ethers as E2P and M2P, as has been showed in the Chapter 4, Section 4.4.2. So E2P is transformed to ethanol and acetone. Considering that the main intermediate to be degraded was ethanol, a primary alcohol, these two microorganisms, *Methanospirillum* and *Pelobacter*, probably played an important role in the solvent degradation of this study, thus corroborating by their progressively increase of abundance during the trial.

B5, B6 and B7 were identified as species belonging to *Geobacter* genus. They have been identified in different anaerobic reactors treating brewery wastewater (Shrestha et al., 2014), these species can use different substrates as ethanol or acetate. Lovley (2011) demonstrated that *Geobacter* species are able to use direct interspecies electron transfer (DIET). This electron exchange between *Geobacter* and syntrophic partners seems to be an important process in anaerobic wastewater treatment (Commault et al., 2015). It has been demonstrated in laboratory scale digesters that *Methanosaeta* is one of this syntrophic partners, and one third of the methane production in an UASB is produced due to DIET between these two species (Rotaru et al., 2014). In our study *Methanosaeta* and *Geobacter* were the predominant microorganisms in archaeal and in bacterial populations respectively, indicating that these types of interactions occur in the reactor treating a mixture of alcohols and ethers.

7.4.5 Pilot scale vs laboratory scale

In spite of the differences in the operational conditions between the anaerobic reactor of the bioscrubber prototype and the laboratoy scale reactors presented in the Chapter 4, as upflow velocity (around 10 m h^{-1} for laboratory scale reactors and 3 m h^{-1} for the pilot plant reactor) or the work conditions (continous for the laboratory scale reactors and intermitent for the anaerobic reactor at pilot scale), performance in terms of RE was very similar at both scales. The reactor of the anaerobic bioscrubber prototype achieved high RE during all the trial (Figure

7.2) with an average RE of 93%, while laboratory reactors performed also at high RE along all the experimental period (Figure 4.2) with an average RE of 95%, and only the global RE only decreased below 90% during the first days of exporsure to E2P due to the incapacity of the microorganisms to degrade this compound in that time because they needed an adaptation period to be able to metabolize this kind of compounds. In this regard, in Figures 7.7 and 7.8 the OL and the E2P concentration of the effluent of the anaerobic reactor at pilot and laboratory scale reactors can be seen, respectively. In the effluent of the pilot reactor (Figure 7.7) the E2P concentration was higher during the first days of operation achieving its maximum concentration (3525 mg COD L^{-1}) at day 37, and then the E2P concentration decreased to around 500 mg COD L^{-1} from day 75 to day 130. Unfortunately, there were not values between days 40 to 75 to estimate the adaptation period needed by the microorganisms to degrade E2P. The same findings were observed in the effluent of laboratory reactors (Figure 7.8) during the stage S-II when E2P was introduced into the feed of the reactors, with higher concentrations at the first days and then the E2P concentrations remained stable around 300 mg COD L^{-1}. In the case of the laboratory reactors the adaptation period was 42 days to keep constant the oulet concentration of E2P, so this fact confirmed the same behavior for E2P degradation at laboratory and pilot scales.

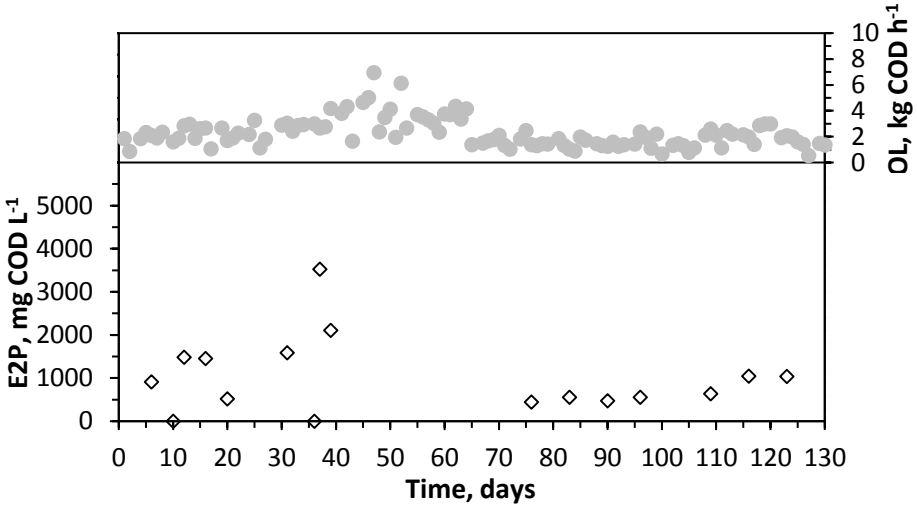

Figure 7.7. OL and E2P concentration at the effluent of the reactor of the anaerobic bioscrubber prototype. Adapted from Bravo (2017).

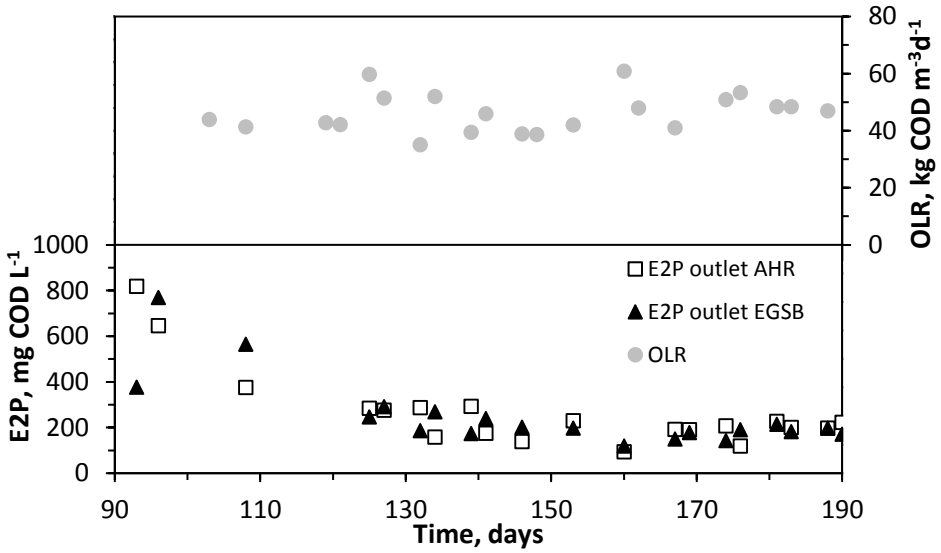

Figure 7.8. OLR and E2P concentration at the effluent of laboratory scale reactors. Adapted from data of Chapter 4.

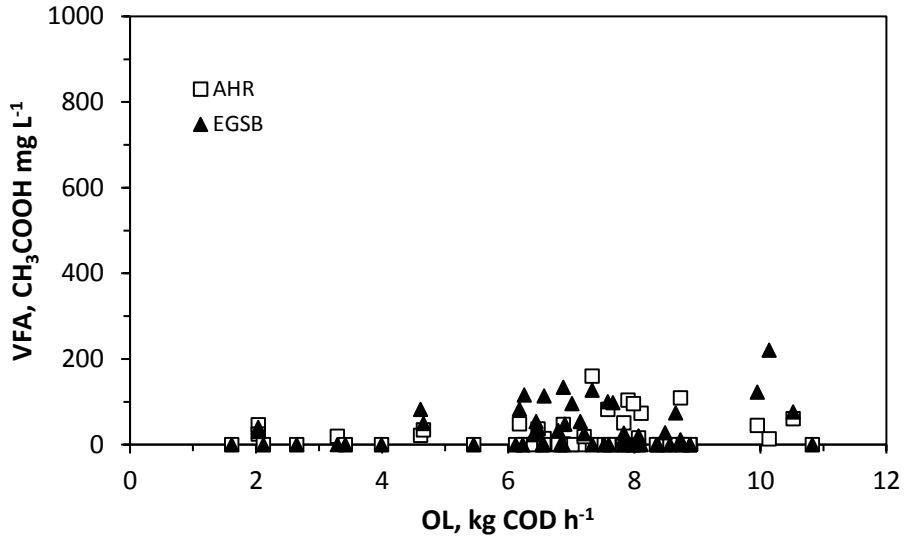

Figure 7.9. Effect of the organic load (OL) on the water effluent VFA concentration of the laboratory scale reactors studied in Chapter 4. Adapted from Figure 4.3.

Pilot plant reactor showed a stable behaviour with VFA contrations below 300 mg L^{-1} when the OL applied to the reactor was up to 3 kg COD h^{-1} (Figure 7.3) but with higher OL, accumulation of VFA was observed. In contrast, as can be seen in Figure 7.9., this behaviour was not observed in laboratory reactors as VFA concentration remained stable even with the highest OL applied to the reactors.

This difference in the VFA accumulation could be due to the stable conditions in which laboratory reactor were operated as the OL was increased slowly and step by step and then maintained along the experimental period. These controlled operational conditions could favour the adaptation of the methanogenic communities to high OL coupling the high VFA production with the methanogenesis. While the reactor of the anaerobic bioscrubber prototype was not able to be operated in the same stable conditions as laboratory scale reactors, the OL varied from day to day due to changes in production facilities (Figure 7.2). These variations in the OL could stress the methanogenic population giving as a result a VFA accumulation.

Comparing the microbial community from the pilot plant (Figures 7.5 and 7.6) and the laboratory scale reactors from the Chapter 5 (Figures 5.1 and 5.2) a stable community along the experimental periods can be observed in the archaeal populations with only a few predominant bands. In both cases, the predominant band in the archaeal population was identified as *Methanosaeta* specie which is in accordance due to the low VFA concentrations observed in the reactors, with only few days with higher VFA concentrations in the pilot scale reactor due to the unstable conditions related with the OL. Also hydrogenotrophic methanogens as *Methanobacterium* species were detected in both experiments, revealing that both pathways of methane formation were present in the reactors (laboratory scale and industrial prototype). Similar findings were observed in the microbial communities from the Chapter 6 (Figure 6.8), where high loads of E2P were used, *Methanosaeta* was a predominant archaea and hydrogenotrophic archaeas as *Methanobacterium* and *Methanospirillum* were found. The only disparity is that in Chapter 6 other archaea was predominant, *Methanomethylovorans uponensis*, a methylotrophic archaea (Cha et al., 2013), and no one methylotrophic archaea was found in the industrial prototype. This difference could be associated to the lack of M2P in the emission of the flexographic industry where the industrial prototype was installed, because methanol, which is need for the apparition of these kind of species, has been identified as an intermediate of M2P degradation is methanol.

Respect to the bacterial communities, an evolution was found from the initial sludge to the final sample indicating the adaptation of the sludge (obtained from a brewery wastewater treatment plant in both cases) to the new environment and the new compounds to degrade. Some predominant bands of samples from the pilot unit and the laboratory scale reactors from (Chapter 5) were associated to the same microorganisms as *Geobacter* and *Pelobacter* species which could indicate that these microorganisms played an important role in the degradation of solvents. Furthermore, as it was described in Section 7.4.4, *Methanosaeta* and *Geobacter* species are syntrophic partners using direct interspecific electron transference (DIET) and both microorganisms has been identified in both types of reactor, so this kind of interaction seems to be an important mechanism for the anaerobic degradation of solvents from flexographic industry as ethanol, ethyl acetate and E2P. Comparing the bacterial communities with the microorganisms identified in the Chapter 6, also was observed an evolution from the inoculum to the final sludge, and the predominant band in the study from that chapter was also a *Geobacter* specie which confirm that this specie played an important role to degrade the solvents from the flexographic industry. However, *Clostridium methoxybenzovorans* was also identified in the Chapter 6, this specie is able to cleave ether bonds from methoxylated aromatic compounds, and bacterias with these capabilities was not found in the industrial prototype. This fact could be associated to the characteristics of the emission, because in Chapter 6 the reactor was treating high OL of M2P (a methoxylated compound) and in the industrial prototype M2P was not presented in the emission.

7.5 CONCLUSSIONS

The reactor of the anaerobic bioscrubber prototype performed stable with high RE (93%) in spite of the variations in the conditions as operational temperature and OL. The limit in OL for a stable conversion of solvents into methane without VFA accumulation was stablished in 3 kg h^{-1}, equivalent to 24 kg $m^{-3}d^{-1}$. The use of granular sludge from a brewery wastewater treatment plant as initial sludge resulted in a good strategy to achieve high RE since the startup. The change in the carbon source to only a few solvents caused an impact in the microbial communities reflected in the biodiversity, which had an initial decrease especially in *Archaea*. The predominant microorganisms identified in the reactor can be associated with the carbon source and operational parameters such as temperature and organic load. Laboratory scale reactors and the reactor from the pilot unit performed similar in

terms of RE and the same adaptation period was found to degrade E2P. Also, a similar microbial community dinamics was found at both scale reactors, with the predominance of *Methanosaeta* and *Geobacter* species, syntrophic partners using DIET, which suggests that DIET is an important mechanism to transform solvents from printing industries into methane.

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8 CONCLUSIONS AND PERSPECTIVES

This thesis has been focused in the anaerobic degradation of solvents used in the flexographic industry. The principal aims were: i) to evaluate alternatives to the EGSB reactor in order to improve the biomass retention and the global performance; ii) to study the impact or the toxic effects of the solvents from flexographic industries in the microbial community structure, iii) to evaluate the effect of the ethanol / glycol ethers ratio in an EGSB reactor treating mixtures of these compounds, and iv) to analise the microbial community structure of an on-site pilot plant reactor and to compare results obtained with the microbial populations developed in the laboratory scale reactors. The main conclusions of this work are:

I. The anaerobic treatment of ethanol-glycol ethers mixtures at high OLR (45 kg COD m^{-3} d^{-1}) with high rate anaerobic reactors has been demonstrated as a feasible technology, achieving global RE higher than 95%. Furthermore, the reactor hybridation by installing packing material in the upper part of an EGSB reactor has been showed as a good alternative for improving biomass retention but not the reactor performance.

II. The detection and identification of acetone, isopropanol and methanol in the effluent of the reactors as intermediate compounds of the anaerobic degradation of glycol ethers revealed the possible mechanism for the ether cleavage.

III. The use of molecular tools as the DGGE, qPCR and high throughput sequencing techniques has been showed as important tools to monitor and to evaluate the possible impacts or negative effects of the solvents over the microbial population. DGGE and high throughput sequencing technologies revealed a succession in the predominant bacterial populations when the E2P and M2P were added as substrates, changing from a dominance of *Proteobacteria* phylum (more specifically of *Geobacter* genus) to a dominance of *Firmicutes* phylum (more specifically of *Clostridium* genus). While, by the use of qPCR a negative impact and a toxic effect was observed when E2P was introduced to the feeding with an important decreased in the densities of every population analised and then, when the RE of this compound increased, the densities of the analised populations were recovered up to their initial values. In contrast, the introduction of M2P as a substrate did not cause any effect in the bacterial population, indicating that M2P is

degraded by the same mechanism than E2P, and the adaptation of biomass to E2P favoured the inmediate degradation of M2P. But, M2P caused a negative impact in the methanogenic population decreasing its densities when it was added to the feeding and when its OLR was increased.

IV. DGGE showed that no microbial stratification was found in the sludge bed of the EGSB and the AHR, as the same predominant bands were observed in the samples taken from the bottom and the top ports of the reactors. While, the microorganisms developed in the support material of the AHR considerably differed from the microbial population of the sludge bed, having all the microorganisms developed in the support material a metabolism related with the VFA.

V. The diminution of ethanol proportion in ethanol-glycol ethers mixtures showed that ethanol had not an effect in the RE of glycol ethers, but it had a role in the maintenance of the structure of the granular sludge, as a partial degranulation of the sludge bed was observed when only glycol ethers were fed to the reactor being ethanol completely eliminated in the feeding. Additionally, EPS content revealed that the proteins / polysaccharides (PN / PS) ratio could be used as a good indicator of the health and stability of the granular sludge, as a decrease in this parameter (PN / PS ratio) was observed in the degranulated sludge.

VI. Treating ethanol-glycol ethers mixtures with high content in glycol ethers, RE of M2P reached a complete removal, while RE of E2P reached 65% and when OLR of glycol ethers was increased RE of E2P decreased showing a kinetic limitation, with an apparent K_s of 8 kg COD m^{-3}, which suggest an enzymatic competitive inhibition mechanism for the degradation of both glycol ethers, and M2P was favoured due to the smaller size of its functional group.

VII. DGGE technique revealed that the changes in the physico-chemical properties of the granular sludge was not transferred directly in sudden changes in the microbial community structure, as almost identical fingerprintings of the granulated and degranulated sludge were found at the end of experiment. Furthermore, a close relationship between the microbial communities identified and the substrates used was found. *Methanomethylovorans* and

Methanosaeta species were the predominant methanogenic archaeas due to the presence of methanol, an intermediate product of degradation of M2P, and the low accumulation levels of VFA. While in the bacterial communities *Geobacter* species were predominant due to the presence of ethanol, as these microorganisms are known as ethanol degraders.

VIII. In the anaerobic bioscrubber prototype, a limit of 24 kg COD m^{-3} d^{-1} in the OLR for the treatment of these emissions was found to keep a balance between the acetogenic and methanogenic stages maintaining the VFA in low levels without accumulation. However, this limit was not observed at laboratory scale reactors. Besides, a similar adaptation period (around 40 days) to degrade E2P was observed in the anaerobic bioscrubber prototype and in the laboratory scale reactors.

IX. Microbial community analysis of the reactor of the anaerobic bioscrubber prototype showed an evolution from the inoculum to the end of the experiment, which revealed an adaptation of the microorganisms to the new conditions of the reactor, such as substrates, OLR or temperature. In the archaeal, these new conditions led to a predominant population that persisted over the time, being *Methanosaeta concilii* the predominant methanogenic archaea in this reactor. While the bacterial populations change, decreasing the intensity of predominant bands in the inoculum and increasing its intensity other bands better adapted to the solvents, being the predominant bands *Geobacter* and *Pelobacter* species. Besides, direct interspecific electron transference should be an important mechanism for the solvent degradation as the presence in all the reactors analysed of *Methanosaeta* and *Geobacter* species revealed.

This thesis is the first work with mixtures of glycol ethers at high content, and it is the first study where the degradation mechanism of these compounds has been demonstrated. Furthermore, it has been revealed a toxic effect of the glycol ethers over the microbial community structure and a potential enzymatic inhibition competition of these compounds. From this thesis, in order to obtain a deeper knowledge about the degradation of these solvents the following studies are going to be carried out:

I. The degradation mechanism of glycol ethers showed in this thesis suggest that enzymes are required for the ether cleavage, so the searching of the responsible enzymes of this mechanism appears as an interesting study in order to improve the performance and to know the conditions, as cofactors necessities, for a better function and kinetics of these enzymes.

II. Microbial community analysis of reactors treating solvents from flexographic industries revealed *Methanosaeta* and *Geobacter* as predominant microorganisms playing an important role in the solvent degradation. These microorganisms are syntrophic partners able to use direct interspecific electron transference. Therefore, the use of conductive materials, such as activated carbon, where these microorganisms could be attached seems to be an attractive alternative to promote the direct interspecific electron transference and the growth of these microorganisms involved in the anaerobic degradation of solvents from the flexographic industry.

9 LIST OF FIGURES

10 **LIST OF TABLES**

11 **NOMENCLATURE AND ABBREVIATIONS**

AHR	Anaerobic Hybrid Reactor
ARC	Archaea
ATP	Adenosine Triphosphate
b	Decay coefficient
bp	Base pair
BP	Bottom Port
BAC	Bacteria
BLAST	Basic Local Alignment Search Tool
BMP	Biomethane Potential
COD	Chemical Oxygen Demand
CRT	Cellular Retention Time
CSTR	Continuous Stirred Tank Reactor
C_t	Cycle Threshold
DGGE	Denaturing Gradient Gel Electrophoresis
DIET	Direct Interspecific Electron Transference
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double Stranded Deoxyribonucleic Acid
E2P	1-Ethoxy-2-propanol
EC	Elimination Capacity
EDTA	Ethylenediaminetetraacetic acid
EEA	European Environmental Agency
EGSB	Expanded Granular Sludge
EPS	Extracellular polymeric substances
FRET	Fluorescence Resonance Energy Transfer
GC	Gas Chromatography
GLS	Gas-Liquid-Solid

H'	Shannon Index
HRT	Hydraulic Retention Time
IPA	Isopropanol
M2P	1-Methoxy-2-Propanol
MBT	Methanobacteriales
MMB	Methanomicrobiales
MSL	Methanosarcinales
N	Sum of intensities of the bands of each sample
$\mathbf{n}_i$	Intensity of the band
NMVOC	Non Methane Volatile Organic Compound
OL	Organic Load
OLR	Organic Loading Rate
PCR	Polymerase Chain Reaction
PN/PS	Ratio Proteins/Polysaccharides
PRTR	Pollutant Release and Transfer Register
qPCR	Quantitative Polymerase Chain Reaction
RE	Removal Efficiency
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Desviation
SM	Support Material
SMA	Specific Methanogenic Activity
SS	Suspended solid
t	time
TOC	Total Organic Carbon
TP	Top Port
TSS	Total Suspended Solid
UASB	Upflow Anaerobic Sludge Bed

VFA	Volatile Fatty Acid
VOCs	Volatile Organic Compounds
VSS	Volatile Suspended Solid
$\mathbf{V_{up}}$	Upflow Velocity
X	Biomass
$\mathbf{X_0}$	Initial biomass

12 APPENDICES: RESUMEN EXTENDIDO AND PUBLISHED PAPERS

12.1 APÉNDICE A: RESUMEN EXTENDIDO

Antecedentes

La emisión de compuestos orgánicos volátiles (COVs) puede causar diferentes problemas en la salud pública y en el medio ambiente, actuando como contaminantes primarios y permitiendo la formación de contaminantes secundarios como el ozono troposférico. Debido a estos problemas, la emisión de COVs está limitada en muchos países tales como EEUU o los pertenecientes a la Unión Europea, en este caso regulado por la directiva 2010/75/EU. Debido al uso de disolventes en su proceso productivo, la industria flexográfica es uno de los sectores que más contribuye a la emisión de este tipo de compuestos, y las técnicas biológicas han sido consideradas como una de las mejores disponibles para el tratamiento de emisiones de COVs en este sector industrial. En este sentido, la transformación de emisiones de COVs en bioenergía podría ser considerada como un proceso emergente y viable que para llevar a cabo este tratamiento, pudiéndose destacar una nueva tecnología para ello: el biolavador anaerobio. En este proceso, los COVs son transferidos de la fase gas (emisión gaseosa) a la fase líquida (agua) y después son transformados en biogás en un biorreactor, pudiéndose destacar el uso del reactor anaerobio de lecho fluidizado (EGSB, por sus siglas en inglés).

Sin embargo, el utilización de reactores EGSB en este proceso presenta algunas barreras inherentes al proceso, como la escasa información existente acerca de la degradación anaerobia de algunos compuestos típicamente utilizados en la industria flexográfica, o la pérdida de biomasa en el efluente debido al uso de una elevada velocidad ascensional. Con relación a este último aspecto, el estudio de la operación empleando una configuración alternativa al reactor EGSB, conocida como configuración hibrida, para evitar la pérdida de biomasa ha mostrado resultados prometedores en estudios previos. Así por ejemplo, una configuración de reactor híbrido anaerobio, consistente en la instalación de un filtro en la zona superior del reactor donde se ubica el separador gas-líquido-sólido, podría llegar a ser una buena alternativa para mejorar la capacidad de retención de la biomasa en los reactores EGSB. Por otro lado, la industria flexográfica emplea con frecuencia disolventes orgánicos sintéticos del tipo éteres glicólicos pudiéndose destacar entre ellos el 1-etoxi-2-propanol (E2P) y/o el 1-metoxi-2-propanol (M2P). No obstante, la biodegradación anaerobia de mezclas con estos compuestos era prácticamente desconocida. Además, apenas hay información disponible en la bibliografía sobre el posible efecto negativo o los efectos tóxicos que estos éteres glicólicos pueden tener en la población microbiana anaerobia. En este sentido, la aplicación de diferentes técnicas moleculares, como la electroforesis en gel con gradiente desnaturalizante (DGGE), la reacción en cadena de la polimerasa cuantitativa (qPCR)

o técnicas de secuenciación masiva, pueden ser de utilidad para caracterizar las comunidades microbianas y para analizar su evolución en sistemas biológicos como los reactores anaerobios.

Alcance y objetivos

Los objetivos generales de esta tesis doctoral son: i) evaluar una alternativa al reactor EGSB para mejorar la capacidad de retención de biomasa y el rendimiento del reactor tratando mezclas de éteres glicólicos y etanol; ii) estudiar las rutas de la degradación anaerobia de los éteres glicólicos, M2P y E2P, utilizados en la industria flexográfica y el posible impacto que podrían causar en la comunidad microbiana; iii) evaluar el efecto de la relación etanol / éteres glicólicos en un reactor EGSB tratando mezclas de estos compuestos; y iv) comparar el rendimiento y la evolución las estructuras de las comunidades microbianas entre los reactores a escala de laboratorio y un prototipo industrial.

Para alcanzar estos objetivos generales se han establecido los objetivos específicos descritos a continuación, correspondientes a los cuatro capítulos de resultados en los que se ha estructurado la tesis (capítulos del 4 al 7).

Los objetivos específicos del capítulo 4 titulado “Degradación anaerobia de mezclas de etanol y éteres glicólicos en reactores EGSB e híbrido: Comparación del rendimiento y mecanismo de ruptura del enlace éter” son:

- Evaluar la viabilidad de la degradación anaerobia de una mezcla típica de disolventes presentes en la emisión de la industria flexográfica, especialmente los éteres glicólicos como M2P y E2P en un reactor EGSB.
- Determinar el comportamiento de un reactor EGSB híbrido modificado con un lecho de anillos de polietileno en la zona superior del reactor donde se lleva a cabo la separación gas-líquido-sólido y comparar la capacidad de retención de biomasa de este reactor con la de un reactor control EGSB.
- Profundizar en el estudio de los mecanismos implicados en la ruptura del enlace éter en la degradación anaerobia de los éteres glicólicos M2P y E2P.

Los objetivos específicos del capítulo 5 titulado “Degradación anaerobia de mezclas de etanol y éteres glicólicos en reactores EGSB e híbrido: Estudio de la comunidad microbina” son:

- Investigar la dinámica de la comunidad microbiana utilizando técnicas de biología molecular para mejorar la comprensión del proceso.

- Evaluar la estratificación microbiana en reactores anaerobios de alta carga con elevadas velocidades de flujo ascendente (≈10 m h^{-1}).
- Determinar el impacto de la introducción de los éteres glicólicos M2P y E2P en las poblaciones microbianas.

Los objetivos específicos del capítulo 6 titulado "Análisis del comportamiento, estabilidad y comunidad microbiana de un reactor EGSB tratando mezclas de disolventes de éteres glicólidos a concentraciones elevadas en mezclas con etanol" son:

- Determinar la influencia de la disminución de la proporción de etanol en la estabilidad del fango granular anaerobio y en las comunidades microbianas.
- Evaluar el rendimiento y la capacidad de eliminación de los éteres glicólicos M2P y E2P cuando estos son mayoritarios en el alimento.
- Analizar la comunidad microbiana en el reactor al tratar relativamente altos valores para las M2P y E2P.

Los objetivos específicos del capítulo 7 titulado "Análisis de la comunidad microbiana de un prototipo de biolavador anaerobio tratando emisiones de COVs de una empresa del sector flexográfico" son:

- Evaluar las comunidades microbianas en un prototipo de reactor instalado en una industria flexográfica.
- Vincular la evolución microbiana con las condiciones de operación y la fuente de carbono presente en las emisiones industriales.
- Comparar el rendimiento de la operación y las poblaciones microbianas presentes en los reactores a escala de laboratorio y el prototipo industrial.

Materiales y métodos

En la presente tesis doctoral se realizaron ensayos a escala de laboratorio simultáneamente en diferentes reactores EGSB, todos ellos con idénticas dimensiones. El volumen efectivo de lecho de fango de cada reactor era de 4 L (1.205 m de altura con un diámetro de 0.065 m) y un volumen total de 19 L incluyendo el elemento de separación Gas/Líquido/Sólido (ubicado a una altura de 1.20-1.60 m) e instalado en la parte superior. La temperatura de los reactores se mantuvo a 25 °C, empleando un sistema de camisa externa con recirculación de agua de un baño termostatizado. En uno de estos reactores se introdujo un relleno compuesto por anillos de polietileno en la parte superior donde se producia la

separación de las fases (situado entre 1.40 y 1.57 m en el reactor). Este reactor modificado constituye una configuración alternativa a la convencional, a la que se ha denominado como reactor híbrido anaerobio (AHR, por sus siglas en inglés). Cada uno de estos reactores anaerobios estaba equipado con dos bombas peristálticas que proporcionaron los caudales volumétricos establecidos tanto del alimento, habitualmente 10 L dia^{-1}, y de recirculación deseados, fijado en 32 L h^{-1}, para proporcionar una velocidad ascensional de alrededor de 10 m h^{-1} en el lecho de fango y de alrededor de 1.0 m h^{-1} en la zona superior de separación Gas/Líquido/Sólido. En todos los estudios realizados, la puesta en marcha se llevó a cabo inoculando al reactor 4 L de un fango anaerobio granular procedente de una planta de tratamiento de una empresa de fabricación de cerveza. Se incrementó paulatinamente la carga orgánica alimentada manteniendo constante el caudal y aumentando progresivamente la concentración de los compuestos orgánicos (expresada como demanda química de oxigeno, kg DQO m^{-3} d^{-1}). El alimento al reactor estaba tamponado con bicarbonato sódico a una concentración de 5 g $NaHCO_3$ L^{-1} y contenía una concentración de magnesio y calcio de 40 mg L^{-1}, además de la ajustada cantidad de los diferentes micronutrientes necesarios. Las primeras etapas de la puesta en marcha se hicieron alimentando únicamente etanol, y posteriormente se incorporaron los éteres glicólicos, E2P y M2P, en diversas proporciones según el experimento diseñado. Periódicamente se analizaron las principales características del efluente líquido de los reactores: concentraciones de DQO, de los distintos COVs, de ácidos grasos volátiles (AGV), y sólidos suspendidos principalmente. El caudal y composición del biogás producido también fue regularmente monitoreado.

En el capítulo 4 de esta tesis, el funcionamiento a escala de laboratorio de un reactor EGSB convencional (control) se comparó con el de un reactor con la configuración alternativa que pudiese mejorar la capacidad de retención de biomasa, un AHR. Ambos reactores fueron operados durante más de 300 días bajo idénticas condiciones y el experimento fue dividido en siete etapas donde se varió la carga y/o la composición del alimento (Tabla A1). En las primera etapas, la velocidad de carga orgánica (OLR, por sus siglas en inglés) se aumentó paulatinamente hasta 45-55 kg DQO m^{-3} d^{-1} utilizando un sustrato fácilmente biodegradable como etanol, para después introducir en posteriores etapas un éter glicólico, el E2P, dando lugar a una mezcla binaria de etanol y E2P, y la OLR total fue mantenida alrededor de 45 kg DQO m^{-3} d^{-1}. En las siguientes etapas, se introdujo también M2P como nuevo sustrato en el alimento de los reactores (mezcla terciaria de etanol, E2P y M2P) manteniendo la OLR constante. Después, la alimentación se interrumpió para simular una parada larga del proceso productivo, típica en las

industrias de impresión flexográfica, y así poder comprobar cómo afecta al reactor un largo periodo de ausencia de sustrato. Después, los reactores fueron puestos en marcha otra vez aumentando la OLR paulatinamente con la mezcla terciaria, y finalmente, en la última etapa la proporción de M2P fue ligeramente aumentada. La proporción de éteres glicólicos nunca superó el 30% en peso en estos experimentos. Para elucidar los mecanismos de reacción se empleó de forma paralela otro reactor EGSB convencional, donde tras la puesta en marcha, alimentando únicamente etanol hasta una OLR de 45 kg DQO m^{-3} d^{-1}, se empleó una mezcla de etanol y acetona.

Tabla A1. Condiciones de operación de los reactores EGSB control e híbrido (AHR) (Capitulo 4).

Etapa	S–I	S–II	S–III	S–IV	S–V	S–VI	S–VII
Tiempo (d)	0–90	91–202	203–221	222–230	231–271	272–305	306–335
DQO (g L^{-1})	3.9–18.8	18.9±3.0	21.6±3.8	19.5± 2.1	–	6.3–16.3	18.5±1.9
OLR (kg DQO m^{-3} d^{-1})	9.1–47.0	47.4±7.4	54.1±9.5	48.8±5.1	–	15.9–40.7	46.2±4.8
E2P (g DQO L^{-1})	0	2.0±0.3	4.2±0.3	2.4±0.9	–	0.7–2.1	1.9±0.2
E2P OLR (kg DQO m^{-3} d^{-1})	0	5.1±0.8	10.6±0.7	6.1±2.3	–	1.7–5.3	4.8±0.6
M2P (g DQO L^{-1})	0	0	0	1.9±0.1	–	0.6–1.9	3.3±0.4
M2P OLR (kg DQO m^{-3} d^{-1})	0	0	0	4.8±0.2	–	1.5–4.7	8.3±1.1

En el capítulo 6 de esta tesis, y con el objetivo general de determinar la influencia en la degradación anaerobia de la relación etanol/éteres glicólicos, especialmente cuando estos últimos son mayoritarios, se describe el estudio experimental realizado empleando un reactor EGSB convencional a escala de laboratorio. El experimento fue dividido en 8 etapas (Tabla A2). Entre la etapa I y la etapa III, se llevó a cabo la puesta en marcha del reactor empleando etanol como única fuente de carbono, aumentando la OLR en las distintas etapas hasta alcanzar un valor de 45 kg DQO m^{-3} d^{-1}. Después, en las siguientes etapas, el etanol fue sustituido progresivamente por éteres glicólicos, E2P y M2P, manteniendo constante la OLR en 45 kg DQO m^{-3} d^{-1}, hasta la última etapa donde los éteres glicólicos fueron la única fuente de carbono alimentada al reactor.

Tabla A2. Condiciones operacionales del reactor EGSB en diferentes etapas.

Etapas	I	II	III	IV	V	VI	VII	VIII
Duración, d	4	7	7	32	17	7	11	3
EtOH: M2P: E2P*	10:0:0	10:0:0	10:0:0	8:1:1	7:1.5:1.5	5:2.5:2.5	2:4:4	0:5:5
OLR, kg COD m^{-3} d^{-1}	15	32.83	40.05	41.01	45.01	39	47.16	44.05
C_S, g COD L^{-1}	6	13.13	16.02	16.4	18	15.6	18.86	17.62

* Relación másica; C_s Concentración total de disolventes

En el capítulo 7, se llevó a cabo el estudio de la comparativa de la evolución y estructura de la comunidad microbiana presente entre los reactores a escala de laboratorio y un reactor prototipo instalado en una planta industrial del sector flexográfico. El funcionamiento operativo de este reactor prototipo, con unas características operacionales semejantes a las del reactor de laboratorio, no ha sido objeto de estudio o análisis en esta tesis, pero sí las características y dinámica de las poblaciones microbianas presentes en el mismo durante su puesta en marcha y operación.

A lo largo de todos estos experimentos, tanto a escala de laboratorio como del prototipo, se tomaron muestras de biomasa en los reactores anaerobios. Estas muestras biológicas se caracterizaron empleando diversas herramientas moleculares principalmente, i) la electroforesis en gel con gradiente desnaturalizante (o *denaturing gradient gel electrophoresis* (DGGE) en inglés); ii) la técnica de reacción en cadena de la polimerasa cuantitativa (o *quantitative polymerase chain reaction* (qPCR) en inglés); y ii) tecnología de secuenciación masiva (en inglés *high throughput sequencing technologies*). El uso combinado de estas técnicas permitió determinar la biodiversidad microbiana, las abundancias de los diferentes órdenes y filos (bacterias, archaeas, metanosarcinales, metanomicrobiales y metanobacteriales), así como la identificación de las especies predominantes en las diferentes muestras, permitiendo relacionar el rendimiento de los reactores anaerobios con la estructura de las comunidades microbianas presentes, tal como se describe en los capítulos 5, 6 y 7.

Resultados y Discusión

En el estudio comparativo entre el reactor EGSB convencional (control) y el AHR (Tabla A1), los resultados mostraron un alto rendimiento en ambos reactores con eficacias de eliminación (EE) globales mayores al 92% incluso tratando OLR de hasta 54 kg DQO m^{-3} d^{-1} y solo bajó por debajo del 90% en ambos reactores durante los primeros días después de la introducción de E2P en el alimento (S-II), indicando que la biomasa no estaba adaptada a este disolvente y se necesitaba un periodo de adaptación para poder de metabolizar este compuesto. Este periodo de adaptación fue también observado en la evolución de la EE del E2P, ya que ésta solo alcanzó un 20% en ambos reactores en el momento de la introducción de este compuesto, pero tras 40 días la EE del E2P aumentó hasta el 80% y se mantuvo a lo largo de todo el periodo experimental. Tras la introducción del M2P (S-IV), su eliminación fue completa (100%) desde el mismo momento de su introducción en el reactor, no apreciándose ningún periodo de adaptación lo que sugeriría que ambos éteres glicólicos presentan el mismo mecanismo de degradación. Además, la eliminación de M2P fue casi completa durante todo el periodo experimental (desde S-IV hasta S-VII), incluso después de aumentar su proporción en el alimento (S-VII). Durante los primeros días de introducción de los éteres glicólicos se detectaron e identificaron algunos productos intermedios de su degradación como son el metanol, la acetona y el isopropanol, lo que se pudo relacionar con la ruta de degradación anaerobia de estos compuestos. En cuanto a la capacidad de retención de biomasa, el AHR mostró una mayor capacidad de retención de biomasa que el reactor EGSB, como se comprobó a partir de los sólidos acumulados en el efluente de cada reactor (563.2 g en el reactor EGSB y solo 293.7 g en el AHR). Sin embargo, el filtro instalado en el AHR finalmente se colmató y los anillos del filtro tuvieron que ser reemplazados por nuevos anillos de polipropileno para mantener su capacidad de retención de biomasa. A pesar de la mayor concentración de biomasa en el AHR, ambos reactores mostraron un rendimiento similar a lo largo de todo el estudio.

Estos resultados, que se recogen en el capítulo 4 de esta tesis, han sido publicados en la revista Journal of Environmental Management (Ferrero, P., San-Valero, P., Gabaldón, C., Martínez-Soria, V., Penya-roja, J.M., 2018. Anaerobic degradation of glycol ether-ethanol mixtures using EGSB and hybrid reactors: Performance comparison and ether cleavage pathway. J. Environ. Manage. 213, 159–167).

A partir de las muestras de biomasa del estudio anterior, se analizó también la dinámica de la comunidad microbiana de ambos reactores usando diferentes

técnicas de biología molecular, como la DGGE, la qPCR y la secuenciación masiva. Estos análisis revelaron un importante impacto en las poblaciones microbianas causadas por la introducción de ambos éteres glicólicos (E2P y M2P). La técnica DGGE mostró una evolución en la comunidad bacteriana desde el principio hasta el final del experimento. De modo que algunas bandas inicialmente predominantes (en el inóculo y/o en etapas tempranas) disminuyeron su intensidad en etapas posteriores, y lo contrario ocurrió con otras bandas, que aparecieron o aumentaron su intensidad, e incluso se convirtieron en predominantes con la evolución del experimento. Además, la DGGE también evidenció que el volumen ocupado por el lecho de ambos reactores se comportó como un reactor de mezcla completa, ya que se encontraron comunidades microbianas con la misma estructura tanto en la parte inferior como en la superior del reactor. Los resultados de la qPCR indicaron un efecto tóxico de los éteres glicólicos sobre las poblaciones de archaeas y bacterias, ya que las concentraciones de las poblaciones disminuyeron después de la introducción de E2P. Los valores de las concentraciones de ambas poblaciones se recuperaron tras 30 días de exposición a este disolvente, lo que corroboró la necesidad de un periodo de adaptación de los microorganismos para degradar anaerobiamente el E2P. Además, cuando posteriormente se introdujo el M2P en el alimento, no se observó ningún efecto negativo sobre la concentración de la población bacteriana demostrando que el M2P era degradado por la misma ruta que el E2P, pues la población bacteriana ya estaba adaptada a este sustrato (M2P). Por contra, la concentración de las poblaciones de archaeas como *Methanobacteriales* y *Methanomicrobiales* disminuyeron después de la introducción de M2P y también después de aumentar la OLR de este sustrato, lo que sugiere un efecto tóxico del M2P sobre estas poblaciones de archaeas. Los resultados de la secuenciación masiva enfatizaron la importancia del tipo de sustrato sobre la predominancia y evolución de las comunidades microbianas a lo largo del periodo experimental. A través de esta técnica, el filo *Proteobacteria* se identificó como predominante cuando los reactores estaban siendo alimentados solo con etanol como fuente de carbono, y la predominancia de este filo fue reemplazada por el filo *Firmicutes* cuando se introdujeron los éteres glicólicos como sustrato.

Este estudio, que se ha incluido en el capítulo 5 de esta tesis, se desarrolló en colaboración con el *Laboratoire du Génie de l'Environment Industriel* del *École des mines d'Alès* (Université de Montpellier) durante una estancia de investigación bajo la supervisión del profesor Luc Malhautier. Recientemente, los resultados de esta investigación han sido enviados para su publicación a la revista Bioresource Technology (Ferrero et al. Link between the anaerobic degradation of glycol ether-

ethanol mixtures using EGSB and hybrid reactors and the dynamics of the microbial community structure. Bioresource Technology, pendiente de decisión)

Posteriormente, en el estudio llevado a cabo para determinar la influencia de elevadas proporciones de los éteres glicólicos en mezclas con etanol en la degradación anaerobia (Tabla A2), de nuevo el reactor EGSB convenional de laboratorio presentó un alto rendimiento (EE>95%) durante su puesta en marcha con etanol como única fuente de carbono. Cuando los éteres glicólicos fueron introducidos la EE global de nuevo cayó inicialmente, hasta el 80%, y después se recuperó hasta el 90%, reflejando que la biomasa necesitó un periodo de adaptación para degradar estos compuestos (E2P y M2P), tal como se había obtenido en los experimentos previos. Cuando el etanol era sustituido progresivamente por éteres glicólicos en el alimento como fuente de carbono, la EE global disminuía ligeramente en consonancia, y en la última etapa (VIII) donde solo se alimentó E2P y M2P (sin etanol) al reactor, la EE redujo hasta el 80% indicando que la eliminación de los éteres glicólicos no podría ser completa, en las condiciones de operación utilizadas. Con respecto a la eliminación individual de cada compuesto, se registró una baja EE durante los primeros días de exposición a cada compuesto (≈20%), pero posteriormente se registraron sus máximas EE: la máxima EE del E2P de alrededor del 70% alcanzada tras 15 días de exposición, y la máxima EE del M2P del 100% tras 24 días de exposición. Además, en la última etapa donde no se introdujo etanol en el reactor, se observó una desgranulación parcial del lecho, lo que podría sugerir que el etanol es un sustrato necesario para mantener la estructura granular del fango. Se estudió el contenido en sustancias poliméricas extracelulares (SPE) y la estructura de la comunidad microbiana de ambos tipos de fangos finales (granulado y desgranulado), revelando que la comunidad microbiana de ambos fangos fue casi idéntica, lo que indica que los rápidos cambios en las propiedades físico-químicas del fango granular no causaron ningún cambio en la estructura de las poblaciones microbianas. Sin embargo, el análisis del contenido en SPE mostró una menor concentración de proteínas y polisacáridos en la biomasa desgranulada que en la granulada, lo que indica que la pérdida de estos compuestos está relacionada con el proceso de desgranulación. Adicionalmente, se determinó la actividad metanogénica especifica (SMA, por sus siglas en inglés) de cada fango utilizando diversos sustratos (únicamente etanol o una mezcla entre etanol y los éteres glicólicos). Los resultados del ensayo SMA demostraron: 1) la necesidad de un periodo de adaptación a los éteres glicólicos; y 2) que el fango granular llevó a cabo la degradación anaerobia del etanol más rápidamente que el fango desgranulado.

Este estudio, que corresponde con el capítulo 6 de esta tesis, ha sido preparado para ser enviado a la revista Applied Microbiology and Biotechnology (Ferrero et al., Behaviour, stability, and microbial community analysis of EGSB reactor at hihg content of glicol ether solvents in mixtures with etanol, Applied Microbiology and Biotechnology, preparado para su envío).

En el último capitulo de esta tesis doctoral, se presenta la comparación de las comunidades microbianas en reactores a escala de laboratorio y a escala real. Diversas muestras de biomasa de un reactor prototipo industrial instalado y operado en una industria flexográfica fueron recogidas, analizadas, y posteriormente comparadas con las obtenidas en los reactores de laboratorio. El reactor industrial trataba principalmente etanol, acetato de etilo y E2P, bajo condiciones fluctuantes de OLR derivadas de los cambios en el proceso productivo de la planta industrial. A pesar de estas fluctuaciones, se alcanzó un rendimiento estable del reactor (EE media de 93%) y sólo se acumularon ácidos grasos volátiles cuando la carga orgánica fue superior a 3 kg h^{-1}, lo que sugirió un límite para la carga orgánica que puede ser tratada asegurando una operación estable del reactor. La estructura de la comunidad microbiana se analizó a través de la técnica DGGE y mostró una evolución durante los primeros meses de operación en los dominios de *Archaea* y *Bacteria*. Teniendo en cuenta que el inóculo inicial fue obtenido de una depuradora de aguas residuales de una industria de fabricación de cerveza, la limitación de la fuente de carbono a solo unos pocos disolventes orgánicos propios de las emisiones de la industria flexográfica pudo ser la causa de este cambio. Las poblaciones del dominio A*rchaeas* fueron las más afectadas por este cambio en la fuente de carbono, resultando en una disminución de la biodiversidad como muestra el índice de Shannon (de 1.07 hasta 0.41 en los primeros 123 días de experimento). *Methanosaeta* fue el microorganismo predominante en este dominio y su predominio persistió a lo largo de todo el periodo experimental. La proporción de la archaea *Methanospirillum* y *Methanobacterium* aumentó a lo largo del periodo experimental, lo que fue asociado a variaciones en la temperatura y en la carga orgánica, respectivamente. En cuanto al dominio de *Bacteria*, los microorganismos predominantes fueron especies del genero *Geobacter* y *Pelobacter*, que son microorganismos especializados en la degradación de etanol. Además, *Methanosaeta* y *Geobater* son socios sintróficos capaces de usar la transferencia de electrones interespecífica para la producción de metano, así que el predominio de estas especies indicaba la aparición de este fenómeno en el reactor prototipo. Comparando los reactores de laboratorio y el prototipo industrial, ambos tuvieron un rendimiento similar con altas EE (> 90%) y un periodo de adaptación similar para la degradación de E2P, y sólo se encontró la diferencia en el valor límite

de carga orgánica asociada a la acumulación de ácidos grasos volátiles. La estructura de la comunidad microbiana mostró una tendencia similar en ambos reactores, con una evolución desde el fango inicial durante el principio de la operación en *Archaea* y *Bacteria*. Finalmente, se obtuvo una comunidad estable en el dominio de *Archaea* con la predominancia de *Methanosaeta*. En el dominio de *Bacteria* la predominancia de los microorganismos mostró una dependencia del sustrato empleado.

Estos resultados, que se recogen el el capítulo 7, han sido publicados en la revista Journal of Environmental Management (Bravo, D., Ferrero, P., Penya-roja, J.M., Álvarez-Hornos, F.J., Gabaldón, C., 2017. Control of VOCs from printing press air emissions by anaerobic bioscrubber : Performance and microbial community of an on-site pilot unit. J. Environ. Manage. 197, 287-295).

Conclusiones y perspectivas

Después de un relativamente corto período de aclimatación, se obtuvieron elevadas eficacias de eliminación de las mezclas de etanol con glicoles éter, tales como E2P y M2P, demostrándose que el tratamiento anaerobio con fango granular es una tecnología viable para la biodegradación de estos solventes típicos de la industria flexográfica. De las dos configuraciones de reactores anaerobios probadas, el AHR mostró una capacidad de retención de sólidos mayor que el EGSB, pero ésto no se vió reflejado en una mayor eficacia de eliminación. Ambas configuraciones de reactores eran capaces de eliminar completamente el M2P (con una OLR de M2P de 8.3 kg DQO m^{-3} d^{-1}) y el 70% del E2P (con una OLR de E2P de 10.6 kg DQO m^{-3} d^{-1}). Los subproductos detectados (acetona, isopropil alcohol, y metanol) en algunas fases del experimento y los resultados obtenidos con la mezcla etanol-acetona permitieron esclarecer las rutas de degradación de los éteres de glicol estudiados.

La evolución microbiana de los fangos dentro de los biorreactores tanto de AHR como de EGSB utilizados para el tratamiento de las mezclas de etanol-glicol éter fue similar, excepto en el material de relleno ubicado en la parte superior del AHR en el que se identificó una población degradante de ácidos grasos volátiles (AGV). No se observó estratificación a lo largo del lecho de fango en ninguno de los reactores indicando un efecto de mezcla elevado dentro de los mismos. Se observó un efecto tóxico de E2P en las densidades bacterianas y de arqueas, mientras que la adición de M2P solo afectó temporalmente a la población de archaeas. La secuenciación masiva resaltó un cambio de predominio desde *Proteobacteria* a *Firmicutes* cuando los biorreactores anaeróbicos se alimentaron con M2P.

El tratamiento anaerobio de etanol y mezclas de éteres glicólicos, con elevada proporción en éstos últimos, también mostró EE globales relativamente

altas (≈ 90%). Sin embargo, se ha demostrado que el etanol es un sustrato importante para el mantenimiento de la estructura granular de los granulos en la degradación anaerobia, ya que se produjo una desgranulación parcial cuando no se introdujo etanol en la alimentación. El M2P tuvo una RE mayor que el E2P, probablemente debido a una inhibición competitiva enzimática. Los análisis de la comunidad microbiana revelaron una evolución tanto desde el inóculo al fango granular final, como por la adaptación al sustrato a degradar. Esto se debe a que los microorganismos predominantes identificados al final del experimento tenían un metabolismo basado en los sustratos utilizados durante el ensayo. Los análisis de sustancias poliméricas extracelulares mostraron que el contenido de proteínas y carbohidratos de la biomasa desgranulada fue menor que en la biomasa granulada. La relación entre las proteínas y los carbohidratos también disminuyó en la biomasa degranulada, lo que sugiere que este parámetro podría ser un buen indicador de la salud y la estabilidad de los gránulos.

El uso de fango granular de una planta de tratamiento de aguas residuales de una fábrica de cerveza como inoculo inicial resultó en una buena estrategia para lograr una alta EE desde el inicio en la operación del reactor prototipo de biolavador anaerobio. Este reactor se mantuvo estable con una elevada EE (93%) a pesar de las variaciones en las condiciones de operación típicas de las emisiones de la industria flexográfica, como la temperatura y la OLR. El cambio en la fuente de carbono a solo unos pocos solventes causó un impacto en la biodiversidad de las comunidades microbianas, que disminuyó inicialmente, especialmente en *Archaea*. Los microorganismos predominantes identificados en el reactor pueden asociarse a la fuente de carbono y los parámetros operativos, como la temperatura y la carga orgánica. Los reactores a escala de laboratorio y el reactor prototipo funcionaron de manera similar en términos de EE y se encontró que se necesitaba el mismo período de adaptación para degradar el E2P. Además, se encontró una dinámica en la comunidad microbiana similar en ambas escalas, laboratorio y prototipo, con el predominio de las especies *Methanosaeta* y *Geobacter*, socios sintróficos que usan el mecanismo basado en la transferencia directa de electrones interespecies, lo que sugiere que éste es importante para transformar en metano los disolventes de las industrias flexográficas.

A partir de esta tesis, a fin de obtener un conocimiento más profundo sobre la degradación de estos éteres gicólicos, se podría proponer llevar a cabo los siguientes estudios:

I. El mecanismo de degradación de los éteres de glicol mostrado en esta tesis sugiere que se requieren enzimas para la escisión del éter, por lo que la

búsqueda de las enzimas responsables de este mecanismo se muestra como un estudio interesante para mejorar el rendimiento y conocer las condiciones, como las necesidades de cofactores, para una mejor función y cinética de estas enzimas.

II. El análisis de la comunidad microbiana de los reactores que tratan solventes de industrias flexográficas reveló que *Methanosaeta* y *Geobacter* son microorganismos predominantes que juegan un papel importante en la degradación del solvente. Estos microorganismos son socios sintróficos que pueden utilizar la transferencia directa de electrones interespecífica. Por lo tanto, el uso de materiales conductores, como el carbón activado, donde podrían adherirse estos microorganismos parece ser una alternativa atractiva para promover la transferencia de electrones interespecífica directa y el crecimiento de estos microorganismos involucrados en la degradación anaerobia de disolventes de la industria flexográfica.

12.2 APPENDIX B: PUBLISHED PAPERS

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Research article

Control of VOCs from printing press air emissions by anaerobic bioscrubber: Performance and microbial community of an on-site pilot unit

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ABSTRACT

A novel process consisted of an anaerobic bioscrubber was studied at the field scale for the removal of volatile organic compounds (VOCs) emitted from a printing press facility. The pilot unit worked under high fluctuating waste gas emissions containing ethanol, ethyl acetate, and 1-ethoxy-2-propanol as main pollutants, with airflows ranging between 184 and 1253 m^3 h^{-1} and an average concentration of 1126 ± 470 mg-C Nm^{-3}. Three scrubber configurations (cross-flow and vertical-flow packings and spray tower) were tested, and cross-flow packing was found to be the best one. For this packing, daily average values of VOC removal efficiency ranged between 83% and 93% for liquid to air volume ratios between $3.5 \cdot 10^{-3}$ and $9.1 \cdot 10^{-3}$. Biomass growth was prevented by periodical chemical cleaning; the average pressure drop was 165 Pa m^{-1}. Rapid initiation of anaerobic degradation was achieved by using granular sludge from a brewery wastewater treatment plant. Despite the intermittent and fluctuating organic load, the expanded granular sludge bed reactor showed an excellent level of performance, reaching removal efficiencies of 93 ± 5% at 25.1 ± 3.2 °C, with biogas methane content of 94 ± 3% in volume. Volatile fatty acid concentration was as low as 200 mg acetic acid L^{-1} by treating daily average organic loads up to 3.0 kg COD h^{-1}, equivalent to 24 kg COD m^{-3} bed d^{-1}. The denaturing gradient gel electrophoresis (DGGE) results revealed the initial shift of the domains Archaea and Bacteria associated with the limitation of the carbon source to a few organic solvents. The Archaea domain was more sensitive, resulting in a drop of the Shannon index from 1.07 to 0.41 in the first 123 days. Among Archaea, the predominance of *Methanosaeta* persisted throughout the experimental period. The increase in the proportion of *Methanospirillum* and *Methanobacterium* sp. was linked to the spontaneous variations of operating temperature and load, respectively. Among Bacteria, high levels of ethanol degraders (*Geobacter* and *Pelobacter* sp.) were observed during the trial.

1. Introduction

The flexographic sector represents 17% of the European printing sector, contributing around 1.7% of the total turnover in 2003 (Ernst and Young, 2007). The consumed solvents are mainly oxygenated compounds, such as ethanol, ethyl acetate, 1-propanol, 2-propanol, 1-methoxy-2-propanol, n-propyl acetate, 1-methoxy-2-propyl acetate, acetone, and 1-butanol (Granström et al., 2002). Flexographic air emissions are characterized by high flow rates and low volatile organic compound (VOC) concentrations (Sempere et al., 2012), with temperatures ranging from 40 to 70 °C and relative humidity varying from 5 to 15% (Rothenbuhler et al., 1995). According to the European Directive on Industrial Emissions (Council Directive 2010/75/EC), these air emissions must be controlled.

Biotreatments represent well-developed air pollution control techniques for removing VOCs in these conditions (Deshusses, 1997). Among biotreatments, bioscrubbers can handle higher gas loads than biotrickling filters and biofilters, and their capacity is up to 3000–4000 m^3 m^{-2} h^{-1} (Kennes et al., 2009). However, there are few available studies on aerobic bioscrubbers. Le Cloriec et al. (2001) reported removal efficiencies of 90.1–100% in a laboratory-scale bioscrubber, with liquid to air ratios ranging

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between $0.6 \cdot 10^{-3}$ and $2 \cdot 10^{-3}$, and with ethanol concentration in waste gas from 18.8 to 291.7 mg-C m^{-3}. Granström et al. (2002) investigated an onsite pilot-scale system for the treatment of waste gas from printing processes. In this study, the major VOC of the waste air was ethanol, with smaller amounts of ethyl acetate, 1-propanol, 2-propanol, 1 methoxy-2-propanol, and 3-ethoxy-1-propanol. The flow of the waste gases varied from 1.68 to 3.73 m^3 h^{-1}, with 99.6% VOC removal efficiency, excluding evaporation losses. Nevertheless, aerobic bioscrubbers are still not widespread within the biotreatment market due to the high energy consumption of aerobic bioreactors. In contrast, anaerobic bioscrubber could be an alternative for recycling waste gases into bioenergy, thereby resulting in a positive net energy balance.

To the best of our knowledge, no previous literature exists on the use of anaerobic bioscrubbers for the treatment of VOC waste gases, although the anaerobic degradation of solvents, such as alcohols (Eichler and Schink, 1985; Widdel, 1986; Zellner and Winter, 1987) or esters (Oktem et al., 2008; Yanti et al., 2014) is well documented. Recently, Lafita et al. (2015) demonstrated that anaerobic degradation of glycol ethers is feasible by reporting the treatment of synthetic packaging wastewater, which contains a mixture of ethanol and 1-methoxy-2-propanol in a mass ratio of 4:1. These authors achieved removal efficiencies of up to 94% at 18 °C and 97% at 25 °C in an expanded granular sludge bed (EGSB) reactor, with organic loading rates of methoxy-2-propanol of 6.4 and 9.3 kg COD m^{-3} d^{-1}, respectively.

The anaerobic degradation of organic solvents in granular sludge reactors relies on the microbial population developed in the anaerobic granules, which should in turn maintain its physical integrity. Leclerc et al. (2004) studied the microbial populations of 44 anaerobic digesters treating effluents from several sectors. These authors indicated that the occurrence and prevalence of the different species are influenced by the running and environmental conditions. Anaerobic granulated sludge coming from breweries is a common source of biomass for other industrial sectors. In this sense, the study of the evolution of the microbial population is an interesting tool to investigate the effect that a change in the substrate composition could have on the feasibility and robustness of the anaerobic degradation of solvents.

The characterization of microbial populations can be carried out using molecular biology tools, such as denaturing gradient gel electrophoresis (DGGE). This is based on the electrophoretic separation of polymerase chain reaction (PCR) products of the same length, but with different sequences, on a linear denaturing gradient polyacrylamide gel (Muyzer and Ramsing, 1995). DGGE has been applied to evaluate the microbial diversity of anaerobic reactors, such as an upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater; this study showed that the dominant archaeal bands were closely related to *Methanosaeta* and *Methanobacterium* (Chan et al., 2001). The DGGE technique has also shown that the microbial population of a UASB treating wastewater from an unbleached pulp plant persisted throughout the experimental period (Buzzini et al., 2006). DGGE studies can also demonstrate the importance of environmental conditions in the diversity of microbial populations; for example, LaPara et al. (2000) indicated that a thermophilic reactor showed less biodiversity than a mesophilic one by treating wastewater from a pharmaceutical facility.

The present study provides the first successful example of an on-site pilot plant of anaerobic bioscrubbers controlling VOC emissions from a flexographic printing facility (Waalkens et al., 2015). The purposes of our work were as follows: (1) to evaluate the best scrubber configuration to achieve high VOC removal efficiencies, and at same time, control pressure drop; (2) to determine the maximum organic load that the EGSB can handle under intermittent and variable waste gas emissions; and (3) to study the dynamics of the microbial community of the EGSB reactor inoculated with granular sludge from a brewery anaerobic reactor using the DGGE technique.

2. Material and methods

2.1. Anaerobic bioscrubber setup

The pilot plant was provided by Pure Air Solutions (Heerenveen, The Netherlands) and was operated on-site in Altacel Transparant Verpakkingsind (Weesp, The Netherlands) by treating a fraction of its air emissions. The flexographic site operates on a two-shift (16 h) basis from Monday to Friday and on a one-shift (8 h) basis on Saturday. The pilot plant comprises a variable-speed fan with a maximum flow of 1500 m^3 h^{-1}, as well as several centrifugal pumps. The two main units were the scrubber and the anaerobic reactor (see Graphical Abstract). The scrubber unit had a total height of 3.06 m and a diameter of 0.5 m. The available height for the packing material was 2.0 m. The scrubber unit was assembled onto a bottom tank of 2 m^3 in volume. The anaerobic reactor had a total height of 5.08 m and diameter of 1.59 m, with an effective water volume of 8.7 m^3. Two intermediate tanks completed the setup; resulting in 16 m^3 of total effective water volume.

The scrubber was operated in the countercurrent mode during the working hours of the facility; VOC-polluted air coming from the factory was introduced to the bottom by the blower, and the water was sprayed from the top and collected in the bottom tank. From there, it flowed to an intermediate tank for supplementation with macronutrients (N, P, S, K) and sodium carbonate for pH control prior to pumping it to the anaerobic reactor for solvent degradation. Ca, Mg, trace metals (B, Co, Cu, Fe, Mn, Mo, Ni, Se, Zn), and yeast extract were discontinuously supplemented. The anaerobic reactor consisted of an EGSB operated at 3 h of hydraulic residence time. The EGSB was filled with granular sludge from an internal circulation (IC) reactor treating brewery wastewater (Heineken, The Netherlands) without further acclimation to simulate operational protocols at the industrial scale. The expansion of the granular bed to 3 m^3 was achieved by mixing the influent water with 50% of the effluent of the reactor; the upflow velocity was kept constant at 3 m h^{-1}. The pilot unit worked in water-closed recirculation, with <10% daily water renewal. The daily purge was done overnight when no biogas production occurred. The plant setup was equipped with a programmable logic controller (PLC) with Twinsoft software (Servelec Technologies, United Kingdom) to monitor and control the parameters, such as the air and liquid flowrates, water and air temperatures, pH, conductivity, and water level in the tanks.

A flame ionization detector (FID) analyzer (model RS 53-T, Ratfisch Analysensysteme, Germany) continuously monitored the VOC concentration in the inlet and outlet of the gas phase. The composition of the inlet and outlet gases was measured by carbon sorbent tubes and post Gas Chromatography analysis. Biogas production was continuously measured by a gas meter (Bellows-BG 4 Gasmeters, Ritter, Germany), and its composition was determined by a dual-wavelength optical infrared analyzer (Combimass GA-m, Binder, Germany). The main parameters of the liquid phase were monitored twice a week with photometric commercial kits as follows: chemical oxygen demand (COD); volatile fatty acids (VFAs); nutrients ($N-NH_4^+$ and $P-PO_4^{3-}$) with LCK 014, LCK 365, LCK 303, and LCK 348 kits from HACH Lange GmbH (Germany); and alkalinity with a titrimetric kit (MColortest™, Merck Millipore, Germany).

The pilot unit was operated for 484 days. The experimentation was divided into five stages characterized by a change in the

Table 1
Experimental set-up of anaerobic bioscrubber pilot unit.

Scrubber unit					
Stage	1	2	3	4	5
Days of operation	0–95	96–130	131–180	181–265	266–484
Configuration	Packing A	Packing B	Spray	Packing B	Packing A
Specific surface area ($m^2\ m^{-3}$)	150	125	–	125	150
Liquid/air vol. ratio·10^3	3.5–9.1	7.6–10.1	1.9–3.7	3.8–8.0	4.3–7.9
EGSB reactor					
Biomass sampling event (day)	S-1 (0)	S-2 (123)		S-3 (238)	S-4 (334) S-5 (413) S-6 (430)

scrubber configuration with the aim to evaluate the best one in terms of VOC removal. Table 1 summarizes the main operational conditions. Two packing materials were used, as follows: a cross-flow packing material, Packing A, with a 150 $m^2\ m^{-3}$ specific surface area (cross-fluted flow fills, KFP 319/619, GEA, Germany); and a vertical-flow packing material, Packing B, with 125 $m^2\ m^{-3}$ specific surface area (vertical-flow fills, KVP 323/623, GEA, Germany). The scrubber unit was also tested as a spray column, removing the packing material and installing three nozzles (MP156N 60°, BETE, USA) spaced 55 cm apart. Packing material A was used in stages 1 and 5; packing material B was used in stages 2 and 4; and the spray column was tested in stage 3. Several liquid to air volume ratios in the range of $1.9\cdot10^{-3}$–$10.1\cdot10^{-3}$ were tested with the aim of minimizing the recirculated water flow to the anaerobic reactor. The organic load (OL) to the anaerobic reactor was set by the operation of the scrubber. Six biomass samples were taken from a port located 1.05 m from the bottom of the EGSB. Sampling events are shown in Table 1.

2.2. Microbial community analysis

DNA from each sample was extracted with a Power Soil Isolation Kit (Mo Bio Laboratories, USA) using the supplier's protocol. DNA concentration and purity were measured using NanoDrop® (Thermo Scientific, USA). Extracted DNA was stored at −20 °C for the analysis. To amplify 16S rDNA, two universal primer sets were used, as follows: F357-GC and R518 for bacterial 16S and F787-GC and R1059 for archaeal 16S. The PCR amplification was conducted according to the following protocol: 20 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 0.5 min, 10 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 0.5 min, and a final extension at 72 °C for 10 min. After amplification, the PCR products were electrophoresed in 0.5% (w/v) agarose gel to evaluate the extent of amplification. The PCR product generated from each sample was separated on an 8% acrylamide gel with a linear denaturant gradient increasing from 20% to 35% using the KuroGel Verti 2020 DGGE System (VWR International Eurolab, Spain). DGGE was performed using 20 μl of PCR product in 1 × Tris-acetate-EDTA buffer at 60 °C with a sequence of 50 V for 5 min, 150 V for 120 min, and 200 V for 60 min. The DGGE gels were visualized in the MiniBIS Pro System (DNR Bio-Imaging System Ltd., Spain). Predominant bands were excised and resuspended in 30 μl of sterilized Mili-Q water, and then bands were stored at 4 °C, allowing DNA to migrate to the liquid. The eluted DNA was reamplified by PCR with the same conditions as the previous PCR to the DGGE. The PCR product was purified with a High Pure PCR Product Purification Kit (Roche, Spain). Successfully reamplified and purified PCR products were sequenced by using an automated DNA analyzer (3730 KL DNA Analyzer, Applied Biosystems, Spain). Sequences were analyzed with MEGA 5.0 and then compared with those available from the NCBI GenBank using BLAST software.

2.3. Granule size distribution

The particle size distribution of biomass samples was measured to monitor the evolution of the granule size and to check the granule integrity by a Malvern Mastersizer 2000 instrument (Worcestershire, UK) with a detection range of 0.02–2000 μm. Samples from days 0, 238, and 430 (S-1, S-3, and S-6, Table 1) were analyzed.

3. Results and discussion

3.1. Characterization of air emissions

The air emissions showed a high variability associated with the number of printing presses in operation (see Fig. 1a: Daily average for the whole trial and Fig. 5a: Instantaneous pattern). Airflow ranged between 184 and 1253 $m^3\ h^{-1}$, and the average daily VOC concentration was 1129 mg-C Nm^{-3} with a standard deviation of 460 mg-C Nm^{-3}. The detected compounds in major proportions by weight were ethanol (60–65%), ethyl acetate (20–25%) and ethoxy propanol (10–15%); other minor compounds were 2-propanol (0.5–1%), 2-propyl acetate (0–0.5%), 1-propyl acetate (0–3%), 4-hydroxy-4-methyl-2-pentanone (0.2–5%) and 1-propanol (0–2%).

3.2. The scrubber unit

Scrubber performance was evaluated in terms of VOC removal efficiency (RE_{VOC}). Results of the online monitoring of the scrubber are shown in Fig. 1a, where the daily average RE_{VOC} is plotted along with the daily averages of the inlet and outlet VOC concentrations in the gas phase. High efficiencies were reached in stage 1 with packing A, which was usually over 83%. A maximum RE_{VOC} of 97% was achieved on day 75, at the maximum tested liquid to air volume ratio ($9.1\cdot10^{-3}$), when the inlet concentration was 2000 mg-C Nm^{-3}. The change to packing B (stage 2) caused a decrease in RE_{VOC}; the maximum value was 88% (day 129), even though a higher liquid to air ratio ($10.1\cdot10^{-3}$) was applied than in stage 1. The outlet emissions with packing B (stages 2 and 4) reached values up to 516 mg-C Nm^{-3} (day 227), while the maximum leak in stage 1 was 310 mg-C Nm^{-3} (day 52). Regarding the removal of the main pollutants, ethanol and 1-ethoxy-2-propanol were almost fully removed with packing A, while with packing B, the removals decreased to values between 80% and 94% (higher values for higher tested liquid to air ratios). In both packings, ethyl acetate was removed to a lesser extent due to its lower water solubility.

By testing the spray tower configuration (stage 3), RE_{VOC} dropped to values of 49–65% with outlet concentrations higher than 135 mg-C Nm^{-3}, even for inlet emissions as low as 310 mg-C Nm^{-3} (day 157). In this case, no effective removal of ethyl acetate was observed (<60%), with moderate transfer to water of ethanol and 1-ethoxy-2-propanol (70–88%).

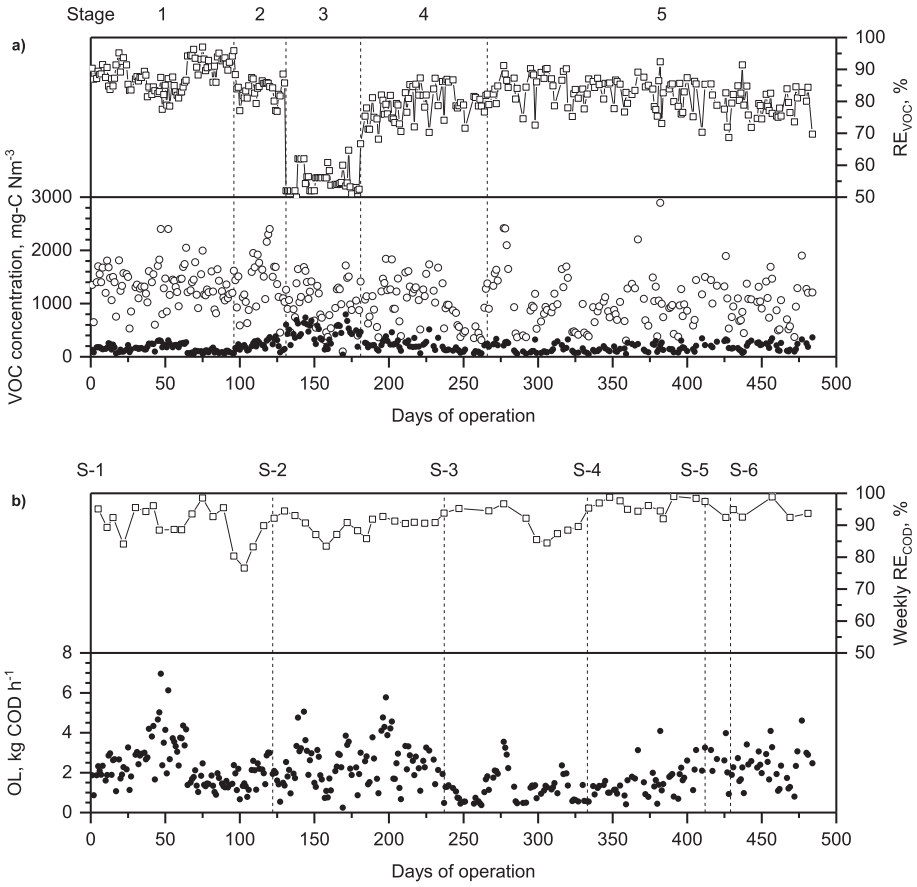

Fig. 1. Performance of the pilot unit. a) Daily averages of (□) RE_{VOC}, (○) Inlet VOC concentration and (●) Outlet VOC concentration in the gas phase of the scrubber. Broken lines represent the days when the configuration of the scrubber was changed. b) (●) Daily average OL and (□) weekly RE_{COD} of the EGSB. Broken lines indicate biomass sampling event.

The achieved RE_{VOC} at the maximum liquid to air ratio ($3.7 \cdot 10^{-3}$) in stage 3 was 40% lower than that obtained with packing A and 33% lower than that obtained with packing B at similar liquid to air volume ratio. The experimental results indicated that unfeasible high water flow rates would be required for the spray tower configuration to fulfill the compliance levels compared with the use of a packing bed. In the case of packing A, a drop of about 9% in RE_{VOC} was achieved in stage 5 in comparison with stage 1 at similar liquid to air ratios. This result was attributed to the creation of preferential pathways, probably due to the self-assembly of the packing on day 266. In stage 5, a periodical chemical cleaning of the packing material was set up, allowing the pressure drop to maintain at 165 Pa m^{-1} (average value). The long-term testing period of stage 5 demonstrated that it is feasible to work with the cross-flow packing material by avoiding the attachment and biomass growth on the packing surface.

The average RE_{VOC} versus the applied liquid to air to ratio for packing A (stage 1) and packing B (stages 2 and 4) is plotted in Fig. 2. In the case of packing A, stage 5 was discarded due to the reinstallation problem. A positive effect of increasing the liquid to air ratio can be observed for both packings. In case of packing A, RE_{VOC} increased from 83 to 93% by increasing the ratio from $3.5 \cdot 10^{-3}$ to $9.1 \cdot 10^{-3}$. For packing B, RE_{VOC} increased from 75 to 85% as the ratio increased from $3.9 \cdot 10^{-3}$ to $10.1 \cdot 10^{-3}$. Comparing both packing materials, higher removals were achieved with packing A due to the higher specific surface and the more complex water path, which favored the contact between both phases. With packing B, a liquid to air ratio higher than $6 \cdot 10^{-3}$ was required to achieve RE_{VOC} over 80%, while this value could be reached by applying nearly half this ratio ($3.5 \cdot 10^{-3}$) in packing A. The results indicate that packing A is the best alternative for industrial applications if wall biomass growth is prevented by active control of pressure drop.

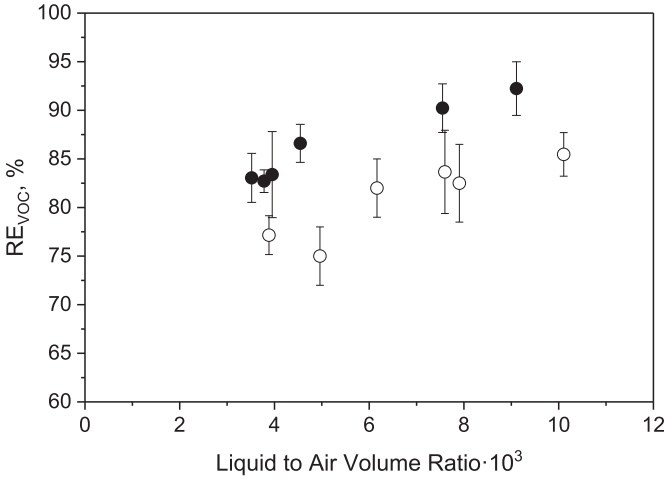

Fig. 2. Influence of the liquid to air volume ratio on the VOC removal efficiency of the scrubber unit. (●) Packing A, stage 1, (○) Packing B, stages 2 and 4.

3.3. The EGSB reactor

The water with the solvents was pumped to the EGSB reactor for solvent degradation prior to recirculation to the scrubber. The daily average OL was derived from the difference between the inlet and outlet VOC concentrations in the gas phase during production time (continuously monitored) and expressed as kg COD h^{-1}. The removal efficiency of the soluble organic matter (RE_{COD}) was calculated on a weekly basis from the mass balance (expressed in COD units):

$$RE_{COD}(\%) = \frac{OL_W - ACUM - PURGE}{OL_W} \times 100 \quad (1)$$

where OL_W is the cumulative organic load applied to the EGSB during a week, ACUM is the intra-week accumulated solvents in water, and PURGE is the total amount of purged solvents during a week. ACUM and PURGE were derived from COD water analysis. The OL (daily average) applied to the EGSB is shown in Fig. 1b along with the weekly RE_{COD}. The daily average organic load to EGSB fluctuated quite a bit due to modifications in the facility's production and the performance of the scrubber, with values ranging from 0.37 (day 262) to 6.96 kg h^{-1} (day 47). Despite the organic load fluctuations, the weekly COD removal efficiency was maintained at very high values for the whole experiment, with an average value of 93 ± 5%, verifying the anaerobic biodegradation of a mixture of solvents containing mainly ethanol, 1-ethyl acetate, and 1-ethoxy-2-propanol. Methane content in the biogas was stable at 94 ± 3% (n = 18). The methane yield was 0.32 Nm^3_{CH4} $kg^{-1}_{COD\ removed}$, which was close to the stoichiometric value (0.35 m^3_{CH4} $kg^{-1}_{COD\ removed}$; Grady et al., 1999). The growth yield coefficient derived from the methane yield resulted in 0.06 mg-VSS mg-COD^{-1}.

Table 2 summarizes the VFA concentration, temperature, and pH of the water effluent of the anaerobic reactor grouped between biomass sampling events. The average temperature for all intervals was in the mesophilic range, although it was below 20 °C (psychrophilic conditions) some days during the first 330 days of operation. Variations were associated with air emission temperature. With the aim of preventing any day from being below 20 °C, a temperature control system was installed on day 334. VFA concentration was normally kept at values lower than 300 mg acetic acid L^{-1} for organic loads lower than 3.0 kg COD h^{-1}, indicating a good balance between acidogenesis and methanogenesis, although some VFA accumulation occurred at high OL. The pH was chemically controlled by adding sodium carbonate, keeping the pH above the minimum value for optimal growth of methanogens (6.8; Grady et al., 1999). The minimum pH values were reached on days when organic loads were high and VFA accumulated in water; for example, a daily average pH of 6.83 was measured on day 199 after 3 days running with OL higher than 3.9 kg COD h^{-1} and resulting in the maximum VFA concentration (1143 mg acetic acid·L^{-1}).

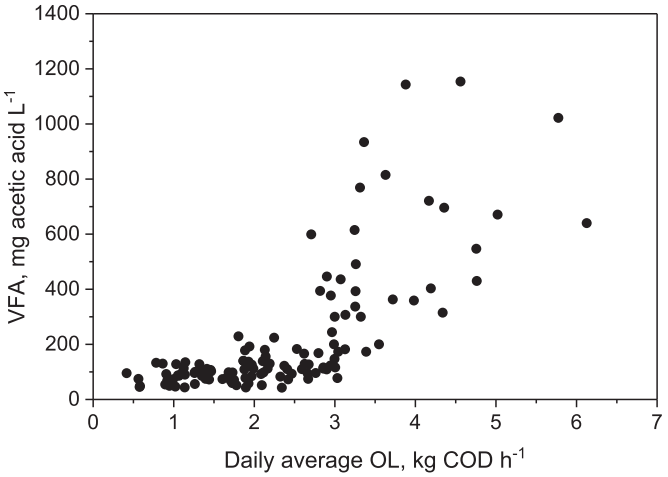

Fig. 3. Effect of the organic load on the water effluent VFA concentration of the EGSB.

Fig. 3 shows the variation of VFA concentration with the organic load. An accumulation of VFA in water can be observed for an average daily OL higher than 3.0 kg COD h^{-1}. The accumulation of VFA indicates that the slowly growing methanogens cannot sufficiently and rapidly metabolize the intermediate products from VFA producers (acidogenic and acetogenic populations). If it continues over time, this imbalanced situation could result in the destabilization of the reactor. Considering the biomass volume of the reactor, the design organic loading rate should be less than 24 kg COD m^{-3} bed d^{-1} to ensure stable removal over 94%.

Fig. 4 shows the granule size distribution of the samples taken during the trial (S-1, S-3, and S-6) based on volume. A narrow range of size distribution was observed in all samples, showing large mean diameters (0.88 mm for S-1, 0.95 mm for S-3, and 1.03 mm for S-6). The results demonstrated that the shift of the substrate from ethanol (S-1) to a mixture of ethanol, ethyl acetate, and 1-ethoxy-2-propanol, in which ethanol was the major component, did not show a marked difference in the granule size; a small increase in particle size was observed. This result contrasts with that previously reported by Lafita et al. (2015). These authors indicated a progressive deterioration in methane production and granule disintegration by working at 35 kg COD $m^{-3}d^{-1}$ with a mixture of ethanol and 1-methoxy-2-propanol (4:1 in mass) applied intermittently (16 h per day, 5 days a week) to a 4-L reactor. Although the carbon source and the type of sludge were similar for both studies, the fluffy granule formation reported by these authors could be related to an excessive granular growth, with abundant extracellular polymeric substance (EPS) production that inhibited the release of gases. This type of granule cannot accommodate extremely high OLs or variations of organic strength (Fukuzaki et al., 1995). In our study, the hydraulic conditions maintained a stable biomass bed volume for

Table 2
Daily average parameters of the water effluent of the EGSB reactor.

Biomass sampling events	Days of operation	VFA concentration, mg-acetic acid L^{-1}			Temperature, °C			pH		
		Average	Min	Max	Average	Min	Max	Average	Min	Max
S-1	0–122	176 ± 183	43	934	22.8 ± 2.0	18.5	26.4	7.50 ± 0.21	7.09	8.41
S-2	123–237	365 ± 327	58	1154	24.9 ± 2.8	19.7	30.6	7.38 ± 0.20	6.83	7.96
S-3	238–333	156 ± 175	44	615	23.0 ± 1.6	18.8	26.7	7.36 ± 0.35	6.85	8.75
S-4	334–412	133 ± 102	49	393	27.2 ± 1.7	23.8	30.8	7.55 ± 0.44	6.88	8.63
S-5	413–429	218 ± 199	78	359	29.9 ± 0.7	28.9	30.9	7.32 ± 0.27	7.10	7.74
S-6	430–484	122 ± 53	80	200	27.5 ± 1.1	25.5	29.3	7.38 ± 0.31	6.85	8.45

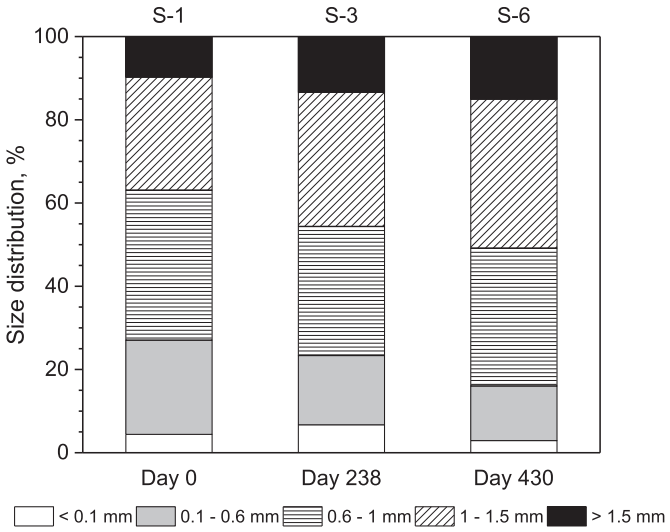

Fig. 4. Variation of the granule size distribution of biomass over time.

the whole trial without excessive biomass accumulation, and the EPS required for granulation was produced in sufficient amounts to handle interruptions and variations of high OLs.

To demonstrate the performance of the EGSB under fluctuating and oscillating feeding of substrate, continuous monitoring of a typical working day (day 481) is depicted in Fig. 5. The inlet and outlet VOC concentrations at the gas phase (scrubber unit) are plotted in Fig. 5a, and the moving hourly average OL and cumulative biogas production (EGSB reactor) are shown in Fig. 5b. The gas emission pattern varied depending upon the printing orders being processed, with an inlet VOC concentration ranging between 430 and 1900 mg-C·Nm^{-3} during production time (from 6:30 to 22:30), with an average RE_{VOC} at the scrubber of 84%. The variations of the VOC air emissions changed the organic load fed to the reactor from 1.7 to 4.7 kg COD h^{-1}, but the biogas production was kept at a nearly constant rate (0.67 m^3-biogas h^{-1}), indicating the capacity of the reactor to absorb these instantaneous shock loads. Biogas production started 1.5 h after the facility production began and stopped 1.5 h after facility production ended. Both shifts indicated that no solvent was accumulated in the water, corroborating the robustness of the process to recycle VOC emissions into bioenergy.

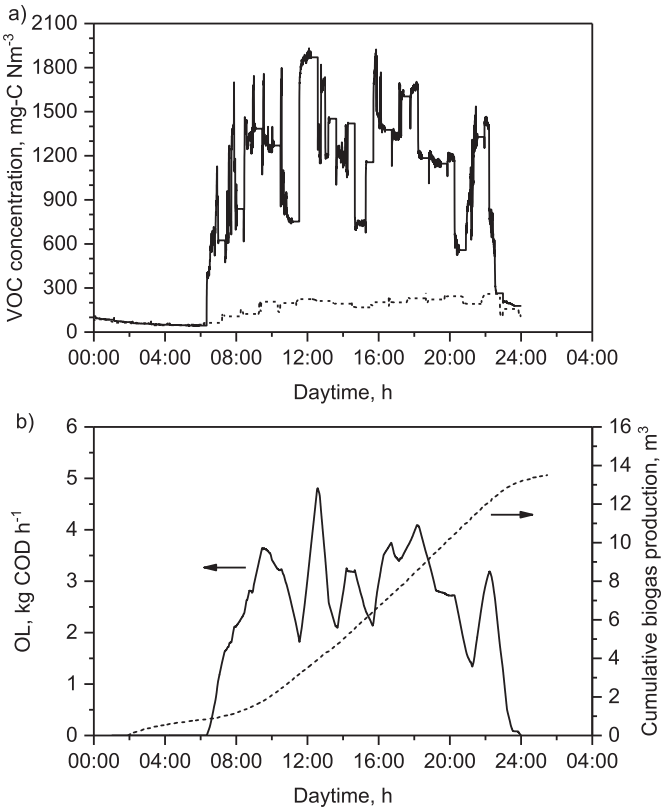

Fig. 5. Plant monitoring data, day 481. a) (▬) Inlet VOC concentration and (■■■) Outlet VOC concentration in the gas phase in the scrubber, b) (■■■) Cumulative biogas production and (▬) Moving hourly average OL in the EGSB.

3.4. Microbial community analysis

The result of the DGGE for archaeal and bacterial population is presented in Fig. 6 for all biomass samples collected during the trial. The predominant bands of the samples, which are labelled in Fig. 6, were excised and sequenced. In addition, Shannon's index (H′) for each sample was indicated. Table 3 summarizes the DGGE band designation, the level of similarity to related GenBank sequences, and the phylogenetic group of each strain.

Archaeal DGGE (Fig. 6a) showed a shift in population during the first 123 days, where the biodiversity decreased as the Shannon index decreased from 1.07 (S-1) to 0.41 (S-2). After this initial shift in the archaeal population, it remained stable, with no high variations for more than a year (S-2 to S-6). The developed archaeal population after the shift in the EGSB reactor presented low archaeal biodiversity; A2 was the predominant band. Bacterial DGGE (Fig. 6b) also showed a smooth shift in the bacterial diversity at the beginning of the trial, where the Shannon index revealed a drop from 2.14 (S-1) to 1.86 (S-2) in the diversity of the bacterial population. This decrease in the biodiversity from the initial sludge seems to have been due to differences in the operational and environmental conditions of the reactor. As the granular sludge came from a reactor treating complex brewery wastewater, the microorganisms had to adapt to a defined wastewater containing only organic solvents as carbon source, with few major compounds (ethanol, ethyl acetate, and 1-ethoxy-2-propanol). The increase in the Shannon index from 1.86 (S-2) to 2.00 (S-5) from day 123 to day 413 indicates that the biodiversity increased because new microorganisms were slowly becoming abundant, such as in bands B4, B6, and B7. At the end of the experiment, the Shannon index slightly decreased to 1.85 (S-6) because bands that initially were predominant, B1 and B2, progressively decreased in intensity and finally, in this sample, disappeared. As a conclusion, the use of granular sludge from a brewery wastewater treatment plant seemed to be a good choice for treating oxygenated solvents coming from VOC emissions of the flexographic industry. The predominant bands (A2, B5, B6, and B7) initially came with the sludge, and the change of the carbon source to pure solvents resulted in a population with less biodiversity in which some microorganisms prevailed.

The predominant band found in the archaeal DGGE in all samples along the operation period was band A2; this band was identified as *Methanosaeta concilii*. This is a well-known acetotrophic archaea, and it is the most abundant microorganism in anaerobic granular processes like EGSB and UASB (Díaz et al., 2006).

Methanospirillum species, band A1, are hydrogenotrophic archaea. Tsushima et al. (2010) found that *Methanospirillum* species were able to proliferate under psychrophilic conditions, in line with that *Methanospirillum* were found in some anaerobic reactors working at low temperatures (Xing et al., 2009). Initially, the

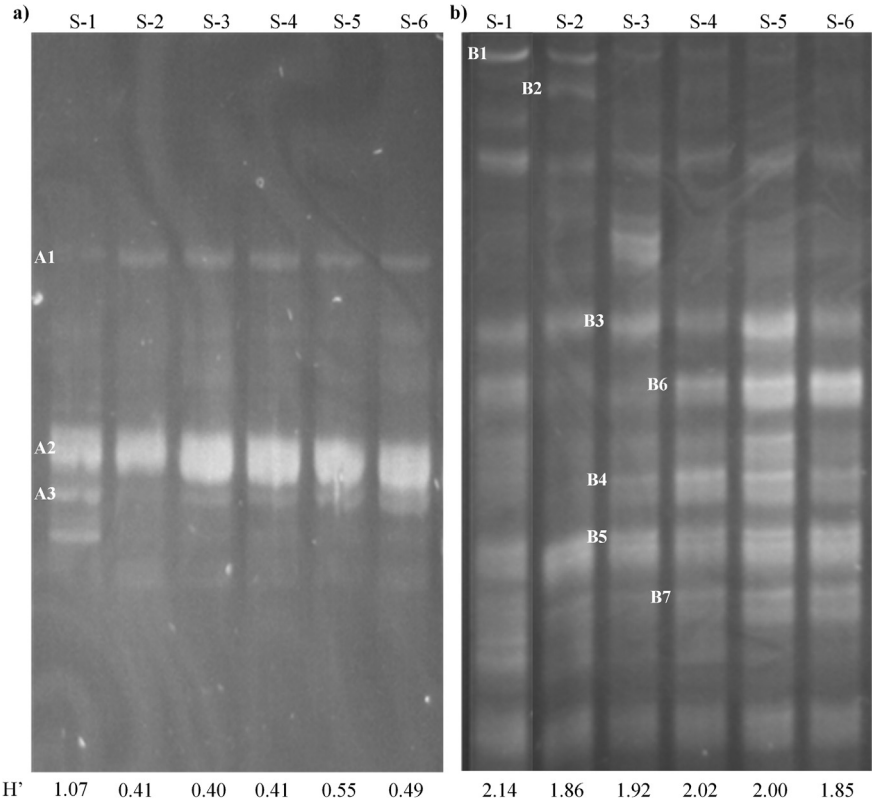

Fig. 6. DGGE profiles of biomass samples from the EGSB reactor including their Shannon index (H′). a) Archaeal DGGE profiles, b) Bacterial DGGE profiles.

intensity of this band was lower than in the rest of the samples; the development of *Methanospirillum* in the reactor can be attributed to the operational temperature of the reactor, especially from days 0–334. During the first year of the experiment, the temperature evolved spontaneously in association with the temperature of the air emissions in the facility, and the daily average temperature reached values as low as 18.5 °C (Table 2), with 8 days lower than 20 °C; this favored the development of *Methanospirillum*. At any rate, the smooth variation of temperature during the whole trial (average of 25.1 ± 3.2 °C) did not seem to effect the removal efficiency of the process, showing that microbial functionality was not adversely influenced.

Methanobacterium species, band A3, were hydrogenotrophic archaeas. Wang et al. (2015) found that *Methanobacterium* species became predominant in the reactor when a drastic increase in the organic load was applied. In our study, the intensity of this band was high in the brewery granular sludge, which was taken for a reactor working at high organic load; then, the intensity of *Methanobacterium* increased again in S-3 (day 238). This could be because working at average daily organic loads higher than 3.5 kg COD h^{-1} from day 195 to day 202 seemed to be an advantage to this species.

No *Methanosarcina* was found in the reactor; this could be explained by the competition with *Methanosaeta* for acetate.

Table 3
DGGE band designation, accession.version numbers in GenBank and levels of similarity to related organisms according to Fig. 6.

DGGE band	Closest organism in the GenBank (accession.version number)	Similarity	Phylogenetic group
A1	*Methanospirillum lacunae* (NR_112981.1)	99%	Methanospirillaceae [a]
A2	*Methanosaeta concilii* (NR_102903.1)	100%	Methanosaetaceae [a]
A3	*Methanobacterium formicicum* (NR_115168.1)	99%	Methanobacteriaceae [a]
B1	*Candidatus Cloacamonas acidaminovorans* (CU466930.1)	89%	Cloacimonetes [b]
B2	*Candidatus Cloacamonas acidaminovorans* (CU466930.1)	87%	Cloacimonetes [b]
B3	*Sulfurovum* sp (AP009179.1)	97%	Epsilonproteobacteria [c]
B4	*Pelobacter propionicus* (NR_074975.1)	100%	Pelobacteraceae [a]
B5	*Geobacter argillaceus* (NR_043575.1)	92%	Geobacteraceae [a]
B6	*Geobacter psychrophilus* (NR_043075.1)	97%	Geobacteraceae [a]
B7	*Geobacter toluenoxydans* (NR_116428.1)	83%	Geobacteraceae [a]

[a] Family.
[b] Phylum.
[c] Class.

Methanosaeta has a higher affinity to acetate than *Methanosarcina* (Jetten et al., 1990); hence, *Methanosaeta* can be predominant against *Methanosarcina* in stable reactors with low acetate concentrations (McMahon et al., 2001). In our study, the EGSB reactor showed quite stable performance, and the acetate concentrations were mostly lower than 200 mg L^{-1}. Furthermore, the archaeal diversity found in our study was extremely similar to that found by Xing et al. (2009), who ran an EGSB treating a synthetic brewery wastewater at 15 °C. In their study, *Methanosaeta*, *Methanobacterium*, and *Methanospirillum* reached 95% of the archaeal population, and *Methanosaeta* was the most predominant archaea. Furthermore, *Methanosaeta* has been described as an important microorganism in anaerobic granulation processes, and it has a key role in granule integrity (Xing et al., 2009). Therefore, its dominance facilitated the maintenance of the granule integrity throughout the trial, as shown in Fig. 4. In conclusion, methane in this anaerobic reactor was produced by hydrogenotrophic and acetotrophic pathways, as the presence of hydrogenoclastic and acetoclastic species in the reactor revealed.

In terms of the bacterial community, B1 and B2 were identified as *Candidatus Cloacamonas acidaminovorans*. Previous studies suggested that these bacteria are probably syntrophic (Pelletier et al., 2008). *Sulfurovum agreggans*, band B3, was a strictly chemolithoautotrophic bacteria (Mino et al., 2014). This bacterium was previously found in an EGSB working with high sulfate concentration. In our reactor, the sulfate average was low (values < 10 mg L^{-1}), so this bacterium probably came in the brewery sludge and was able to survive under low sulfate concentrations. *Pelobacter propionicus*, band B4, produce acetate and propionate from ethanol with sulfate presence (Schink et al., 1987), so it is involved in VFA production. It was found in anaerobic reactors treating winery wastewater (Cresson et al., 2009). Furthermore, it is remarkable that this bacterium can be associated with *Methanospirillum species*, to which it transfers H_2, to degrade primary alcohols and diols (Eichler and Schink, 1985). Yanti et al. (2014) proposed that the mechanism of ethyl ester degradation is the same as the mechanism for methyl ester degradation, so ethyl acetate is probably transformed into acetate and ethanol. In the case of 1-ethoxy-2-propanol, its degradation mechanism is not yet known, but Lafita et al. (2015) proposed that after enzymatic ether cleavage, 1-methoxy-2-propanol decomposes to acetone and methanol. By analogy, 1-ethoxy-2-propanol should also be transformed into acetone and ethanol. Considering that the main intermediate to be degraded was ethanol, a primary alcohol, these two microorganisms—*Methanospirillum* and *Pelobacter*—probably played an important role in the solvent degradation of this study, a claim that is corroborated by their progressive increase in abundance during the trial.

B5, B6, and B7 were identified as species belonging to the *Geobacter* genus. These organisms have been identified in different anaerobic reactors treating brewery wastewater (Shrestha et al., 2014); they can use different substrates as ethanol or acetate. Lovley (2011) demonstrated that *Geobacter* species can use direct interspecies electron transfer (DIET). This electron exchange between *Geobacter* and syntrophic partners seems to be an important process in anaerobic wastewater treatment (Commault et al., 2015). It has been demonstrated in laboratory-scale digesters that *Methanosaeta* is one of these syntrophic partners, and one-third of the methane production in an UASB is produced due to DIET between these two species (Rotaru et al., 2014). In our study, *Methanosaeta* and *Geobacter* were the predominant microorganisms in archaeal and bacterial populations, respectively, indicating that these types of interactions occur in the reactor treating a mixture of alcohols and ethers.

4. Conclusions

The anaerobic bioscrubber was shown to be an effective solution for VOC control emission coming from the flexographic sector. The optimization of a pilot unit composed of a packed scrubber and an expanded granular sludge bed reactor ensured high VOC elimination removal and efficient control of pressure drop in the scrubber. Despite the high fluctuations in the waste gas emissions, with interruptions during nights and weekends and temperature oscillations, stable conversion of alcohols, esters, and glycol ethers to enriched methane biogas was demonstrated. The use of granular sludge from a brewery wastewater treatment plant has been proven to be an adequate strategy to achieve consistently high efficiencies since startup. The limitation of carbon sources to a few organic solvents caused an initial decrease in biodiversity, especially in the domain Archaea, and then the predominant population persisted over time. The predominant Archaea and Bacteria species can be associated with the carbon source and operational parameters, such as temperature and organic load.

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Research article

Anaerobic degradation of glycol ether-ethanol mixtures using EGSB and hybrid reactors: Performance comparison and ether cleavage pathway

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ABSTRACT

The anaerobic biodegradation of ethanol-glycol ether mixtures as 1-ethoxy-2-propanol (E2P) and 1-methoxy-2-propanol (M2P), widely used in printing facilities, was investigated by means of two laboratory-scale anaerobic bioreactors at 25°C: an expanded granular sludge bed (EGSB) reactor and an anaerobic hybrid reactor (AHR), which incorporated a packed bed to improve biomass retention. Despite AHR showed almost half of solid leakages compared to EGSB, both reactors obtained practically the same performance for the operating conditions studied with global removal efficiencies (REs) higher than 92% for organic loading rates (OLRs) as high as 54 kg of chemical oxygen demand (COD) m^{-3} d^{-1} (REs of 70% and 100% for OLRs of 10.6 and 8.3 kg COD m^{-3} d^{-1} for E2P and M2P, respectively). Identified byproducts allowed clarifying the anaerobic degradation pathways of these glycol ethers. Thus, this study shows that anaerobic scrubber can be a feasible treatment for printing emissions.

1. Introduction

Solvents are commonly used in packaging printing industries as ink components that are dried through evaporation, which are the sources of volatile organic compounds (VOCs) in air emissions throughout the printing process. These emissions show ethanol as the main solvent, which is used in many solvent-based inks, but other solvents such as glycol ethers are also predominantly identified (Bravo et al., 2017; Sempere et al., 2012). The glycol ethers that are present in these emissions are 1-ethoxy-2-propanol (E2P) and/or 1-methoxy-2-propanol (M2P) that are frequently used as retarders in the printing process (Cheremisinoff, 2003). Several regulations and guidelines have been established in many countries for the control of the emissions of VOCs in printing industry. In the European Union, VOC concentrations of these emissions must comply with limit values ranging from 20 to 100 mg C Nm^{-3} (Council Directive, 2010/75/EU), depending on printing activity (offset, rotogravure, flexography, etc.) and solvent consumption threshold (>15 or >25 tonnes per year). USA Environmental Protection Agency also recommends levels of control for these emissions ranging from 65 to 80% for overall control efficiency and from 90 to 95% for control device efficiency, depending on installation date of equipment and printing activity. Many states and local agencies have adopted regulations for controlling emissions from printing industry consistent with these control levels (USEPA, 1978; USEPA, 2006). To fulfill these regulations and guidelines, the printing industry frequently needs end-of-pipe treatments of these emissions. In this regard, biological treatments such as biofiltration have been studied to remove volatile organic compounds from air emissions (Malhautier et al., 2005). A new alternative treatment based on an anaerobic bioscrubber has recently been described (Bravo et al., 2017; Waalkens et al., 2015). In this process, solvents are initially transferred from the gas phase to the liquid phase and subsequently degraded in an anaerobic reactor, where solvents are transformed into bioenergy in the form of biogas. The anaerobic biodegradability of M2P was demonstrated by Lafita et al. (2015) when working with an expanded granular sludge bed (EGSB) reactor at 25 °C. The removal efficiency (RE) of M2P reached up to 87% and showed a long lag phase that took 34 days to remove more than 50% of M2P. A first evidence of anaerobic degradation of M2P

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was reported by the European Chemicals Bureau (2006). In this study, a batch test using inoculum from a municipal digester, only a removal efficiency of 38% was observed after 81 days of operation.

Regarding the mechanism for the ether cleavage, it is not yet well understood, but the most accepted mechanism for glycol ether cleavage includes a double H/OH interchange (*trans*hydroxylation), resulting in the *gem*-diol intermediate (Speranza et al., 2002; White et al., 1996), which rapidly collapses to the carbonyl or the keto group. In the anaerobic degradation of polyethylene glycol (PEG), migration of hydroxyl group generates an intermediate hemiacetal leading to acetaldehyde. Thus, degradation of the polymer occurs by successive eliminations of acetaldehyde units catalyzed by a cobalamin-dependent intracellular enzyme (PEG acetaldehyde lyase), according to the following reaction (Frings et al., 1992):

$$R\text{-}O\text{-}CH_2\text{-}CH_2\text{-}OH \rightarrow R\text{-}O\text{-}CH(OH)\text{-}CH_3 \rightarrow R\text{-}OH + CH_3\text{-}CHO$$

Kawai (2002) hypothesized that the metabolism of polypropylene glycol (with identical terminal OH group as M2P and E2P) is the same as that of PEG. By analogy, Lafita et al. (2015) proposed the same mechanism for the degradation of M2P, in this case leading to methanol and acetone or propionaldehyde.

Biological treatments have been shown to be capable to remove slowly biodegradable industrial compounds (Zhang et al., 2016; Huang et al., 2017). Among these, anaerobic reactors based on granular sludge technology have become interesting options for the cost-effective and sustainable treatment of industrial wastewaters (Petta et al., 2017; Delforno et al., 2016). In this sense, the high contact between wastewater and biofilm that is promoted by expanding the sludge bed makes the EGSB a suitable technology to treat high stress, less biodegradable or toxic components in wastewater. Expanding the granular sludge requires high upflow velocity (v_{up}) that can produce the wash-out of granular biomass from the reactor (Dries et al., 1998). This wash-out phenomenon can also be caused by a low-concentrate substrate (Puñal et al., 2003); wastewaters containing fat, oil, and grease (Jeganathan et al., 2006); or the combination of organic overload and daily interruption in the substrate supply (Lafita et al., 2015).

To improve biomass retention, Guiot and van den Berg (1985) used an anaerobic hybrid reactor (AHR) in treating synthetic soluble sugar wastewater, where an upflow anaerobic sludge bed (UASB) reactor was modified by adding an anaerobic filter in the upper zone to replace the gas-liquid-solid (GLS) separator. The results showed the filter as an inexpensive method to enhance biomass retention. This type of AHR offers different advantages, such as higher biomass retention capacity, a polishing effect to the effluent due to the biomass accumulated in the filter (Shivayogimath and Ramanujam, 1999), or a higher buffering effect against shock loading (McHugh et al., 2006). Because of these characteristics, the AHR seems a promising and emerging alternative that can be considered feasible for the treatment of (especially high-strength) wastewaters from different industries. Thus, for wastewater from a distillery spent wash, Shivayogimath and Ramanujam (1999) demonstrated that a hybrid reactor combining a UASB reactor in the lower part with polypropylene pall rings filter media in the upper part could treat high organic loading, such as 36 kg of chemical oxygen demand (COD) $m^{-3}d^{-1}$, and it was a very efficient GLS separator. The AHR also showed high REs (up to 90.3%) in the treatment of a dairy industry effluent (Belançon et al., 2010). Treating a chemical synthesis-based pharmaceutical wastewater, Oktem et al. (2008) revealed that the AHR presented strong stability to changes in organic loadings. McKeown et al. (2009) showed that the AHR had a suitable configuration for long-term low-temperature treatment of acidified wastewaters.

The main purposes of this work were (i) to evaluate the feasibility of the anaerobic biodegradation of wastewater containing solvents from a flexographic printing industry by working with an AHR, including common glycol ethers such as M2P and E2P, and (ii) to compare the operation performance and biomass retention capacity between the AHR and the EGSB reactor. Furthermore, (iii) the study of the degradation of two different glycol ethers such as E2P and M2P might be useful for an in-depth understanding of the mechanisms involved in the anaerobic ether cleavage. Additionally and to the best of the authors' knowledge, this research is the first study about E2P degradation in an anaerobic reactor.

2. Materials and methods

2.1. Reactors' setup

Most of the experiments were performed in two anaerobic bioreactors, an EGSB reactor (R1) and an AHR (R2). Fig. 1 shows a schematic diagram of both systems. The EGSB diagram reactor was equivalent to the AHR but without the presence of the packing material (Fig. 1, part 4 in R2). The upper part of the AHR, from 1.40 m to 1.57 m in height, was randomly packed with polyethylene rings (0.0254 m in diameter). Both reactors had the same dimensions, with a sludge bed (Fig. 1, part 3) of 4 L (height of 1.205 m and internal diameter of 0.065 m) and a total volume of 19 L. In this paper, organic load rate is calculated on the basis of this sludge bed effective volume (4L). The supernatant reactor samples were taken at the recirculation port installed at a 1.60-m height. The gas was conducted to a gas seal (Fig. 1, part 8) with a solution of NaOH to remove H_2S and CO_2. The reactors' temperature was maintained at 25 °C, with an external jacket with water recirculation connected to a thermostat system (Polyscience SD15R-30, USA). Each anaerobic system was equipped with two peristaltic pumps that provided feeding and recirculation flows (Fig. 1, part 7) through the reactor. The feeding (Fig. 1, part 1) and the recirculation were maintained at constant flows of 10 L day^{-1} and 32 L h^{-1}, respectively, to provide a v_{up} of 9.80 m h^{-1} in the sludge bed and a v_{up} of 0.98 m h^{-1} in the fixed film section. The increment in the organic loading rate (OLR) was carried out by increasing the COD concentration, keeping the feeding flow constant. A second EGSB (R3), identical to R1, was also utilized in the last part of this study to elucidate the reaction mechanism. For this purpose, an ethanol-acetone mixture was used as the substrate.

2.2. Reactor operation

The reactors were started up by using anaerobic granular sludge (4 L) taken from a local brewery wastewater treatment plant (Font Salem S. L., El Puig, Spain). Both R1 and R2 were operated for more than 300 days (Table 1), with influent buffered with 5 g of $NaHCO_3$ L^{-1}; magnesium and calcium were maintained at 40 mg L^{-1}, and macronutrients and micronutrients were added in proportion to the COD (Table 2). All chemicals used were of analytical grade. The startup (stage S-I) of the reactors was carried out in the first 90 days of the experimental period (Table 1). At this stage, the OLR was increased from 9.1 to 47.0 kg COD $m^{-3}d^{-1}$, with a readily biodegradable substrate such as ethanol. From day 91 to day 202 (stage S-II), the influent composition was changed to a binary mixture of ethanol and E2P with a mass ratio of 9:1, and the average OLR was kept at 47.4 kg COD $m^{-3}d^{-1}$. At stage S-III, from day 203 to day 221, the OLR of E2P was increased to an ethanol-E2P mass ratio of 4:1, resulting in an OLR of 54.1 kg COD $m^{-3}d^{-1}$. At stage S-IV, from day 222 to day 230, the composition of the influent was changed to a ternary mixture of ethanol, E2P, and M2P with a mass ratio of 8:1:1 to check if there would be no need for a period of adaptation for M2P degradation. At this stage, the average OLR was 48.8 kg COD

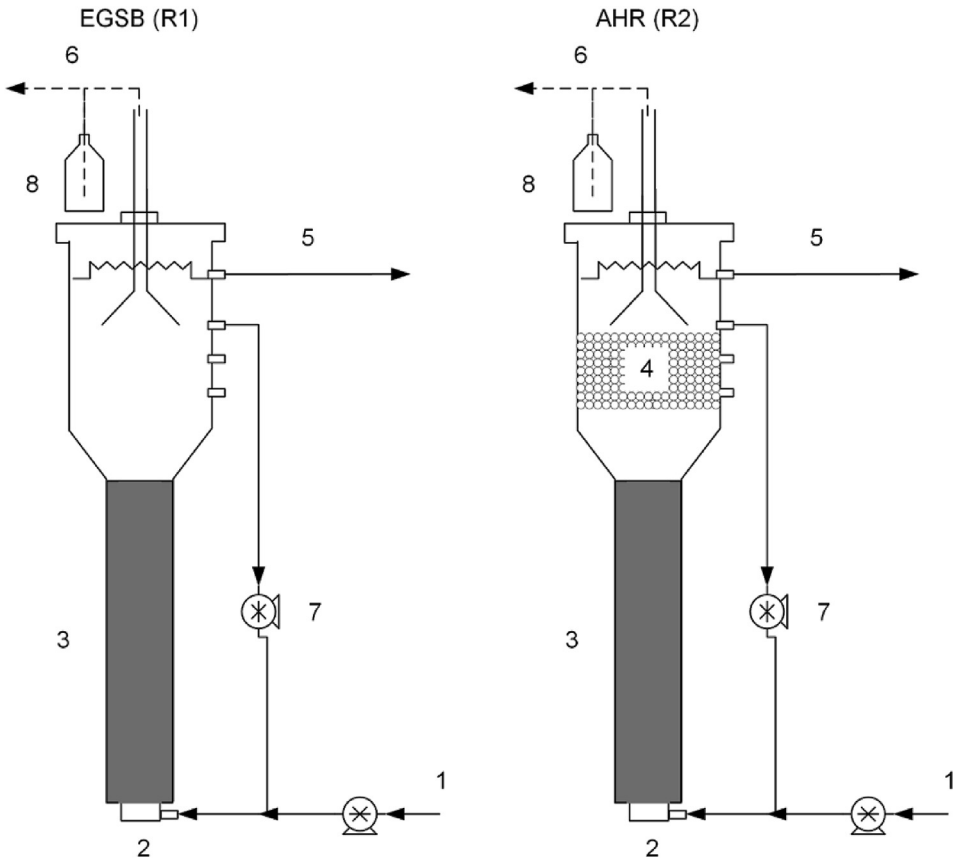

Fig. 1. Diagram of the EGSB reactor (R1) and AHR (R2) with numbered parts: 1) Feed, 2) Glass balls, 3) Effective volume, 4) Packing material, 5) Effluent, 6) Biogas, 7) Recirculation, 8) Gas seal.

Table 1
Operating conditions for the EGSB reactor (R1) and AHR (R2). Average values and Standard deviation.

Stage	S-I	S-II	S-III	S-IV	S-V	S-VI	S-VII
Time, d	0–90	91–202	203–221	222–230	231–271	272–305	306–335
COD, g L^{-1}	3.9 to 18.8	18.9 ± 3.0	21.6 ± 3.8	19.5 ± 2.1	–	6.3 to 16.3	18.5 ± 1.9
OLR, kg COD m^{-3} d^{-1}	9.1 to 47.0	47.4 ± 7.4	54.1 ± 9.5	48.8 ± 5.1	–	15.9 to 40.7	46.2 ± 4.8
E2P, g COD L^{-1}	0.0	2.0 ± 0.3	4.2 ± 0.3	2.4 ± 0.9	–	0.7 to 2.1	1.9 ± 0.2
E2P OLR, kg COD m^{-3} d^{-1}	0.0	5.1 ± 0.8	10.6 ± 0.7	6.1 ± 2.3	–	1.7 to 5.3	4.8 ± 0.6
M2P, g COD L^{-1}	0.0	0.0	0.0	1.9 ± 0.1	–	0.6 to 1.9	3.3 ± 0.4
M2P OLR, kg COD m^{-3} d^{-1}	0.0	0.0	0.0	4.8 ± 0.2	–	1.5 to 4.7	8.3 ± 1.1

Table 2
Macronutrient and micronutrient supplementation influent.

Compound	Dose, mg g^{-1} COD	Compound	Dose, mg g^{-1} COD
NH_4Cl	15.7	H_3BO_3	0.1143
Yeast extract	7.5	EDTANa	0.100
KCl	6.1	$(NH_4)_2Mo_7O_{24}·4H_2O$	0.0625
$(NH_4)_2HPO_4$	5.6	Al_2O_3	0.0595
$FeCl_3·6H_2O$	0.4208	$NiSO_4·6H_2O$	0.0447
$CoCl_2·6H_2O$	0.1615	$CuCl_2·2H_2O$	0.0134
$MnCl_2·4H_2O$	0.1441	$ZnSO_4·7H_2O$	0.132

$m^{-3}d^{-1}$. At stage S-V, from day 231 to day 271, no feeding and no recirculation were applied to evaluate the effect of a long starvation period that typically occurs in industrial facilities. At stage S-VI, from day 272 to day 305, a restartup was carried out with the same ternary mixture used at stage S-IV and with the OLR range from 15.9 to 40.7 kg COD $m^{-3}d^{-1}$. Finally, at stage S-VII, from day 306 to day 335, the average OLR was 46.2 kg COD $m^{-3}d^{-1}$, and the mass ratio of the mixture of ethanol, E2P, and M2P was changed to 7:1:2.

The influent of the R3 reactor was buffered, and the micronutrients and the macronutrients were added in the same way as in the previously described reactors. The startup was carried out with ethanol until it reached almost complete substrate degradation for an OLR of 25.0 kg COD $m^{-3}d^{-1}$. Then, maintaining the OLR, the feeding composition was changed to an ethanol-acetone mixture with a mass ratio of 1:1, and the reactor was operated for 27 days under these conditions.

2.3. Effluent and biogas analyses

The COD, as well as volatile fatty acid (VFA), total suspended solid (TSS), and volatile suspended solid (VSS) concentrations were analyzed according to Standard Methods (American Public Health Association, 1999). The NH_4^+, Ca^{2+}, Mg^{2+}, K^+, PO_4^{3-}, and SO_4^{2-} concentrations were measured by ionic chromatography (883 Basic IC Plus, Metrohm, Switzerland). The effluent's byproducts and

solvents were identified by gas chromatography-mass spectrometry (5973 N MS/GC Agilent Technologies, Spain). The solvents in the effluent were determined by gas chromatography (Agilent GC 7890A, Spain) equipped with flame ionization detector; the solvents and the byproducts of the solvent degradation were separated on a Restek Rtx-VMS (USA) column (30 m long × 0.25 mm i.d. × 1.4 μm film thickness), and helium was used as the carrier gas. The biogas composition before the gas seal was determined by using a portable biogas analyzer (COMBIMASS® GA-m, Binder, Germany). The methane flow rates of both reactors were measured after H_2S and CO_2 in the gas seal were removed by a gas meter (MGC-10 PMMA, Ritter, Germany). Conductivity and pH were determined by a precision handheld meter (Multi 340i, WTW, Germany).

The monitoring of bioreactors has been carried out from individual determinations, average values and standard deviation have been considered for overall operation conditions for each stage (Table 1).

3. Results and discussion

3.1. Reactor performance

The evolution of the global performance of both reactors, R1 and R2, is plotted in Fig. 2, where stages S-I to S-VII are indicated. The detailed evolution of the main parameters of the system (pH, conductivity, COD inlet concentration, etc.) can be found as supplementary material (Figs. S.1–S.6). REs higher than 95% were achieved during all startup periods (S-I), including when the OLR reached the maximum value of 47.0 kg of COD $m^{-3}d^{-1}$, as can be observed in Fig. 2. This was a predictable behavior since the organic substrate was ethanol, and the sludge came from a brewery wastewater treatment plant, so the biomass was well adapted to this substrate. The VFA concentrations were below 70 mg L^{-1} in the first 50 days, but when the OLR was increased to 47.0 kg COD $m^{-3}d^{-1}$, the VFA concentrations reached values of 221.9 and 172.0 mg CH_3COOH L^{-1} for R1 and R2, respectively. The minimal accumulation of VFA can be attributed to the faster metabolism of acidogenic bacteria compared to methanogenic archaea, but the VFA concentrations decreased when the high OLR was maintained over time, reflecting the methanogenic population's adaptation to these operational conditions. In any case, the VFA concentrations were always relatively low (<250 mg CH_3COOH L^{-1}) in both reactors, indicating no significant kinetic decoupling between the acidogenic and the methanogenic communities.

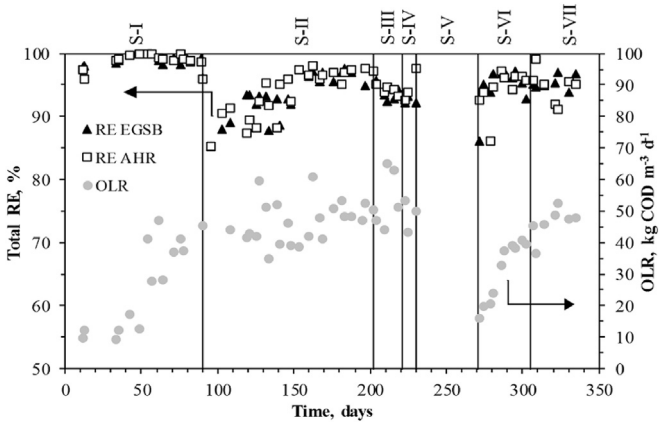

Fig. 2. Evolution of total OLR and COD removal efficiency of EGSB reactor (R1) and AHR (R2).

When E2P was included as a component of the influent at stage S-II (Table 1), the total RE in both reactors dropped from 99% to 85%, but the RE was progressively recovered to more than 95% (Fig. 2). This behavior can be explained by assuming that the biomass was not adapted to E2P, so the biomass needed an acclimatization period to be able to metabolize this substrate. This period could be related to the development of ether-cleavage enzymes to degrade this substrate as suggested by Lafita et al. (2015). This approach is shared by Chen et al. (2008) who point the induction of specific enzymes as one of the mechanisms through which adaptation can occur. Various anaerobic bacteria yielding rearrangements of carbon-carbon bonds via *trans*hydroxylation and depending on cobalamin were described for the degradation of polyethylene glycol (Schink et al., 1992; Frings et al., 1992). It would seem plausible that this same catalytic system governs the degradation of ether glycols as E2P, M2P, but no empirical evidences have been reported so far.

Fig. 3a illustrates the evolution of the OLR and the RE of E2P in both reactors. The RE of E2P was very low in the beginning, at 21% and 16% for R1 and R2, respectively, but gradually increased to around 80% for both reactors after 40–50 days of exposure to E2P. Generally, longer adaptation periods had been reported for similar solvents. Traverso-Soto et al. (2016) needed 169 days to reach 99.7% of anaerobic biodegradation of alcohol ethoxylates in anaerobic degradation assays with marine sediments. A longer adaptation period for another glycol ether such as M2P was also observed by Lafita et al. (2015), who reported a 34-day acclimatization period for degrading more than 50% of M2P. In a recent study (Lafita et al., 2017), the time required to obtain 80% of removal of M2P was shortened to 22 days when chitosan was supplemented. In the present study, the acclimatization period for removing over 50% of E2P lasted only 11 and 16 days for R1 and R2, respectively. This shorter acclimatization period could be explained by taking into account the different substrate and the slightly lower OLR of the glycol ether used in comparison with previous studies. Besides,

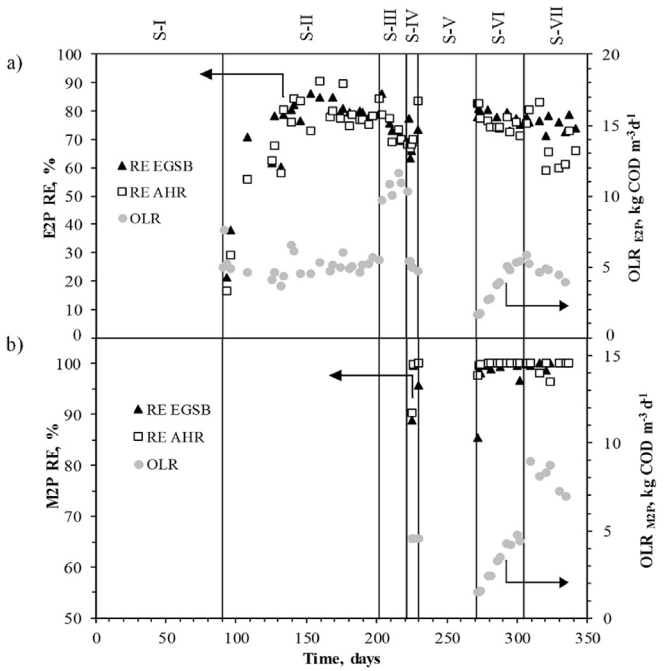

Fig. 3. Evolution of glycol ethers OLR and COD removal efficiency of EGSB reactor (R1) and AHR (R2), a) E2P OLR and E2P removal efficiency, b) M2P OLR and M2P removal efficiency.

during these first weeks of exposure to E2P, acetone and isopropanol were transitorily observed in the effluents of both reactors. The appearance of these byproducts indicates the amenability of E2P to anaerobic treatment and sheds some light on the mechanism of degradation as discussed further below.

The OLR of E2P was doubled to 10.6 kg COD $m^{-3}d^{-1}$ at stage S-III (Table 1), reaching an average total load of 54.1 kg COD $m^{-3}d^{-1}$, which resulted in a decrease of the global RE to around 92% for both reactors (Fig. 2). This decline can be essentially attributed to the decrease in the RE of E2P down to 70%, as shown in Fig. 3a, indicating that the presence of E2P in the system has no observable effect on the ethanol's RE.

At stage S-IV, the average of the total OLR was maintained at around 50 kg COD $m^{-3}d^{-1}$ (similar to stages S-II and S-III), but a ternary mixture of ethanol, E2P, and M2P, with a mass ratio of 8:1:1, was used as the organic substrate. At this stage, the performance of both reactors barely changed in terms of the global RE (Fig. 2), as well as the RE of E2P in comparison to the previous stage (S-III). The RE of M2P at the end of S-IV was practically complete as shown in Fig. 3b. Even from the first day of exposure to this compound, the RE of M2P was very high (>85%) in both reactors, indicating no significant adaptation period for metabolizing this compound. This result could be attributed to the fact that both glycol ethers are degraded through the same mechanism; therefore, the adaptation period for M2P degradation is negligible for a system degrading E2P. As in the case of E2P, degradation byproducts were detected in the effluent during the first days of exposure to M2P (acetone and methanol in this case). Regarding the biodegradability of E2P and M2P, the RE is greater for M2P (almost 100%), which could be related to the substrate's accessibility to the active center of the enzyme, as an M2P molecule is smaller than an E2P molecule. Therefore, the results suggest that the use of M2P would be preferable to E2P as a retarder additive if an anaerobic system is considered for the effluent treatment configuration of the packaging printing facility.

A noticeable conductivity increase to 7.48 mS cm^{-1} and 7.65 mS cm^{-1} for R1 and R2, respectively, was observed at the end of S-V, the stage in which no feeding and no recirculation were applied to the system. This conductivity increase above the typical value in both reactors, around 5.50 mS cm^{-1}, was caused by the increase in ammonium and phosphate concentrations as a consequence of a lysis phenomenon during the starvation period. From a mass balance and considering the cell content of nitrogen and phosphorus, the total biomass decay during this period can be estimated: 52.2 g and 66.0 g for R1 and R2, respectively. This higher increase in nitrogen and phosphorus concentration in R2 than in R1 reflects the AHR system's (R2) greater biomass retention capacity. Because during this period (S-V), no feeding was applied, the cell growth can be neglected, and the concentration of microorganisms (X) can be related to the microorganism concentration before the starvation period (X_0) from a mass balance, as shown in the following equation:

$$X = X_0 \exp(-bt)$$

where b is the decay coefficient of biomass, and t denotes the duration of the starvation period. The calculated decay coefficients were 0.0024 d^{-1} and 0.0022 d^{-1} for R1 and R2, respectively. These values are quite similar to the decay coefficients obtained by Wu et al. (1995) with methanogenic granules that previously degraded VFA at 22 °C (between 0.0015 and 0.0028 d^{-1}).

During the restartup, at stage S-VI, conductivity decreased after 4 days to the habitual values, and the OLR was gradually increased from 15.9 to 40.7 kg COD $m^{-3}d^{-1}$, with the same feeding composition as in stage S-IV. Both reactors showed a high total RE, 95% for R1 (EGSB) and 94% for R2 (AHR), indicating that a typical industrial long starvation period (>30 days) does not negatively affect the biomass activity. The glycol ethers' REs were high since the beginning of stage S-VI, 100% for M2P (Fig. 3b) and 77% for E2P (Fig. 3a) in both reactors, so no adaptation period was observed despite the biomass not being exposed to these compounds for more than 40 days.

At stage S-VII, the average total OLR was 45.9 kg COD $m^{-3}d^{-1}$ (Table 1), but the feeding composition was changed to a ternary mixture of ethanol, E2P, and M2P with a mass ratio of 7:1:2. In both reactors, the total RE was maintained at a high value, similar to the previous stage (Fig. 2). Moreover, M2P was completely removed despite its increase in OLR, indicating that the levels of M2P concentration used in the experiment did not affect the performance of the reactors. Regarding the REs of E2P (Fig. 3a), these reached 76% and 80% for R1 and R2, respectively, until day 315 when the pall rings in the hybrid reactor (R2) were replaced by clean ones to avoid clogging problems in the filter media. After the replacement, the RE of E2P for R2 dropped to 65%, and the average concentration of VFA in the effluent increased from negligible values to 68.5 mg CH_3COOH L^{-1}, while in the EGSB reactor, it only amounted to 14.6 mg CH_3COOH L^{-1}. The decrease in the E2P's RE and the increase in VFA after the replacement of the packaging material showed that a not quite significant part of the R2 activity was related to the active biomass associated with the packed fraction of the reactor.

The methane average and standard deviation (SD) yield in R1 was 0.324 ± 0.051 m^3 CH_4 kg $COD^{-1}_{degraded}$ and in R2, it was 0.318 ± 0.049 m^3 CH_4 kg $COD^{-1}_{degraded}$, indicating no significant difference between both reactors in the methane production. The evolution of methane production can be found in the supplementary material (Fig. S.7). The average and SD proportion of methane in the biogas was $82.4 \pm 2.6\%$ and $83.0 \pm 2.9\%$ for R1 and R2, respectively, with a presence of H_2S below 130 ppm_v. Narra et al. (2014) reported a methane yield between 0.12 and 0.16 m^3 CH_4 kg $COD^{-1}_{degraded}$ when working with anaerobic hybrid reactors that treated the wastewater of mild alkali-treated rice straw in ethanol fermentation process. This low yield could be due to the different substrate used. Treating more similar substrates such as solvents that included ethanol, acetone, propanol, and methanol, Enright et al. (2009) obtained methane yields between 0.11 and 0.35 m^3 CH_4 kg $COD^{-1}_{degraded}$, within the range of those obtained in the present study for both reactors.

Since acetone was detected as a key intermediate product of anaerobic biodegradation of glycol ethers, an EGSB reactor (R3) was fed with this solvent and ethanol (1:1 mass ratio) to shed light on

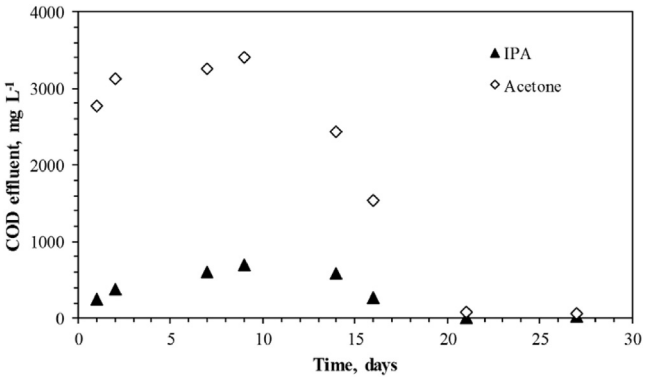

Fig. 4. Evolution of intermediate compounds' concentration found in the effluent in the reactor R3 treating the ethanol-acetone mixture with an average inlet COD concentration of 11.2 ± 1.5 g L^{-1}.

the mechanism. Fig. 4 shows the evolution of the organic compounds detected in the effluent of R3. The acetone concentration was quite high in the beginning of the trial, but since day 9, the concentration gradually decreased, and the system practically reached complete degradation (98%) of this compound on day 21. These results showed that the biomass needed an adaptation period to remove acetone, as what occurred with E2P, but in this case, the adaptation period was shorter than in the case of E2P, indicating that acetone could be more readily biodegradable than E2P. Thus, experimental evidence seems to point out that the first step of the cleavage to alcohol and acetone could be considered the controlling step for the biodegradation of glycol ethers. This hypothesis would be reinforced, considering the detection of negligible amounts of acetone and/or isopropanol in the effluent found in the experimental series with R1 and R2 (these compounds were only briefly detected when the glycol ethers were first fed into the bioreactors). Furthermore, isopropanol was identified in the effluent of bioreactor R3, as shown in Fig. 4. The appearance of isopropanol in a system treating acetone was explained by Tonouchi (2004) and Zellner and Winter (1987), who concluded that isopropanol could be produced from acetone in the presence of hydrogen, with a reversible reaction. Additionally, Vermorel et al. (2017) observed a significant presence of acetone, besides isopropanol, in the effluent of a laboratory-scale anaerobic CSTR that treated synthetic wastewater with ethanol and isopropanol as organic substrates. This observation would support the hypothesis of the reversibility of the transformation between isopropanol and acetone.

3.2. Anaerobic mechanism of ether cleavage

The mechanism of the anaerobic ether cleavage is not well established yet, although it is suggested that intracellular enzymes depending on cobalamin are involved in this process (Frings et al., 1992). The most accepted mechanism implies a double H/OH interchange (hydroxyl shift) resulting in the *gem*-diol that rapidly collapses to a carbonyl or a keto group (Speranza et al., 2002). As previously described, the identified byproducts during the first days of the biomass exposure to E2P were acetone and isopropanol, while acetone and methanol were identified in the case of M2P. By analogy with the hydroxyl-shift mechanism of ether excision for anaerobic ether cleavage (White et al., 1996), the theoretical byproducts should be ethanol and acetone from E2P degradation and methanol and acetone from M2P degradation (Lafita et al., 2015). In the case of E2P, no ethanol was observed in the effluent as a readily biodegradable substrate. In any case, as a main substrate, ethanol could not have been attributed as an intermediate product of degradation. On the other hand, the presence of isopropanol could be related to the formation of acetone as it was corroborated with the R3 reactor performance, as previously described. Thus, acetone together with the hydrogen can make the conversion to isopropanol possible, explaining the appearance of this compound in the effluent.

In the case of M2P, the presence of methanol was briefly detected in the effluent of both bioreactors (R1 and R2) after M2P was added to the influent. However, isopropanol was not observed because at this phase of the study, the biomass was already well adapted after more than 100 days of degrading E2P; thus, the formed isopropanol should be undetectable by the equipment. Therefore, the hypothetical mechanism schematized in Fig. 5 would explain the presence of the detected organics in the effluent of the reactors. Regarding acetone, its degradation to methane and CO_2 was reported to be the first case in which acetate is the only intermediate transferred between a fermenting bacterium and a methanogen (Platen and Schink, 1987). According to these authors, acetone is first carboxylated to acetoacetate by condensation with CO_2, from which acetate is formed and then transferred to *Methanosatea* sp. (formerly *Methanothrix* sp.). Methanol can be directly utilized as a carbon and energy source by several species of methanogens and acetogens.

3.3. Biomass retention capacity of AHR and EGSB reactor

Granular and non-granular accumulated solids of each stage collected from the effluent of reactors R1 and R2 can be found in Table 3, and the evolution of total accumulated solids of both reactors are plotted in Fig. 6, which shows that during stage S-I, the solids in the effluent of R1 were considerably higher than in R2, reaching 73.2 g and 29.0 g of accumulated solids, respectively. In the beginning of S-II, the solids collected from both effluents were similar, but from day 133 to day 161, a granular sludge flotation occurred in R1. Therefore, the accumulated solids in the effluent increased considerably with respect to R2, where no granular sludge flotation was registered. Thus, during this stage, 255.0 g of solids were collected from R1 and only 109.0 g from R2. During stage S-III, the solids in the effluent were also slightly higher in the EGSB reactor (28.3 g) than in the AHR (20.1 g). Anaerobic granular sludge flotation was observed in the systems (stage S-IV), reaching 45.9 g and 36.5 g of accumulated solids for R1 and R2, respectively. It is important to clarify that the sludge flotation observed has not been a total breakdown that had led to a complete wash out as those described extensively in literature (Macarie et al., 2017; Yoda and Nishimura, 1997; Lafita et al., 2015). In fact, if the leakage of solids is quantified in reference to the effluent flow, the average outlet concentration of reactor R1 in stage S-II (stage and reactor most unfavorable in terms of leakage of solids) is 228 g TSS m^{-3} which can be considered as a normal value for this kind of anaerobic systems.

After the shut-down period (S-V), when feeding was resumed, 20.0 g of solids in total were collected from R2, while only 13.8 g were washed out from R1 during the first week of stage S-VI. This unusually larger amount of biomass washed out from R2 could be explained by the lysis that occurred during the starvation period; as more biomass was retained by R2 than by R1, more granules were proportionally broken in R2, yielding greater biomass washout. The last two stages also showed greater washout from R1, in accordance with the global experimental trend. At the end of the experiment, the accumulated solids collected in the effluent were 563.2 g and 293.7 g for R1 and R2, respectively. This difference shows the AHR's (R2) higher capacity to retain biomass, supporting the study of Borja et al. (1995), who reported that the packing of a hybrid anaerobic reactor significantly enhanced the retention of active biomass treating slaughterhouse wastewater. Furthermore, Guiot and van den Berg (1985) demonstrated that the AHR's biomass retention capacity was between 1.5 and 2.8 times higher than those of other bioreactor configurations such as the UASB or the anaerobic baffled reactor.

The significant improvement in the biomass retention capacity of the hybrid system (R2) was not reflected in its performance since no significant differences in the REs of the different compounds were observed over the experimental period. Only slightly more stable and lower concentrations of VFA were observed in the effluent of R2.

In previous research, episodes of biomass flotation in the AHR had been attributed to lipid adsorption in granules (Belançon et al., 2010), but as no lipids were present in the feeding of the present study, the biomass flotation produced during stages S-II and S-IV had to be caused by other factors. Furthermore, in the AHR, no biomass flotation was observed during stage S-II, probably due to the higher capacity to retain biomass. However, the episode of

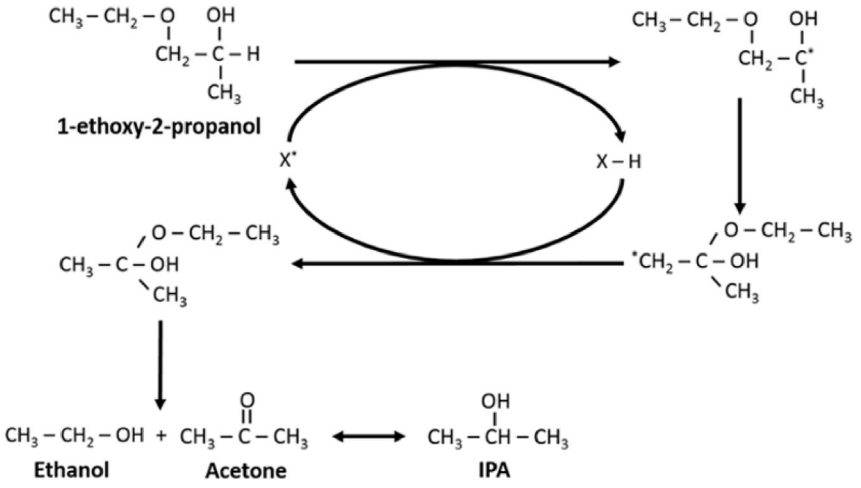

Fig. 5. Proposed mechanism for the anaerobic degradation of 1-ethoxy-2-propanol, including isopropanol (IPA) formation from acetone.

Table 3
Accumulated suspended solids (SS) in the effluent from each reactor in the different stages.

	Stage:	S-I	S-II	S-III	S-IV	S-VI	S-VII
Non-granular solids (g)	EGSB	73.1	101.2	17.3	6.7	15.0	15.4
	AHR	29.0	81.5	16.6	7.1	12.2	27.5
Granular solids (g)	EGSB	<0.1	153.8	11.0	39.2	39.1	91.3
	AHR	<0.1	27.5	3.5	29.4	36.5	22.9
Ratio SS_{EGSB}/SS_{AHR}	Non-granular	2.52	1.24	1.04	0.94	1.24	0.56
	Granular	–	5.59	3.14	1.34	1.07	3.98
	Total	2.52	2.34	1.40	1.26	1.11	2.12

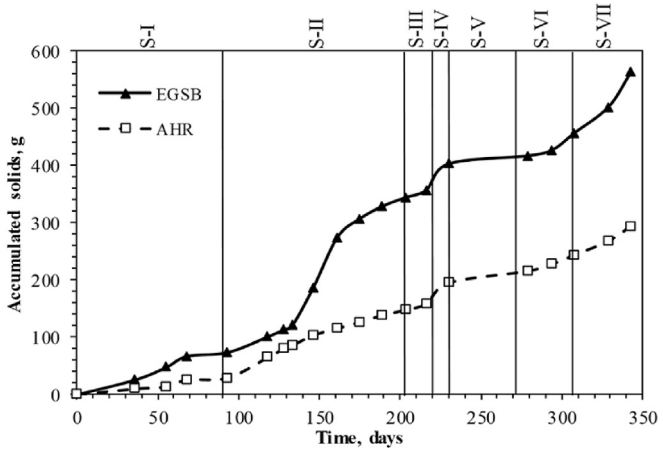

Fig. 6. Accumulated suspended solids in the effluent of EGSB reactor (R1) and AHR (R2).

anaerobic granular sludge flotation observed in the AHR at stage S-IV indicated that the filter was highly clogged and close to its maximum retention capacity. This fact can also be observed in Table 3 where, in addition to the absolute values of the leakage of solids from each reactor, the ratios of the solids in the effluent between control EGSB (R1) and AHR (R2) have been included (granular, non-granular, and total solids). The ratio of the total solids (R1/R2) decreased over the experimental period from 2.5 at stage S-I to 1.1 at stage S-VI, and then, at stage S-VII, it increased again to 2.11 when the packing material was replaced by new and clean polyethylene rings. The decrease in the ratio at every stage was even higher if only granular solids were considered-from 5.6 at stage S-II to 1.1 at stage S-VI, and then, after the replacement of the packing material, the ratio increased again to 4.0 at stage S-VII. In contrast, the non-granular solids (suspended solids) collected from both systems were not significantly different at each of the stages studied (the ratio varied from 1.2 to 0.6). These results indicated that the packing material selectively retained the granular biomass versus the solids in suspension. Furthermore, the recovered capacity to retain the solids after the replacement of the packing material suggested that the filter was clogged; thus, to maintain proper performance, it should be periodically cleaned or replaced. It seems that the leakage of granules would be related with regular discharge of surplus biomass in normal operation as reported by Pereboom (1994). Likewise, Hulshoff Pol et al. (2014) affirmed that light sludge is separated from mature granules using the rapid upward velocity of EGSB reactor. This selective wash-out, resulting in an increased growth of retained (heavier) sludge agglomerates would be crucial for the granulation process (Lim and Kim, 2014). Thus, it is interesting to highlight that the observed leakage throughout the experimental series has not had significant influence with the performance of the EGSB reactor. Even more interesting is that no difference has been observed between the performance of both systems (control EGSB and AHR) for any stage, which would indicate that the activity of the excess sludge (that was retained in the case of the AHR system) was practically negligible and no comparable to the activity of retained granules.

As a resume, the main advantages and disadvantages of the AHR in comparison with the EGSB observed in this long-term study have been synthesized in Table 4.

4. Practical applications and future perspective

The present work is part of a broader research related to the industrial implementation of anaerobic bioscrubber, an innovative system for the control of VOC emissions that is capable to transform solvents into bioenergy (Waalkens et al., 2015). A pilot-scale study of this technology, composed of a packed scrubber and an EGSB reactor of 8.7 m^3 treating on-site emissions from a packaging printing facility, showed to be an effective solution for VOC emission control (Bravo et al., 2017). In this context, the results obtained from the present work, based on the acquired knowledge about the retention of the biomass and the anaerobic degradation of glycol-ethers, will allow extending the horizons of practical application of the anaerobic bioscrubber.

Table 4
Advantages/disadvantages of the AHR in comparison with the EGSB system.

Advantages
- Higher solid retention capacity
Disadvantages
- AHR increases to some extent the operational complexity of the treatment
- Increasing biomass retention in the upper packed filter can lead to clogging
- Slight increase in the cost of capital of the system

With regard to the packing printing sector, given the general tendency of packaging manufacturing firms to move nearer to their customers, investment potentials for developing countries abound in this sector. The appeal of products from the developing Southern economies to their Northern and Western markets will have to be extended to their packaging (FAO, 2014). As a result of this sector's trend to install the facilities at point of origin, the demand for these VOCs control systems is expected to be on the rise.

The current research lines are focused in the study of the dynamics of the microbial communities and their interrelation with the bioreactor operation, including its response to starvation periods. On the other hand, the increase of the feasibility of this technology by using a more available and economic source of anaerobic sludge (e.g. from municipal waste water treatment plant) is being investigated.

5. Conclusions

Granular anaerobic treatment was shown to be a feasible technology for the biodegradation of ethanol-glycol ether mixtures such as E2P and M2P after a short period of acclimatization. Of the two systems tested, the AHR showed a higher solid retention capacity than the EGSB but did not result in the AHR's improved performance. Both systems could completely remove M2P (M2P OLR of 8.3 kg COD m^{-3} d^{-1}) and 70% of the E2P (E2P OLR of 10.6 kg COD m^{-3} d^{-1}). The detected byproducts allowed clarifying the degradation pathways of the studied glycol ethers.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jenvman.2018.02.070.

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