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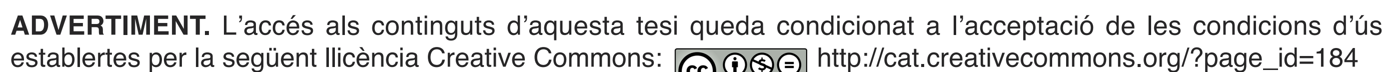

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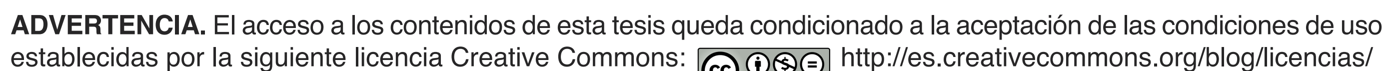

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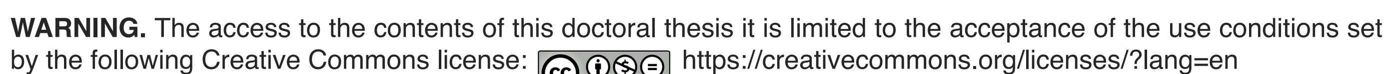

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Escola d'Enginyeria

Departament d'Enginyeria Química, Biològica i Ambiental

Integrated assessment of long-term sulfidogenesis in UASB reactors using crude glycerol as carbon source

PhD Thesis

Programa de Doctorat en Ciència i Tecnologia Ambientals

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Bellaterra, 26 de juny de 2020

Dr. David Gabriel Buguña — Dr. Maria Isabel Mora Garrido

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Thesis abstract

Many industrial activities generate effluents containing sulfur compounds, both as liquid or gaseous emissions, which are mainly treated through physical-chemical processes. Sulfate is generally present in wastewaters coming from paper, pharmaceutical, mining or food processing industries, among others. As such, sulfate is not a harmful compound, but if it is poured into rivers or sewage systems, an imbalance in the overall sulfur cycle can be generated. Inside this cycle, the last product after the reduction of sulfur compounds is hydrogen sulfide (H_2S). This compound is corrosive, odorous and toxic at low concentrations. For these reasons, there is a need to develop environmentally friendly alternatives to valorize not only gaseous emissions, such as SO_2 emissions, but also S-rich liquid effluents. In addition, a further recovery of elemental sulfur from these effluents could be obtained providing an opportunity to recover resources in the framework of the circular economy. With these premises, the SONOVA project, in which this thesis is enclosed, is based in the development of a comprehensive treatment process to valorize SOx and NOx from flue gases by economical, robust and environmentally friendly biological methods. It also takes into account the reuse of energy and resources along the process as well as residues valorization. The proposed process is based on a first double stage for selective absorption of SOx and NOx; a second biological step for reducing the sulfate from the first absorption stage to hydrogen sulfide (which is the focus of this thesis); and a third biological stage for the oxidation of hydrogen sulfide to elemental sulfur and its subsequent recovery.

Biological-based systems, such as Up-flow Anaerobic Sludge Bed (UASB) reactors, have been developed and implemented world-wide to treat many types of wastewaters. In this thesis, the use of an UASB reactor for the treatment of synthetic wastewater with sulfate was studied, specifically selecting crude glycerol as carbon source and electron donor. Both physical-chemical processes

and molecular biology techniques were used to get a broad knowledge of the anaerobic process. The UASB reactor allowed the reduction of sulfate at a COD/S-SO_4^{2-} ratio ranging from 3.8 g O_2 g^{-1} S to 5.4 g O_2 g^{-1} S. The highest average sulfate elimination capacity (S-EC=4.3 kg S $m^{-3}d^{-1}$) was obtained at a COD/S-SO_4^{2-} ratio of 5.4 g O_2 g^{-1} S and an OLR of 24.4 kg O_2 $m^{-3}d^{-1}$ with a sulfate removal efficiency of 94 %. The influence of possible inhibitions and competition between sulfate reducers and methanogens was studied in order to improve sulfate removal and sulfide production. It was observed that in long-term operations (after 200 days approximately) methanogens were washed out from the system and sulfate reducers colonized the reactor sludge. However, acetate accumulation was observed because of the disappearance of methanogens, leading to a loss of carbon source in the outlet of the reactor that could have been used to produce sulfide in the UASB. Long-term performances allow detecting further limitations of the system. A loss of granular structure and the growth of unidentified non-sulfate reducer, non-methanogenic biofilm was observed during UASB operations along this thesis. This biofilm, called slime along this thesis, was found to be a crucial factor affecting our system, conferring properties such as viscosity to the sludge. Consequently, problems related to mass transfer limitations could be observed, affecting as well, the sulfate reducing activity of the granules and leading to failured operations.

Finally, since the accumulation of acetate could not be avoided, experiments were designed to pursue the enrichment of acetate degrading sulfate reducing bacteria in serum bottles, with the final objective of improving sulfidogenesis. In addition, isolation of potential acetate-utilizing sulfate reducers was also pursued. Unfortunately, a culture able to perform sulfate reduction with acetate was not developed during the enrichment experiments. Therefore, further research is needed to enhance the operation in terms of organic matter consumption and sulfide productivity in the long-term.

Resumen de la tesis

Muchas actividades industriales generan emisiones que contienen compuestos de azufre tanto en efluentes líquidos como emisiones gaseosas, que mayoritariamente son tratadas mediante procesos fisicoquímicos. El sulfato se encuentra generalmente en las aguas residuales de estas industrias, como la industria papelera, la farmacéutica, la minera o la alimentaria. Como tal, el sulfato no es un compuesto nocivo, pero si se vierte en los ríos o en los sistemas de alcantarillado, puede generarse un desequilibrio en el ciclo del azufre. Dentro de este ciclo, el producto final de la reducción de compuestos dentro del mismo es el sulfuro de hidrógeno (H_2S). Este compuesto es corrosivo, oloroso y se ha demostrado que es tóxico en bajas concentraciones. Por estos motivos, es necesario desarrollar alternativas respetuosas con el medio ambiente para tratar y valorizar no sólo las emisiones de SO_2 sino también los efluentes líquidos ricos en azufre. Además, podría recuperarse azufre elemental de esos efluentes, lo que brindaría la oportunidad de recuperar recursos en el marco de la economía circular. Con estas premisas, el proyecto SONOVA, en el cual se enmarca esta tesis, desarrolló un proceso integral de tratamiento del SOx y el NOx proveniente de gases de combustión mediante procesos biológicos, económicos, robustos y respetuosos con el medio ambiente que también tiene en cuenta la reutilización de energía y recursos a lo largo del proceso, así como la valorización de residuos. El proceso propuesto se basa en una primera doble etapa para la absorción selectiva de SOx y NOx; una segunda etapa biológica para reducir el sulfato de la primera etapa de absorción a sulfuro de hidrógeno (que es el objetivo de estudio de esta tesis); y una tercera etapa biológica para la oxidación del sulfuro de hidrógeno a azufre elemental y su posterior recuperación.

El desarrollado de sistemas, como el reactor de lecho de lodo anaerobio de flujo ascendente (UASB), han sido implementados para el tratamiento de diversas aguas residuales. En esta tesis, se estudió el uso de este tipo de reactor

UASB para el tratamiento de aguas sintéticas con sulfato, específicamente, se seleccionó el glicerol crudo como fuente de carbono y donador de electrones. Se utilizaron tanto procesos fisicoquímicos como técnicas de biología molecular para obtener un mayor conocimiento del proceso. El reactor UASB permitió la reducción de sulfato con relación DQO/S-SO_4^{2-} entre 3.8 g O_2 g^{-1} S y 5.4 g O_2 g^{-1} S de. La mayor capacidad de eliminación de sulfato (S-EC=4.3 kg S $m^{-3}d^{-1}$) se obtuvo con una relación DQO/S-SO_4^{2-} de 5.4 g O_2 g^{-1} S y una carga orgánica de 24.4 kg O_2 $m^{-3}d^{-1}$ obteniendo una eficiencia de remoción de sulfato del 94 %. Se estudió la influencia de posibles inhibiciones y la competencia entre las bacterias sulfato reductoras y los metanógenos a fin de mejorar la eliminación de sulfato y la producción de sulfuro. Se observó que en las operaciones a largo plazo (después de 200 días aproximadamente) los metanógenos desaparecen del sistema y las bacterias sulfato reductoras son las que lo colonizan. Sin embargo, se observó una acumulación de acetato como consecuencia de la desaparición de los metanógenos, lo que dio lugar a una pérdida de la fuente de carbono en la salida del reactor que podría haberse utilizado para producir sulfuro. Las operaciones a largo plazo permitieron detectar otras limitaciones del sistema. A lo largo de las operaciones del UASB llevadas a cabo en esta tesis, se observó una pérdida de la estructura granular y el crecimiento de una biopelícula no metanogénica ni sulfatoreductora no identificada. Esta biopelícula, llamada slime a lo largo de esta tesis, se consideró como un factor crucial que afectaba a nuestro sistema, confiriendo propiedades como la viscosidad al lodo granular. En consecuencia, se pudieron observar problemas relacionados con la limitación de transferencia de materia, que afectaba también a la actividad sulfato reductora de los gránulos y que condujo a operaciones fallidas.

Por último, como no se pudo evitar la acumulación de acetato, se diseñaron experimentos para perseguir el enriquecimiento de bacterias reductoras de sulfato y capaces de utilizar el acetato para ello en botellas anaerobias, con el

objetivo final de mejorar la sulfidogénesis. Además, se intentó también aislar a las bacterias encargadas de este proceso. Desafortunadamente, no se consiguió la proliferación de un cultivo capaz de reducir sulfato utilizando acetato durante los experimentos de enriquecimiento. Es por eso, que se requiere más investigación para incrementar la utilización de la materia orgánica en las operaciones a largo plazo de este tipo de sistemas.

Resum de la tesi

Moltes activitats industrials generen emissions que contenen compostos de sofre tant en efluents líquids com emissions gasoses, que majoritàriament són tractades mitjançant processos fisicoquímics. El sulfat es troba generalment a les aigües residuals d'aquestes indústries, com la indústria paperera, la farmacèutica, la minera o l'alimentària. Com a tal, el sulfat no és un compost nociu, però si s'aboca als rius o sistemes de clavegueram, pot generar-se un desequilibri del cicle del sofre. Dins d'aquest cicle, el producte final de la reducció de compostos és el sulfur d'hidrogen (H_2S). Aquest compost és corrosiu, olorós i s'ha demostrat que és tòxic en baixes concentracions. Per aquests motius, és necessari desenvolupar alternatives respectuoses amb el medi ambient per a tractar i valoritzar no només les emissions de SO_2 sinó també els efluents líquids rics en sofre. A més, podria recuperar-se sofre elemental d'aquests efluents, la qual cosa brindaria l'oportunitat de recuperar recursos en el marc de l'economia circular. Amb aquestes premisses, el projecte SONOVA, en el qual s'emmarca aquesta tesi, va desenvolupar un procés integral de tractament del SO_x i el NO_x provinent de gasos de combustió mitjançant processos biològics, econòmics, robustos i respectuosos amb el medi ambient que també tenen en compte la reutilització d'energia i recursos al llarg del procés, així com la valorització de residus. El procés proposat es basa en una primera doble etapa per a l'absorció selectiva de SO_x i NO_x; una segona etapa biològica per a reduir el sulfat de la primera etapa d'absorció a sulfur d'hidrogen (que és l'objectiu d'estudi d'aquesta tesi); i una tercera etapa biològica per a l'oxidació del sulfur d'hidrogen a sofre elemental i la seva posterior recuperació.

El desenvolupament de sistemes biològics, com el reactor de llit anaerobi amb flux ascendent (UASB), han estat implementats per al tractament de diverses aigües residuals. En aquesta tesi, s’ha estudiat l'ús d'aquest tipus de reactor UASB pel tractament d'aigües sintètiques amb sulfat, específicament, es va seleccionar

el glicerol cru com a font de carboni i donador d'electrons. Es van utilitzar tant processos fisicoquímics com tècniques de biologia molecular per a obtenir un major coneixement del procés. El reactor UASB va permetre la reducció de sulfat amb una relació DQO/S-SO_4^{2-} entre 3.8 g O_2 g^{-1} S i 5.4 g O_2 g^{-1} S. La major capacitat d'eliminació de sulfat (S-EC=4.3 kg S $m^{-3}d^{-1}$) es va obtenir amb una relació DQO/S-SO_4^{2-} de 5.4 g O_2 g^{-1} S i una càrrega orgànica de 24.4 kg O_2 $m^{-3}d^{-1}$ obtenint una eficiència de remoció de sulfat del 94 %. Es va estudiar la influència de possibles inhibicions i la competència entre els bacteris reductors de sulfat i els metanògens a fi de millorar l'eliminació de sulfat i la producció de sulfur. Es va observar que en les operacions a llarg termini (després de 200 dies aproximadament) els metanògens desapareixen del sistema i els bacteris reductors del sulfat són els que colonitzen. No obstant això, es va observar una acumulació d'acetat a conseqüència de la desaparició dels metanògens, la qual cosa va donar lloc a una pèrdua de la font de carboni a la sortida del reactor que podria haver-se utilitzat per a produir sulfur. Les operacions a llarg termini permeten detectar altres limitacions del sistema. Al llarg de les operacions en UASB dutes a terme en aquesta tesi, es va observar una pèrdua de l'estructura granular i el creixement d'una biopel·lícula no metanogènica ni sulfat reductora no identificada. Aquesta biopel·lícula, anomenada slime al llarg d'aquesta tesi, es va considerar com un factor crucial que afectava el sistema biològic, conferint propietats com la viscositat al llit granular. En conseqüència, es van poder observar problemes relacionats amb la limitació de transferència de matèria, que afectava també l'activitat sulfat reductora dels grànuls i que va conduir a operacions fallides.

Finalment, com no es va poder evitar l'acumulació d'acetat, es van dissenyar experiments per a perseguir l'enriquiment i aïllament de bacteris reductors de sulfat i capaços d'utilitzar l'acetat en ampolles anaeròbies, amb l'objectiu final de millorar la sulfidogènesi. Malauradament, no es va aconseguir la proliferació

d'un cultiu capaç de reduir sulfat utilitzant acetat durant els experiments d'enriquiment. És per això, que es requereix més recerca per a incrementar la utilització de la matèria orgànica i augmentar la productivitat de sulfur en operacions a llarg termini d'aquesta mena de sistemes.

List of abbreviations

AMD	Acid mine drainage
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
CLSM	Confocal laser scanning microscopy
CE-SSCP	Capillary electrophoresis single-strand conformation polymorphism
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EC	Elimination capacity
EDTA	Ethylenediaminetetraacetic acid
EGSB	Expanded granular sludge bed reactor
EPS	Extracellular polymeric substances
FA	Formamide
FAME	Fatty acid methyl ester
FBR	Fluidized bed reactor
FISH	Fluorescence in situ hybridization
FTIR	Fourier-transform infrared spectroscopy
GAG	Glycosaminoglycan
sGAG	Sulfated glycoaminoglycans
GLB	Gas-lift bioreactor
HRT	Hydraulic residence time
HVRs	Hypervariable regions
LCFAs	Long-chain fatty acids
MBR	Membrane bioreactor
OLR	Organic loading rate

ORP	Oxidation-reduction potential
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PS	Polysaccharide content
RE	Removal efficiency
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SAOB	Sulfide antioxidant buffer
SEM	Scanning electron microscope
SLR	Sulfate loading rate
SRB	Sulfate reducing bacteria
SSCP	Single strand conformation polymorphism
TDS	Total dissolved sulfide
TGGE	Thermal gradient gel electrophoresis
TIC	Total inorganic carbon
TOC	Total organic carbon
TSS	Total suspended solids
UASB	Up-flow anaerobic sludge bed
VFAs	Volatile fatty acids
VOCs	Volatile organic compounds
WFGD	Wet flue gas desulfurization

Table of contents

Thesis abstract III

Resumen de la tesis V

Resum de la tesi VIII

List of abbreviations XI

Chapter 1: Research motivations and thesis overview 18

1.1 Research motivations 3

1.2 Thesis overview 3

Chapter 2: Introduction 7

2.1 Biological sulfur cycle 9

2.2 Gaseous effluents and wastewaters containing sulfur compounds 11

2.3 Anaerobic reactors for sulfate-rich effluents valorization 13

2.3.1 Sulfate reduction in sulfidogenic bioreactors 15

2.3.2 Electron donor and carbon source for sulfate reduction 16

2.3.3 Types of bioreactors used for sulfate reduction 18

2.4 Sulfate reducing bacteria (SRB) 20

2.4.1. Effect of sulfide on SRB 21

2.4.2 Competition between sulfate reducers, methanogens and acetogens 23

2.5 Techniques for microbial identification in anaerobic bioreactors 26

Chapter 3: Objectives 31

Chapter 4: General materials and methods 35

4.1. Description of the reactors and experimental set-up 37

4.1.1. Lab-scale UASB reactor 37

4.1.2. CSTR reactor 38

4.2. Analytical methods 39

4.3. Microbial analysis 44

4.3.1. Fluorescence *in situ* hybridization (FISH) 44

4.3.2. 16S Amplicon sequencing analysis (Illumina MiSeq) 48

Chapter 5: Exploring the performance limits of a sulfidogenic UASB during the long-term use of crude glycerol as electron donor 49

Abstract 51

5.1 Introduction 53

5.2. Materials and methods 57

5.2.1. Experimental setup 57

5.2.2 Operating conditions and short-term experiments 57

5.2.3. Illumina sequencing analysis 59

5.2.4. Assessment of COD mass balances 59

5.3. Results 60

5.3.1. Long-term performance of the UASB and short-term experiments 60

5.3.2. Organic matter sink: sulfate reduction and biogas and VFA production 67

5.3.3. Illumina sequencing analysis and bacterial community assessment 68

5.4. Discussion 72

5.4.1 Start-up of a sulfate reducing UASB reactor 72

5.4.2 Shifts in the organic matter sink 73

5.4.3 Long-term UASB performance and microbial diversity changes 75

5.4.4 Potential process limitations 77

5.4.5 Overall performance of the sulfidogenic UASB 79

5.5. Conclusions 81

Chapter 6: Microbial evolution and interpretation of the microbial composition in a sulfidogenic UASB reactor 83

Abstract 85

6.1 Introduction 87

6.2. Materials and methods 90

6.2.1 Reactor set-up and operation (UASB long-term continuous operation)... 90

6.2.2. Illumina sequencing analysis 90

6.2.3. Fluorescence in situ hybridization (FISH) 93

6.3. Results 94

6.3.1. Long-term performance of the UASB at constant loading rate 94

6.3.2. Microbial evolution: 16S rRNA sequencing 98

6.3.3. Fluorescence in situ hybridization (FISH) 108

6.4 Discussion 116

6.4.1 Long-term performance and evolution in microbial populations 116

6.4.2 Different reactor heights and profiles 118

6.5 Conclusions 121

Chapter 7: Operational issues during the long-term performance of a sulfidogenic UASB: from success to failure 123

Abstract 125

7.1 Introduction 127

7.2 Materials and methods 130

7.2.1 Sampling of the slime substance 130

7.2.2. Chemical structure analysis by Fourier transform infrared spectroscopy (FTIR) 135

7.2.3 Scanning electron microscopy 136

7.2.4 FAMEs 137

7.2.5. Elemental analysis 138

7.2.6. 16S rRNA gene amplification of the slime 139

7.2.7. Bottle test with slime substance 140

7.3 Results 142

7.3.1. Comparison of long-term operations and limits of the system 142

7.3.2 Physical-chemical characterization of the slime 146

7.3.3. Biological characterization of the slime 157

7.3.4 Degradability of sulfate and glycerol by the slime.................................... 159

7.4 Discussion 162

7.5 Conclusions 169

Chapter 8: Microbial diversity study towards the improvement of sulfidogenesis in a UASB reactor through the bioaugmentation of acetotrophic-sulfate-reducing bacteria 171

Abstract.. 173

8.1 Introduction 175

8.2 Materials and methods 176

8.2.1 Inoculum source 176

8.2.2 Enrichment experiments 177

8.2.3 Serial dilution bottles 179

8.2.4 Microbial diversity analysis 180

8.3 Results 182

8.3.1 Inoculum source from the long-term UASB operation............................. 182

8.3.2 Assessment of sulfate reduction rates and microbial diversity of enriched cultures.. 183

8.3.3 Serial dilutions experiments 196

8.4 Discussion 200

8.5 Conclusions 204

Chapter 9: General conclusions and future work.. 207

9.1 General conclusions 209

9.2 Future work 211

Chapter 10: References 213

Chapter 1: Research motivations and thesis overview

1.1 Research motivations

This thesis has been conducted in the Department of Chemical, Biological and Environmental Engineering of the UAB, in the Research Group on Biological Treatment and Valorization of Liquid and Gas Effluents (GENOCOV) (www.genocov.com) within the projects "Desarrollo de un proceso integral de tratamiento de SOx y NOx procedentes de gases de combustión orientado a su valorización" (SONOVA) ref. CTQ2015-69802-C2-1-R, and "Enhanced treatment of flue gases in multistage bioscrubbers towards sulfur recovery" (ENSURE) ref. RTI2018-099362-B-C21 funded by Ministerio de Economia of Spanish government. This thesis started on 2016 with the initial goal of valorizing sulfate-rich wastewaters using biological processes in view of elemental sulfur recovery. To demonstrate the feasibility of the process, within the project framework different stages of the whole system were carried out at lab-scale in order to characterize the maximum capabilities of each of the stages and get valuable design data for different operating scenarios. In this thesis the sulfate reduction step was studied using crude glycerol as carbon source, as the potential of this waste organic effluent has been poorly explored in sulfidogenic reactors. Both the operational parameters and the microbial aspects of the operation were addressed in order to obtain a more complete view of the process.

1.2 Thesis overview

This thesis is divided in ten chapters. In the present chapter (**Chapter 1**) the motivations of this thesis and the thesis overview are presented. In **Chapter 2**, the general introduction to the research topic is presented. Information about the sulfur cycle, covering chemical, microbiological and technological aspects of the treatment of sulfur rich streams, is presented. Bioreactors most commonly used in anaerobic digestion are presented, focusing on those types used for sulfate

reduction. A small overview of the different available techniques for microbial identification in anaerobic bioreactors is also shown. This information facilitates the understanding of the following chapters and gives an overview of the different aspects that will be discussed among the different chapters of results.

Chapter 3 states the main objective of the thesis, as well as the specific objectives derived from it. **Chapter 4** describes the general materials and methods used during the experimental work of this thesis; specific materials and methods used in results chapters (Chapters 5 to 8) are described in the corresponding chapter where they were used. **Chapter 5** presents the investigation on the effect of the long-term operation of an up-flow anaerobic sludge bed (UASB) reactor for sulfate reduction using crude glycerol as electron donor, exploring the performance limits of the operation. The investigation focused on the feasibility and constraints of the use of crude glycerol as electron donor, evaluating sulfate and COD removal efficiencies. Moreover, the organic matter sink was calculated as a way of reporting the potential use of COD for sulfate reduction. The knowledge obtained from Chapter 5 is applied in **Chapter 6** to operate once more the sulfidogenic UASB reactor under steady conditions, constant sulfate and organic loading rates. The biomass developed was characterized to assess the microbial evolution and to relate physical and chemical data with microbial diversity and composition. Biomass samples were collected along the whole operation, including the inoculum, to gain more knowledge through molecular biology-based analysis (Illumina sequencing and FISH analysis).

Chapter 7 highlights the operational issues encountered during the long-term operation of both reactors (Chapters 5 and 6). Decrease of sulfate reduction efficiency and excess of TOC present in the effluent, mainly as acetate, are discussed. A further and thorough characterization of the biomass itself is carried out to gain more knowledge about the formation of a slime substance inside the reactor associated with the failure of the long-term operation in both cases.

Chapter 8 approaches the possible improvement of sulfidogenesis related to the acetate uptake by sulfate reducing bacteria (SRB). The cultivation and enrichment of bacteria able to use acetate coupled to sulfate reduction was performed during a research stay at the department of microbiology (Wageningen University) under the supervision of Dr. Irene Sanchez Andrea. An attempt to isolate and characterize the acetate and sulfate-consumers was performed and the possibility and evaluation of their biostimulation in the UASB reactor is discussed. In **Chapter 9** the conclusions extracted from the results obtained in previous chapters are exposed together with the future perspectives of this research field. Finally, **Chapter 10** contains the references used along the thesis.

Chapter 2: Introduction

2.1 Biological sulfur cycle

Sulfur is a pale yellow, odorless and insoluble chemical element with symbol S and atomic number 16. Sulfur is one of the most abundant elements on Earth, mainly present in sediments and rocks in the form of sulfate minerals (gypsum, $CaSO_4$), sulfide minerals (pyrite, Fe_2S) and sulfur deposits (S^0), which have been formed in different geological periods. However, the most significant reservoir of sulfur for the biosphere is in the oceans, in the form of dissolved inorganic sulfate (Muyzer and Stams, 2008).

Biogeochemical cycles represent the motion and the conversion of matter due to the biochemical activities in the ecosystems. There are biogeochemical cycles for the chemical elements: calcium, carbon, hydrogen, mercury, nitrogen, oxygen, phosphorous, selenium and sulfur. The sulfur cycle is more complex than the nitrogen or carbon cycles. The complexity is hidden behind the greater number of sulfur redox states (Walter K. Dodds and Matt R., 2017). Figure 2.1 shows the variety of oxidation states that results from the sequential transformation reactions involving sulfur. Elemental sulfur is the chemical state of sulfur when the valence is 0 and it is normally found in cyclic octatonic molecules (S_8). The other most abundant forms of S present in nature are sulfide (oxidation state -2 or completely reduced) and sulfate (oxidation state +6 or completely oxidized).

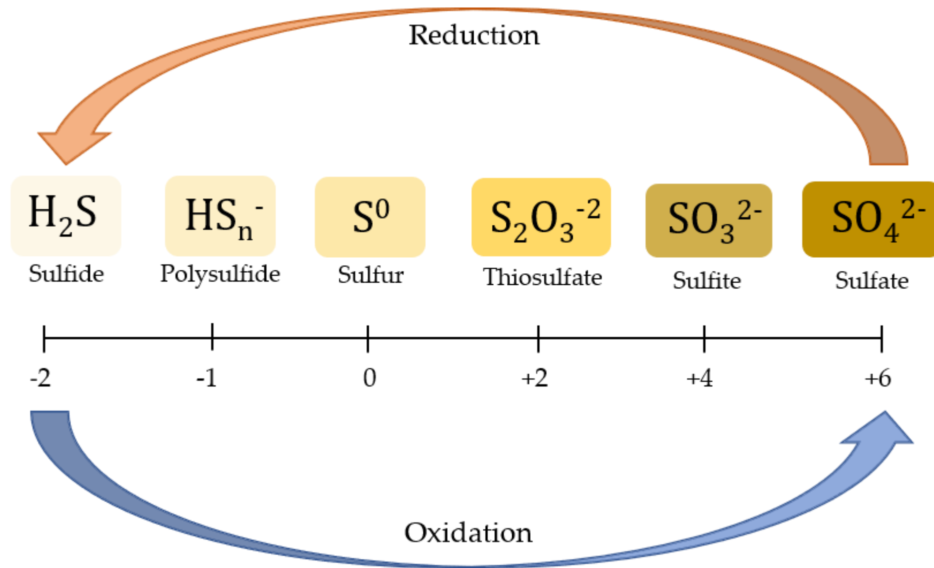


Figure 2.1. Main chemical species of sulfur with their corresponding oxidation states. The upper arrow indicates the reduction path of sulfur compounds leading to the formation of sulfide. The formation of sulfate through oxidation reactions is displayed in the lower part.

It is important to note that H_2S is the last sulfur compound of the reduction path. It is a volatile, toxic, corrosive, malodorous compound that causes an impact on the chemistry of the environment, also due to its reactivity with metals. Furthermore, it can be used by different microorganisms as an electron donor coupled to either oxygen, nitrate or iron reduction (Rabus et al., 2013). Therefore, the formation of this volatile, dissolved or gaseous, end-product is not desirable in the sulfur cycle, contrarily to what happens in both carbon and nitrogen cycles, for example. In those cases, the production of, respectively, CO_2/CH_4 and N_2 is the common method applied for the removal of C and N from liquid streams. Even its toxicity, it has a key role in essential intracellular organization; as an example, it has been proposed as 'the third signaling gas' in mammalian physiology (Kimura et al., 2005).

2.2 Gaseous effluents and wastewaters containing sulfur compounds

Since the Industrial Revolution, the increasing amount of human activities, specially through the combustion of fossil fuels and the processing of metals, have contributed to the amount of sulfur entering into the atmosphere. Combustion of sulfur-containing fuels, such as coal, natural gas, peat, wood and oil, results in SO_2 formation mainly generated in the energetic and industrial sectors (Klimont et al., 2013). One-third of all sulfur reaching the atmosphere, including 90 % of SO_2, comes from human activities. Emissions from these activities, along with nitrogen emissions, react with other chemicals in the atmosphere to produce tiny particles of sulfate salts which fall as acid rain and can cause health impacts, acid deposition in the environment and visibility depletion (EPA, 2019).

Flue gases are usually treated through physical-chemical processes. One of the most implemented treatment to remove SO_2 from flue gases is wet flue gas desulfurization (WFGD). However, these processes are expensive (due to the use of alkaline absorbents) and generate additional effluents requiring further processing and energy inputs (Srivastava and Jozewicz., 2001; Philip and Deshusses., 2003). Apart from the effluents coming from flue-gas scrubbing, several wastewater streams contain sulfate, sulfite or other sulfur compounds. Typical wastewaters containing sulfur are those produced in industries that use sulfuric acid as a cheap and strong acid or sulfite or dithionate as either bleaching or preserving agent in the production process, such as food production. Other examples of industrial wastewaters containing high concentrations of sulfate are those from the fermentation-based, pharmaceutical and mining industries, edible oil or pulp and paper industries (O'Flaherty et al., 1999a; Kaksonen and Puhakka, 2007; Lens and Pol, 2015).

The development of environmentally friendly alternatives to valorize not only SO_2 from WFGD but also S-rich liquid effluents is clearly needed. The most applied technologies are the SANI® process and the SULFATEQ® process. The SANI® process has been implemented at full-scale with excellent results (Jiang et al., 2013). However, sewage and nitrogen sources are required to valorize S-containing effluents. The power of the SULFATEQ® process (Paques B. V., The Netherlands) relies in its ability to simultaneously remove sulfate and recover metals such as copper, nickel and zinc as metal sulfides. Nevertheless, as reported in Schröder-Wolthoorn et al. (2008), a second reactor is needed to partially oxidize sulfide to elemental sulfur under metal limiting conditions to oxidize the exceeding sulfide. The two-stage bioscrubber concept described in Mora et al. (2020) is a promising integrated process to recover elemental sulfur as a value-added product. The process consists of a first scrubbing stage of SO_2 using slightly alkaline absorbents, followed by two stages: the biological reduction of sulfite and/or sulfate to sulfide and the partial oxidation of sulfide to elemental sulfur. This thesis is specially focused on the first biological stage of this process in which the reduction of sulfate to sulfide is involved. In particular, sulfate reduction using an organic waste (crude glycerol) as carbon source and electron donor is studied under different operational conditions. The implementation of a valorization-based process as the one proposed in Mora et al. (2020) pursues treating wastewater much more efficiently in terms of energy consumption. Nowadays, the transition towards a circular economy is becoming a priority in the EU and it can be an opportunity to promote the conversion of wastes to value-added products; and therefore, to enhance the efficiency of resource utilization. In the actual bio-economy era, the establishment of the concept of circular economy would expand and diversify the market of bio-based products as bio-based chemicals, biopolymers, fuels, and bioenergy (Maina et al., 2017).

In general, one of the most implemented application of S-rich effluents valorization is to treat acid mine drainage (AMD) since this type of waste contains metals, commonly precious or heavy metals, and sulfate. Even so, the recovery of elemental sulfur from S-rich streams has gained interest because it can be used in vulcanization, rechargeable batteries and thiol coupling reactions (Boyd, 2016) and also, as a novel feedstock to prepare chemically stable copolymers (Chung et al., 2013). Elemental sulfur is currently obtained from the petrochemical industry, so, its recovery has a strong potential from a sustainable and environmental point of view. The sulfur fertilizers market is projected to reach USD 6.29 billion by 2022, which is driven by increase in demand for higher agricultural productivity and reduction in sulfur emission influencing the demand for added sulfur. Other possible application for the recovered elemental sulfur, could be the pigments industry, which utilizes a wide diversity of chemical compounds, in innumerable formulations. In particular, to manufacture products such as Ultramarine blue and Prussian blue pigments, sulfur and other additives, such as natural clay or copper, respectively, are required (Eastaugh et al., 2007). The Global pigments market size was valued at 20023 million USD in 2015 registering a Compound Annual Growth Rate (CAGR) of 4.1 % during the forecast period 2022 (Nathwani et al., 2016).

2.3 Anaerobic reactors for sulfate-rich effluents valorization

Under anaerobic conditions, organic matter is degraded in several steps (Figure 2.2). Several trophic groups (including fermentatives, acetogens, methanogens and sulfate-reducing bacteria) need to cooperate to carry on these reactions (Colleran et al., 1995). Sulfate reducers hardly ever oxidize complex organic compounds such as lipids, proteins or carbohydrates (Postgate, 1984). This is the reason why synergism among different groups of microorganisms is

necessary to produce substrates that sulfate-reducing bacteria (SRB) can further use (Tuttlee et al., 1969).

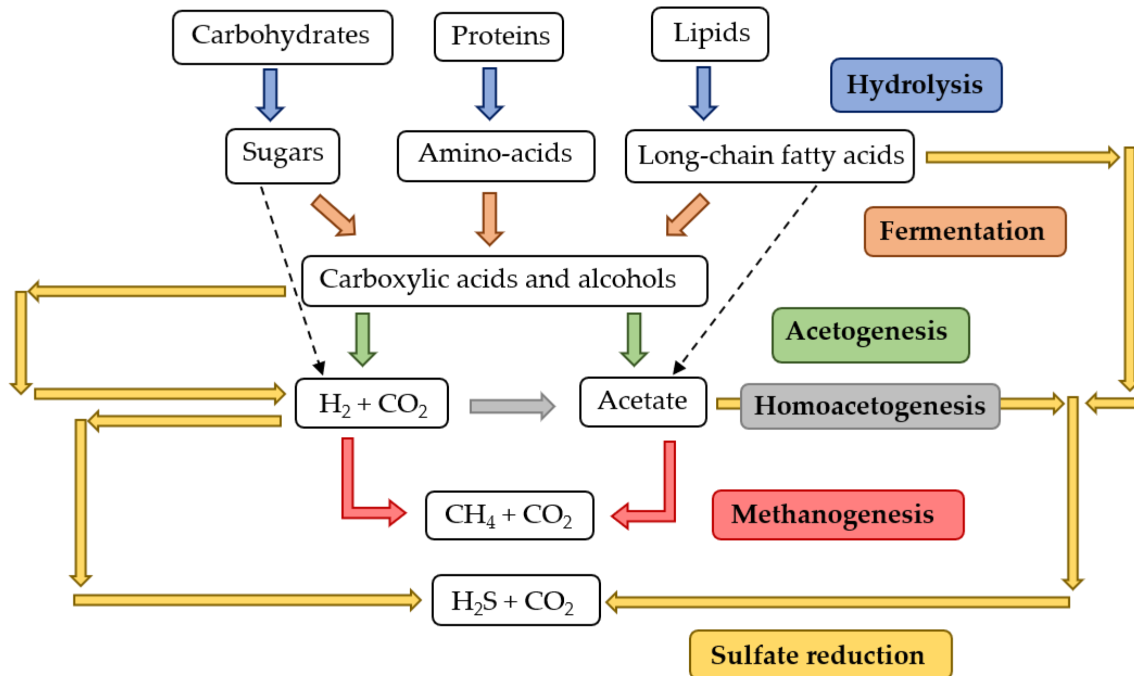


Figure 2.2. Anaerobic degradation of organic compounds in the presence of sulfate (adapted from Visser, 1995).

The presence of biodegradable components coupled with the advantages of the process over other treatment methods, makes anaerobic digestion a convenient alternative when treating high strength organic effluents (Rajeshwari et al., 2000). The advantages of anaerobic biological wastewater treatment for most organic wastewaters have been discussed by sev eral authors (Lettinga, 1996; Rajeshwari et al., 2000; Mchugh et al., 2003). The development of reactors for anaerobic treatment has gained considerable attention in recent years. Important progresses have been made in the development of high rate reaction systems for the conversion of organic molecules into biogas. The development of biosystems, such as the up-flow anaerobic sludge bed (UASB) reactor, is based on the concept of retaining high viable biomass by uncoupling sludge retention time from liquid retention time. This approach has allowed treating a wide

variety of industrial or domestic wastewaters around the world becoming a proven, mature and sustainable technology (Lettinga, 1995; Mccarty, 2001).

2.3.1 Sulfate reduction in sulfidogenic bioreactors

Anaerobic treatment of wastewaters containing significant amounts of sulfate present a challenge due to competition between sulfidogenesis and methanogenesis. Sulfate reduction during the anaerobic treatment of wastewaters is generally an unwanted process because of reduction in methane yield and problems of corrosion and toxicity caused by H_2S. The production of hydrogen sulfide (H_2S) by SRB can be toxic to the various trophic groups of bacteria involved in the process (Oude Elferink et al., 1994). Therefore, research efforts have been commonly focused on its negative role in anaerobic wastewater treatment, studying H_2S toxicity and competition between methanogens and sulfate reducers aiming to suppress sulfidogenesis (Rinzema and Lettinga, 1988). More recently, the potential of sulfidogenesis to treat a wide range of waste streams contaminated with sulfur compounds and/or heavy metals has gained attention. To what extend sulfate reduction will predominate over methanogenesis depends on many factors, including the organic substrate to sulfate ratio (COD/SO_4^{2-}) of the wastewater and the type of organic substrate used, among others.

In process streams with high sulfate concentrations, sulfate can be removed by SRB. Sulfate can act as an external electron acceptor for SRB, which couples the oxidation of organic or inorganic intermediates in the anaerobic degradation to the reduction of sulfate (Colleran et al., 1995). This process (the reduction of sulfate to sulfide) is called dissimilatory sulfate reduction when it is coupled to energy conservation and growth (Sánchez-Andrea et al., 2014). An electron donor is required for this process, which should be added when the organic content of the waste or process water is insufficient.

2.3.2 Electron donor and carbon source for sulfate reduction

If organic material is oxidized via sulfate reduction, 8 electrons can be accepted per molecule of sulfate. Because one molecule of oxygen can only accept 4 electrons, the electron accepting capacity of 2 mols of O_2 equals 1 mol of SO_4^{2-}. This means, 0.67 g of O_2 per g SO_4^{2-} (Lens et al., 1998b). Hence, the theoretical ratio of COD/ SO_4^{2-} is 0.67, which means 1 g of sulfate reduced can remove 0.67 g COD. Considering this, for waste streams with a COD/sulfate ratio of 0.67, there is enough sulfate available to completely remove the organic matter via sulfate reduction. As reported by Omil et al. (1998), methanogenesis is necessary, in addition to sulfate reduction, to achieve a complete removal of the organic matter for wastewaters with COD/sulfate ratios exceeding 0.67. For wastewater that contains any or insufficient electron donor and carbon source for a complete sulfate reduction, the addition of an electron donor is required.

Sulfate reduction, which is catalyzed by SRB (Liamleam and Annachhatre, 2007), can be carried out with a wide range of different compounds acting as electron donors for dissimilatory sulfate reduction. Sulfate reduction can be performed autotrophically, when the electron donor is H_2, or heterotrophically, using organic matter as electron donor. Electron donors can be classified in three groups: waste streams containing organic matter, bulk chemicals (relative concentrated, pure liquids or dissolved salts) and gaseous electron donors (Figure 2.3). Among others, SRB are able to use alcohols, short chain fatty acids or H_2.

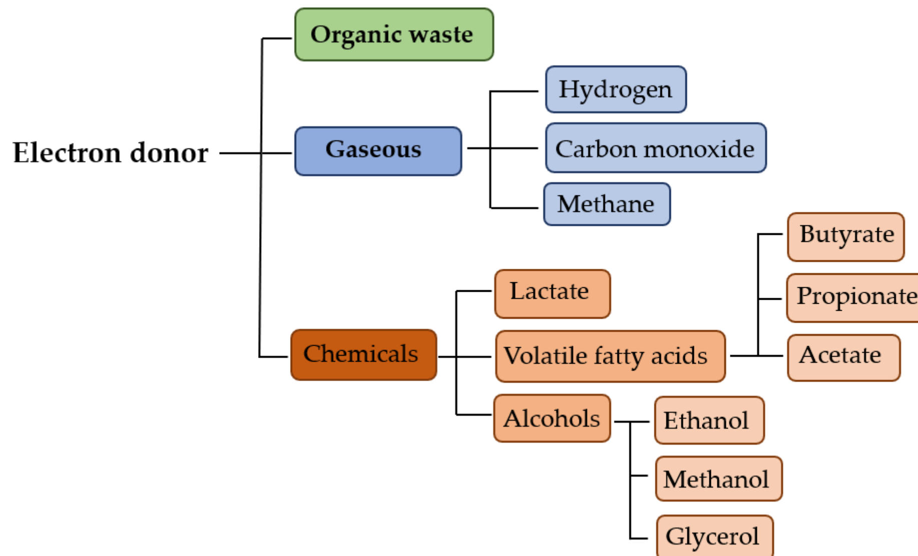


Figure 2.3. Electron donors for sulfate reduction (modified from Bijmans et al. (2011).

When comparing electron donors to choose the most suitable one, several aspects need to be considered: the price of the electron donor, transport and storage costs, the purity of the available electron donor and its suitability for a specific waste or process (depending on its volume, composition, temperature and salinity). As an example, Weijma et al. (2000a) used methanol in lab-scale expanded granular sludge bed (EGSB) reactors; Kimura et al. (2006) selected glycerol as a suitable electron donor to work at low pH (3.8); Weijma et al. (2002) used H_2/CO_2 to study the competition between sulfate reducers and methanogens. Lactate is usually selected in microbial studies as model substrate but becomes too expensive in full-scale operations (Hard et al., 1997; El Bayoumy et al., 1999).

The use of waste streams with organic content, as an electron donor source, would be the cheapest and most sustainable option. However, the sort of this waste is not usually constant in quality and quantity and cannot be available throughout the year. Aiming to find a cheap and appropriate carbon source for sulfate reduction, Mora et al. (2018) performed screening tests with different waste organic sources (obtained as byproducts or waste effluents from industrial

processes) to reduce sulfate heterotrophically. In that study, crude glycerol was found as a competitive substrate to reduce high loads of sulfate to sulfide in batch tests as well as during the start-up of an up-flow anaerobic sludge blanket reactor.

Crude glycerol is a COD-rich by-product produced in large quantities from the soap and biodiesel manufacturing processes (Dinkel et al., 2010), that does not require any additional treatment before its use as carbon source. Several studies can be found among the literature about chemical and/or biological conversion of crude glycerol into more valuable products (Yazdani and Gonzalez, 2007). However, in most of the recent research, crude glycerol has been used either as a suitable substrate for biogas production in anaerobic systems (Siles López et al., 2009; Nakazawa et al., 2015) or as co-substrate to increase biogas production (Nghiem et al., 2014; Athanasoulia et al., 2014). On the contrary, the use of crude glycerol to valorize sulfate-rich effluents has been poorly explored in sulfidogenic reactors. Several researchers have studied the use of crude glycerol to generate sulfide and recover precious metals, immobilize toxic metals, or simply to study and get knowledge about the competition between methanogens and sulfate reducers (Bertolino et al., 2014; Santos et al., 2018). However, to the best of my knowledge, none of them targets the utilization of crude glycerol to assess the long-term performance of a sulfidogenic UASB, which will be studied in this thesis besides the obtention of elemental sulfur, a valued compound, actually obtained from non-renewable resources.

2.3.3 Types of bioreactors used for sulfate reduction

As a consequence of the increasing interest in applying biological processes for sulfate containing-wastewater treatment, many different designs of bioreactors have been developed (Speece, 1983; Hulshoff Pol et al., 2001; Lens et al., 2002; Kaksonen and Puhakka, 2007). Sulfate reduction frequently occurs

spontaneously during anaerobic wastewater treatment whenever sulfate is present. Therefore, bioreactor configurations commonly used in methanogenic wastewater treatment are adopted also in sulfate reduction processes, for example UASB reactors. Figure 2.4 presents several reactor configurations used for the biological reduction of sulfate, each kind of reactor configuration providing its own flexibility in terms of operation and efficiency.

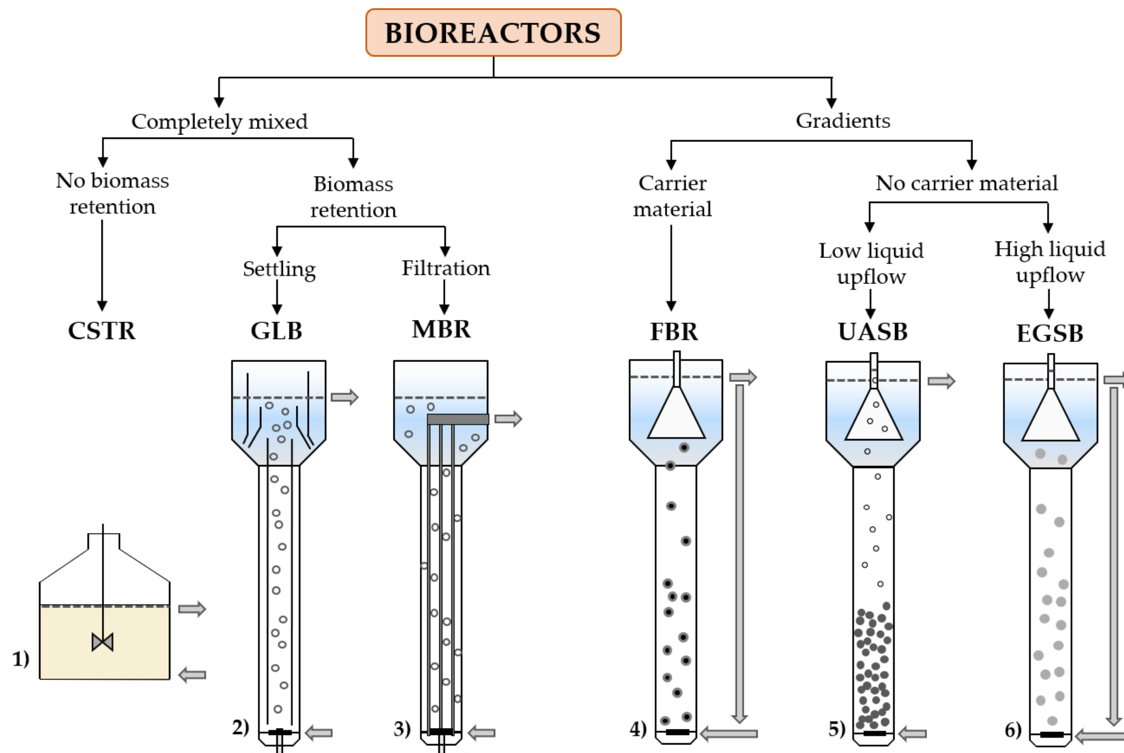


Figure 2.4. Reactor types used for sulfate reduction: 1) continuous stirred tank reactor (CSTR); 2) gas-lift bioreactor (GLB); 3) submerged membrane bioreactor (MBR); 4) fluidized bed reactor (FBR); 5) up-flow anaerobic granular sludge bed (UASB) reactor and 6) expanded granular sludge bed (EGSB) reactor. Modified from Bijmans et al. (2011).

Focusing on UASB reactors, their design was originally developed for methane production from highly concentrated organic wastewater (Hulshoff Pol et al., 2001). In this kind of reactors, biomass retention is based on good settling characteristics of sludge (frequently granular), so no packing or carrier material

is usually needed and there are less problems with clogging. Therefore, start-up and operating costs are appreciably lower if compared to fluidized bed reactors (FBR), for example. The settling efficiency of the biomass is the most important peculiarity of UASB reactors. Microorganisms form a granular sludge-bed and the influent passes through it (Lettinga et al., 1980; Omil et al., 1996). However, as biomass retention depends on granulation and sulfate reducers do not granulate as easily as methanogens, UASB reactors are more suitable to produce methane instead of sulfide. For that reason, many UASBs have been used to study the effect of sulfate reducers in methanogenic reactors, such as those treating synthetic paper mill wastewater (Sipma et al., 1999; Lens et al., 2003). Therefore, the main disadvantages of UASB reactors are the washing out of the biomass during process failures and the high susceptibility to changes in the influent quality if compared to other bioreactor typologies (Jhung and Choi, 1995).

2.4 Sulfate reducing bacteria (SRB)

Sulfate-reducing prokaryotes (SRP) constitute a heterogeneous group of bacteria and archaea capable of using sulfate as terminal electron acceptor during anaerobic respiration. This group can be divided into four groups according to rRNA sequence analysis: Gram-negative mesophilic SRB; Gram-positive spore forming SRB; thermophilic bacterial SRB; and thermophilic archaeal SRB (Castro et al., 2000). In literature, sulfate-reducing prokaryotes have been classified according to many other different properties, including cell shape, electron transfer proteins, guanine cytosine content of DNA, optimal growth temperature and capability to oxidize acetate (Akagi, 1995; Chen et al., 1995; Widdel, 1988). According to optimal growth temperature, most SRB identified are mesophilic. However, Knobtauch et al. (1999) and Jeanthon et al. (2002) also described thermophilic, hyperthermophilic and psychrophilic species. Another classification criterion for SRB important along this thesis, is their ability to

oxidize acetate. Based on this criterion, SRB fall into two categories: those species able to oxidize organic compounds to CO_2, and those that carry out incomplete oxidations, usually with acetate as end-product (Widdel et al., 1988). The genera in the first group include: *Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, *Desulfosarcina*, *Desulfomonile*, *Desulfonema*, *Desulfoarculus* and *Archaeoglobus*. Incomplete oxidizers include *Desulfomicrobium*, *Desulfobulbus*, *Desulfobotulus*, *Thermodesulfobacterium* and most species of the genera *Desulfovibrio* and *Desulfotomaculum* (Widdel et al., 1992). However, these are purely physiological or functional groups that overlap only partly with molecular systematic groups (Rabus et al., 2013).

SRB are important microorganisms not only for the sulfur cycle, but as regulators of a variety of processes in wet-land soils. They are involved in organic matter turnover, biodegradation of chlorinated aromatic pollutants in anaerobic soils and sediments and in mercury methylation, among others (Barton and Tomei, 1995). Most of the described species of SRP are bacteria, therefore throughout this thesis SRP will be simply named SRB.

2.4.1. Effect of sulfide on SRB

The state of sulfide only depends on the pH of the environment. Figure 2.5 presents the prevalent forms of sulfide according to the pH. In a pH ranging from 6 to 8, sulfide exists as a mixture of HS^- and H_2S. At pH lower than 6, undissociated hydrogen sulfide (H_2S) becomes the dominant sulfide species. The relation between the concentrations of undissociated hydrogen sulfide in the liquid and gas phase is based on Henry's law and is governed by equations 2.1 and 2.2

$$H_2S \rightarrow HS^- + H^+ \quad (2.1)$$

$$HS^- \rightarrow S^{2-} + H^+ \quad (2.2)$$

Information available on sulfide toxicity and the mechanism of toxicity is frequently ambiguous. It has been reported that the undissociated sulfide molecule is absorbed into the cell and destroys the bacterial proteins thereby making the cell inactive (Speece, 1983; Postgate, 1984). If this is the case, bacteria should not be able to restart its activity once sulfide is removed. By contrast, it has also been reported that sulfide inhibition is reversible, and the normal cell growth and sulfate reduction rates are attained as soon as sulfide is removed from inoculated bioreactors (Reis et al., 1992; Okabe et al., 1992; Maillacheruvu and Parkin, 1996).

Apart from the undissociated H_2S, it has been shown that total sulfide can be also inhibitory for bacteria depending on the environmental pH. Therefore, two threshold inhibition levels can be considered. At pH lower than 7.2, undissociated H_2S is dominant and it will reach the threshold limit. Above this pH of 7.2, the total sulfide is responsible for the inhibitory effect (O'Flaherty and Colleran, 1999b). In addition to the uncertainty and contradictory information among the literature with respect to inhibitory mechanisms of sulfide, contradictory reports exist with respect to inhibitory effects of various forms of sulfide (Sheoran et al., 2010). Therefore, it is not easy to compare the inhibitory/toxic values reported in different studies, as the inhibition has been assessed based on growth, substrate degradation, sulfate reduction or cellular yield.

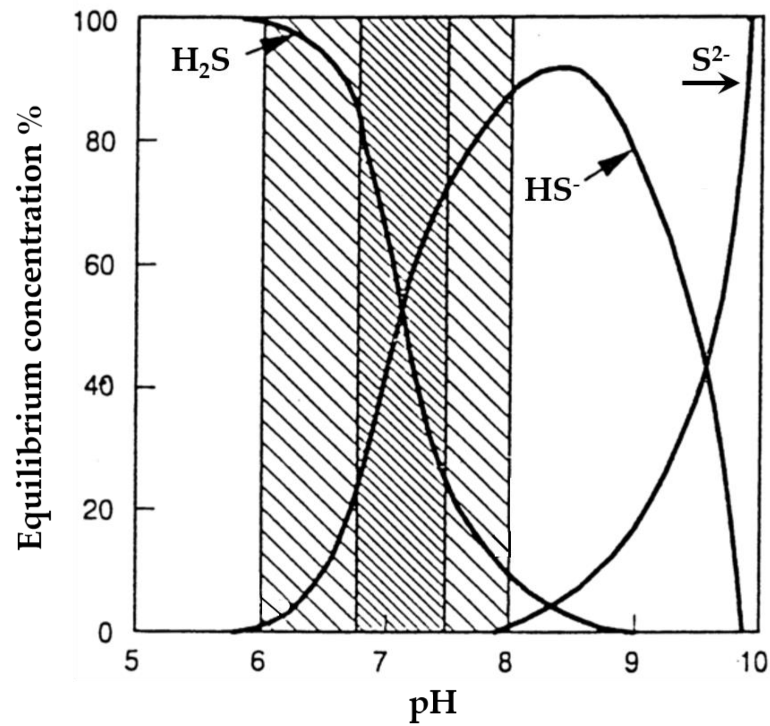


Figure 2.5. Prevalent forms of sulfide at different pH values (adapted from Rintala and Puhakka, 1994).

2.4.2 Competition between sulfate reducers, methanogens and acetogens

In anaerobic environments where sulfate is present, SRB compete with other anaerobes, including fermentative bacteria, acetogenic bacteria and methanogens for the available common substrates. Some important conversions are listed in Table 2.1.

Table 2.1. Sulfate-reducing, methanogenic and acetogenic reactions.

Equation	$\Delta G^{0'}$ (kJ/reaction)*
Sulfate-reducing reactions	
$4\ H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\ H_2O$	-151.9
$Acetate^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$	-47.6
$Propionate^- + 0.75\ SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.75\ HS^- + 0.25\ H^+$	-37.7
$Butyrate^- + 0.5\ SO_4^{2-} \rightarrow 2\ Acetate^- + 0.5\ HS^- + 0.5\ H^+$	-27.8
$Lactate^- + 0.5\ SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.5\ HS^-$	-80.2
Acetogenic reactions	
$Propionate^- + 3\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3\ H_2$	+76.1
$Butyrate^- + 2\ H_2O \rightarrow 2\ Acetate^- + H^+ + 2\ H_2$	+48.3
$Lactate^- + 2\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2\ H_2$	-4.2
Methanogenic reactions	
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	-135.6
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0
Homoacetogenic reactions	
$4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow Acetate^- + 4\ H_2O$	-104.6
$Lactate^- \rightarrow 1.5\ Acetate^- + 0.5\ H^+$	-56.5

*Data from (Thauer et al., 1977)

Compared to methanogens, SRB are much more versatile. Methanogens use a limited number of substrates for growth, being hydrogen, carbon dioxide and acetate the most important and best-known. Therefore, compounds such as lactate, propionate and butyrate, which are common substrates for sulfate reducers, require syntrophic communities to form products that are used by methanogens (Muyzer and Stams, 2008). Kinetic properties of SRB, methanogens and acetogens can be used to predict the outcome of the competition for these common substrates (Lovley et al., 1982; Kristjansson et al., 1982).

When sulfate is present, sulfate reducers compete with methanogens for the common substrates hydrogen and acetate and with syntrophic methanogenic communities (Stams et al., 2003). In the case of hydrogen, both thermodynamic and kinetic data predict that hydrogen-utilizing methanogens and homoacetogens are easily and rapidly outcompeted by hydrogen-utilizing SRB

(Robinson and Tiedje, 1984). On the other hand, literature data on the outcome of competition for acetate in anaerobic reactors are contradictory. Several studies have found that acetate is completely converted into methane even in the presence of excess sulfate (Isa et al., 1986; Yoda et al., 1987; Parkin et al., 1990). However, others have found that acetotrophic methanogens could be outcompeted by sulfate reducers (Alphenaar, 1994; Uberoi and Bhattacharya, 1995). In the competition for the substrates available in anaerobic environments, SRB are generally favored if considering thermodynamic and kinetic values considerations (Colleran et al., 1995). Even so, in practice, many other factors may significantly affect the outcome of the competition. Some of these factors are substrate composition and concentration, pH, temperature, type of reactor and biomass, differential sulfide toxicity, trace metals and other nutrients, etc. (Patidar and Tare, 2005). The composition of the influent COD to sulfate ratio (COD/SO_4^{2-}) contained in the wastewater to be treated is a key parameter on the competition for the electron flow between different microbial communities (Mccartney and Oleszkiewicz, 1993). In theory, all COD can be degraded via sulfate reduction if the ratio is below 0.67. Nevertheless, considerable differences among the values reported in the literature can be found (Choi and Rim, 1991; Mizuno et al., 1994; Annachhatre and Suktrakoolvait, 2001). Although an overall understanding of bioprocesses, like sulfidogenesis and methanogenesis and the competition between SRB and methanogens, has been studied (Mccartney and Oleszkiewicz, 1993), our understanding of its influence on the diversity and dynamics of microbial communities is still limited. To overcome these drawbacks, molecular techniques have provided alternative approaches to overcome the limitations associated with the traditional microbiological techniques (Dar et al., 2008).

2.5 Techniques for microbial identification in anaerobic bioreactors

The major microbial processes that take place in anaerobic bioreactors, such as methanogenesis, sulfidogenesis and acetogenesis, are nowadays well understood. However, there are still many unanswered questions when discussing the diversity and dynamics of the microbial communities responsible for these processes. This is because microbial communities in large-scale biotechnological processes, such as wastewater treatment facilities, have been considered as a "black box". But, since the mid-1980s, the application of molecular biological methods to study the diversity and ecology of microorganisms in natural and engineered environments has been practiced (Head et al., 1998). Limitations of traditional identification and enumeration techniques, such as selective enrichment, pure culture isolation and most probable number estimates, have also helped in the development of new and specific analytical tools, currently available. As reported by Amann et al. (1995) the majority of microscopically visualized cells are viable but do not form viable colonies on plates, so direct microscopic counts is not precise and cannot be fully trusted. It has been also estimated that more than 99 % of microorganisms observable in nature cannot be typically cultivated using standard techniques (Hugenholtz et al., 1998). These problems are even more heighten in studies of anaerobes because of their low growth rates. SRB and methanogens are among the microorganisms that are most difficult to study through culture-based techniques. Fortunately, to study the structure and functioning of sulfate reducing bacteria, molecular techniques have provided alternative procedures to overcome the abovementioned problems associated with culture dependent analysis of complex microbial communities (Amann et al., 1995).

Molecular techniques that can be widespread applied in studies on natural SRB communities include: membrane lipid analysis, immunodetection denaturing or thermal gradient gel electrophoresis (DGGE and TGGE), single strand conformation polymorphism (SSCP), Fluorescence in situ hybridization (FISH), DNA microarrays (Amann et al., 1995; Dabert et al., 2002; Friedrich, 2002) and polar-lipid fatty acid biomarkers (Oude Elferink et al., 1998a). In general, these molecular techniques give more information than chemical methods as for example quinone profiles (Kurisu et al., 2002).

Fluorescence in situ hybridization (FISH)

Using rRNA based probes, the presence of a specific species or a group of bacterial species, in a particular type of granule or biofilm, can be detected. Moreover, if 16S rRNA probes are fluorescently labeled, bacteria can be spatially localized within the granule or biofilm. DeLong et al. (1989) reported for the first time the suitability of fluorescently labeled rRNA-targeted oligonucleotide probes for the identification of microorganisms. DeLong et al. (1989) highlighted the importance of this technique for the study of natural microbial populations, where unknown, often uncultivatable organisms are routinely encountered. Use of rRNA-targeted oligonucleotide probes has become very important in microbial ecological studies including studies focusing on identification and abundance of SRB. Examples in which FISH has been used can be found in the literature (Amann et al., 1992; Manz et al., 1998; Boetius et al., 2000). However, 16S rRNA probes will show the presence of a bacterium but will not always reflect its metabolic activity. Therefore, FISH has been used in combination with many different techniques, such as microelectrodes/microsensors (Ramsing et al., 1993; Santegoeds et al., 1999) or microautoradiography (MAR-FISH) (Ito et al., 2002) to elucidate the ecophysiology of identified SRB.

The essence of FISH is that, fluorescently labeled probes allow the direct identification and specific visualization of microbial populations directly in their natural and complex environment. FISH not only provides insight into microbial community structure, but relative or even absolute numbers of visualized cells can also be determined (Glöckner et al., 1999; Daims et al., 2001). FISH has been used for the identification of SRB in different environments. Manz et al. (1998) monitored the relative abundance and the spatial organization of SRB in activated sludge flocs from a large municipal wastewater treatment plant. Activated sludge reactors at laboratory scale using acetate and peptone as the artificial wastewater were used by Miyazato et al. (2006) to monitor the growth of sulfate reducing bacteria (SRB) and filamentous sulfur bacteria. Bade et al. (2000) applied FISH and digital image analysis to investigate the response of SRB to oxygen stress under oligotrophic conditions in particle-free systems in sterile Berlin drinking water, mineral medium and in co-culture experiments with aerobic bacteria. Molecular techniques together with microsensors (for H_2S and CH_4) was the approach selected in Santegoeds et al. (1999) to study the population structure and the activity distribution in anaerobic aggregates from different reactors (a methanogenic reactor, a methanogenic-sulfidogenic reactor and a sulfidogenic reactor).

16S rRNA sequencing for microbial community analysis

Among the different rRNA molecules of ribosomal RNAs: 5S, 16S (Prokaryotes) or 18S (the counterpart to 16S rRNA in Eukaryotes) and 23S, the most commonly used as a phylogenetic marker in the field of microbial taxonomy is 16S rRNA gene (Stackebrandt and Goebel, 1994; Konstantinidis and Tiedje, 2005; Konstantinidis and Tiedje, 2007) as the other two, 5S and 23S, are very small (120bp) and large (~3,300bp) in size, respectively (Raina et al., 2019). Therefore, the use of 16S rRNA gene sequences has become one of the most common molecular genetic marker to study bacterial phylogeny and taxonomy.

Several reasons can be mentioned, including: (1) its presence in almost all bacteria, often existing as a multigene family; (2) the function of 16S rRNA gene has remained constant over a long period, suggesting that sequence changes are more likely to reflect random changes (a more accurate measure of time) than selected changes, and (3) the length of this gene (~1,500bp) is appropriate for bioinformatic purposes (Janda and Abbott, 2007). Moreover, it contains several regions of highly conserved sequences useful to obtain proper sequence alignments, but also other regions with enough sequence variability to serve as excellent phylogenetic molecular clocks.

The analysis of small subunit ribosomal RNAs provide knowledge of the evolutionary relationship of microorganisms that allow grouping and identifying microorganisms through sequences of the obtained genes (Woese, 1987). Many sequences are nowadays available for the 16S rRNA gene (16S rDNA). More than 200,000 bacterial sequences are available in GenBank, which comprises the DNA DataBank of Japan (DDBJ) (Miyazaki et al., 2004), the European Molecular Biology Laboratory, UK (EMBL) (Kulikova et al., 2004) and GenBank at National Center for Biotechnology Information, USA (NCBI) (Benson et al., 2005). Sequence analysis is conducted by comparing the obtained sequences of the sample with the available sequences in databases. Therefore, information about the identity or relatedness of the new sequences is compared to that of known species. However, even if this technique is quite useful and provides a quite satisfactory framework for prokaryotic phylogeny, classification at species level is usually not possible (Heuer et al., 1997). Rosselló-Mora and Amann (2001) reported several situations in which 16S rRNA gene sequence data cannot provide absolute resolution to taxonomic issues: 1) when different species with identical or nearly identical 16S rRNA sequences are present; 2) if there is micro heterogeneity of the 16S rRNA genes within a single species, or 3) when two or more 16S rRNA genes with relatively high sequence divergence are found

in one organism. Considering all the drawbacks mentioned, the bacterial species definition can never be solely based on sequence similarity of rRNAs. Even so, comparative analysis of 16S rRNA may be highly adequate for a first phylogenetic affiliation approach of both potentially novel and poorly classified organisms (Rosselló-Mora and Amann, 2001). The use of functional genes could be also a powerful approach in the detection of microorganisms such as SRB. For example, genes which encode enzymes that play an important role in the sulfate-reduction pathway, such as dsrAB, which encodes the dissimilatory sulfite reductase (Wagner et al., 1998), or aprBA, which encodes the dissimilatory adenosine-5′-phosphosulfate (APS) reductase (Meyer and Kuever, 2007). However, these methods present also the disadvantage that little or no information on the number of SRB cells that are present is provided (Muyzer and Stams, 2008).

Chapter 3: Objectives

The general goal of this thesis was the treatment of synthetic wastewater with high sulfate content, in a UASB reactor and using crude glycerol as carbon source to maximize the production of sulfide for its further recovery as elemental sulfur. UASB reactors have been operated world-wide demonstrating to be a robust and versatile technology to treat different types of wastewater and to recover energy (as in anaerobic digestion) or other valuable products. However, the use of crude glycerol as carbon source for sulfate reduction has been poorly addressed. Therefore, this thesis aimed at facing several challenges as well as fulfilling knowledge gaps in this process. The following specific objectives were proposed in order to gain knowledge about this process and improve the treatment of sulfate-laden wastewaters using a waste organic source:

- ✓ To demonstrate the technical feasibility of the use of crude glycerol as carbon source for the treatment of sulfate-rich effluents without the supply of additional external electron donors to produce high removal rates.
- ✓ To assess the use of crude glycerol specifically for sulfate reduction through the analysis of C sinks to the main bioprocess occurring in the system.
- ✓ To study the long-term performance of a sulfate-reducing UASB fed with crude glycerol under variable and constant loading rates to assess the influencing parameters for efficient sulfate removal and possible inhibitions or potential process limitations. Therefore, to enhance our insights into the long-term disturbances and mechanisms affecting the UASB operation.
- ✓ To investigate the correlation existing between physical-chemical parameters with microbial diversity evolution along long-term performances by characterizing the biomass through molecular-biology techniques.

- ✓ To study and promote the growth of acetate-utilizing sulfate reducers towards the improvement of sulfidogenesis performing enrichment experiments in serum bottles. In addition, to pursue the isolation of potential acetate-utilizing sulfate reducers.

Chapter 4: General materials and methods

4.1. Description of the reactors and experimental set-up

4.1.1. Lab-scale UASB reactor

A jacketed glass-made up-flow anaerobic sludge bed (UASB) reactor of 2.5 L, with a granular sludge volume of 1 L, was used in this thesis. A detailed scheme of the UASB is presented in Figure 4.1. Oxidation-reduction potential (ORP) and pH were monitored in the UASB with probes (Crison pH5333 and ORP5353, Hach Lange, Spain) connected to a multimeter (Crison MM44, Hach Lange, Spain).

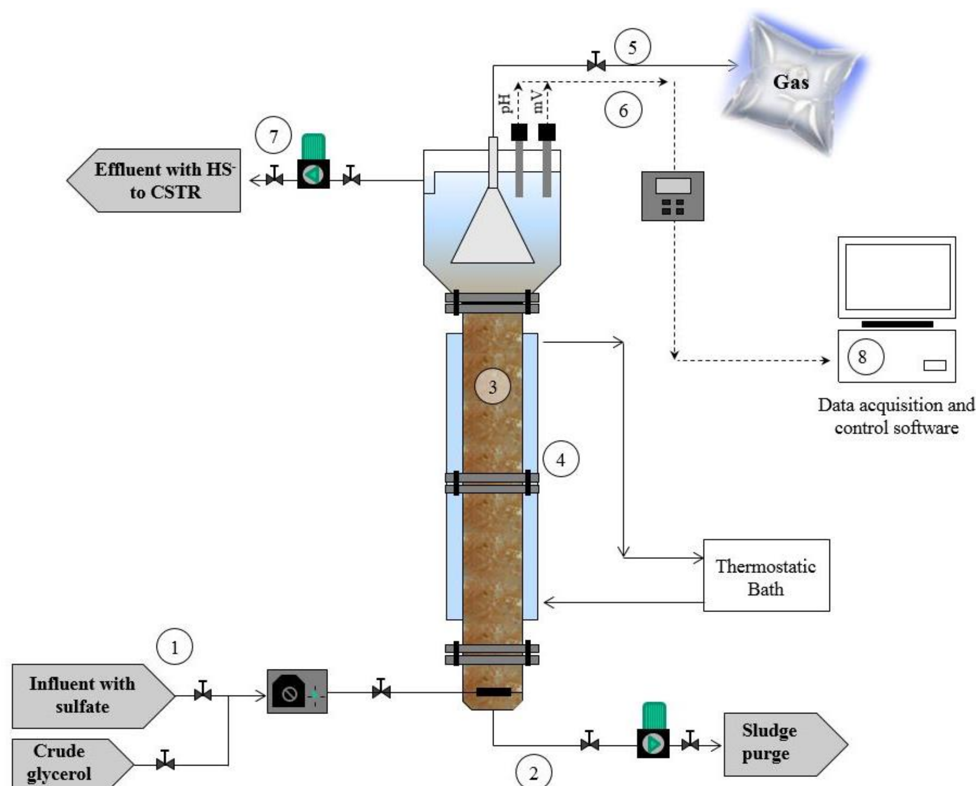


Figure 4.1. Detailed scheme of the UASB where (1) Inlet of mineral medium with sulfate and crude glycerol, (2) Sludge purge line to control the cellular residence time, (3) UASB reactor, (4) Water jacket connected to a thermostatic bath to control the temperature, (5) Biogas collector and storage, (6) pH and ORP monitoring connections, (7) Outlet effluent with dissolved sulfide to feed the CSTR and (8) Data acquisition system.

4.1.2. CSTR reactor

The continuous stirred tank reactor (CSTR) was a glass-made reactor with a volume of 6 L used for biosulfur production from the partial oxidation of sulfide produced in the UASB. Then, the influent of the CSTR was the effluent of the UASB. It was inoculated with 4 L of sulfide oxidizing sludge obtained from a bioscrubber for biogas desulfurization. The partial oxidation of sulfide produced in the CSTR was performed under microaerophilic conditions set only by transferring the oxygen from the headspace to the liquid phase through mechanic agitation. The thermostatic bath connected to the UASB reactor was also connected to the CSTR to set a temperature of 35 ºC. pH and redox potential in the reactor were monitored and registered with probes (Crison pH5333 and ORP5353, Hach Lange, Spain). Both, the pH and the redox probes were connected to a bench top multimeter (Crison MM44, Hach Lange, Spain) for data monitoring. In Figure 4.2 a picture of the bioreactors sequence (UASB-CSTR) is presented.

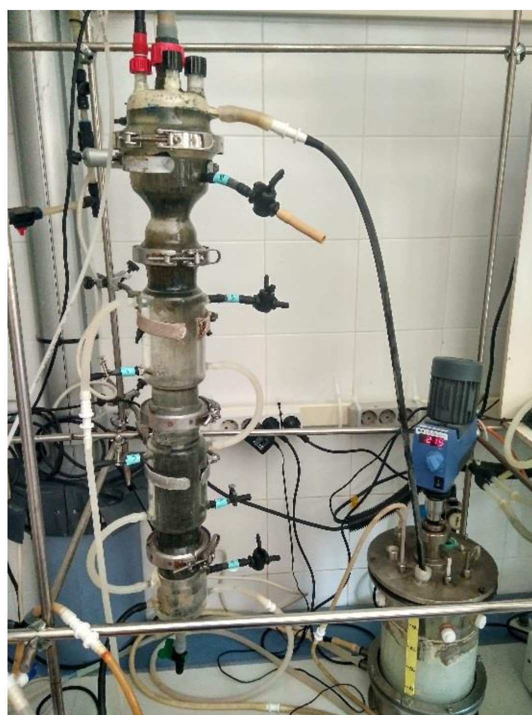


Figure 4.2. Picture of the bioreactors sequence (UASB-CSTR).

4.2. Analytical methods

Inlet and outlet flows of the reactors were sampled every two/three days during the experimental periods to monitor the ionic compounds, dissolved sulfide and volatile fatty acids (VFAs). Hence, different analytical methods were applied. In this section only the common analysis for all the experiments are detailed. More specific analytical methods and techniques used for each experiment will be detailed in the corresponding chapter of results.

- Ionic compounds

Samples were filtered with 0.22 μm Hydrophilic PES filters (Millipore). Sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) concentrations were analyzed by ion chromatography with conductivity detection using a Dionex ICS-2000 equipment with an Ultimate 3000 Autosampler Column Compartment, and an IonPac AS18 column (ThermoScientific, USA) with a detection range from 1 to 100 mg S L^{-1}. This methodology was used for Chapters 5, 6 and 7.

In Chapter 8, sulfate and thiosulfate concentrations were determined using ion chromatography Dionex ICS 2100 (Thermo Scientific, USA) equipped with Dionex™ IonPac™ AS16 column at 30 ºC. Potassium hydroxide (22 %) was used as eluent in a multistep gradient from 1 to 45 mM with a flow rate of 0.4 mL min^{-1} where sodium iodide (0.25 mM) was used as an internal standard. The quantification limit was 0.05 mM. In all cases, samples of 1 mL were taken and immediately centrifuged at 14000 rpm for 5 minutes. Subsequently 30 μL of supernatant were added to 970 μL of internal standard (0.25 mM of sodium iodide).

-Total dissolved sulfide

Along Chapters 5, 6 and 7 a sulfide selective electrode (VWR International Eurolab, S.L) connected to a benchtop meter (Symphony, VWR) was used for the

off-line measurement of total dissolved sulfide (TDS) concentration. Prior to their measurement, samples were diluted and preserved in sulfide antioxidant buffer (SAOB) above pH 12 to convert HS^- and $H_2S_{(aq)}$ to S^{2-}. The SAOB composition was (g L^{-1}): ascorbic acid (35) and EDTA (67) dissolved in NaOH (2 M). Ascorbic acid acts as sulfide antioxidant and EDTA avoids interferences with metallic compounds. This electrode has its own internal reference and presents a high sensitivity to S^{2-}. In Chapter 8, sulfide was measured photometrically by using the methylene blue colorimetric assay (Cline, 1969).

-Volatile fatty acids

In Chapter 5, volatile fatty acids concentrations (VFAs) were measured by gas chromatography (7820-A, Agilent Technologies) equipped with a DB-FFA column and using a flame ionization detector (FID) with helium as carrier gas. Prior to VFA analyses, samples were prepared following the procedure described in Baeza et al. (2017) which consisted of pipetting 0.8 mL of filtered samples together with 0.2 mL of a preserving solution (which also contained hexanoic acid as the internal standard) in a glass vial of 1.5 mL. The VFA species analyzed included acetic, propionic, butyric, isobutyric and valeric acids. All samples were previously filtered at 0.22 μm (Millipore, USA).

In Chapter 6, VFAs were determined by a Dionex 3000 ultimate high-performance liquid chromatography equipped with a UV/visible detector. The VFAs content was determined by UV spectroscopy (210 nm). The chromatographic separation was performed in an ICE-COREGEL 87H3 column (7.8 x 300 mm, Transgenomic, USA), heated at 40 ºC, employing 0.006 mM of H_2SO_4 as a mobile phase at a flow rate of 0.5 mL min^{-1}. Samples were filtered at 0.22 μm (Millipore, USA).

In Chapter 8, organic acids were quantified by high pressure liquid chromatography using a Shimadzu LC-2030C equipped with a Metacarb 67H column (Agilent Technologies, Santa Clara, CA, USA), operated at 45 ºC, with 0.01 N H_2SO_4 as

eluent at a flow rate of 0.8 mL min^{-1}. The detection of the organic acids was performed using both, a refractive index (RI) detector and UV detectors set at 210 nm, with a detection limit for formate and acetate of 0.05 mM. In all cases, samples of 1 mL were taken and immediately centrifuged at 14000 rpm for 5 minutes. Subsequently 0.4 mL of supernatant were added to 0.6 mL of 10mM Arabonise in 0.1 N H_2SO_4 solution for HPLC measurements.

- Gas analysis

CH_4 and CO_2 contained in the biogas were analyzed by gas chromatography (7820-A, Agilent Technologies, USA). The volume of the gas produced in the UASB reactor was calculated following the Gas Bag Method (GBM) as presented in Ambler and Logan (2011). This method is based on 1) measuring the initial composition of the collected gas in the bag, 2) adding a known volume of tracer gas (CO_2 in this case) in order to produce an appreciable change in the area of the tracer gas peak in the GC chromatogram and 3) analyzing the new composition after the injection. The average methane flowrate was calculated based on the volume of gas collected along variable time periods in which biogas was accumulated in the sampling bag located on top of the UASB and the methane concentration in the gas bag. H_2S production was analyzed by gas chromatography (Hewlett-Packard HP 5890 A, Agilent Technologies, USA) using a thermal conductivity detector and a Porapak Q column with helium as carrier gas.

Volatile organic compounds (VOCs) with sulfur (organosulfur compounds) produced in the UASB were determined by thermal desorption gas chromatography mass spectrometry (TD-GC/MS), using the equipment showed in Figure 4.3.

Figure 4.3. TD-GC/MS system used for VOCs characterization.

Gas sampling was performed by means of adsorption tubes (Markes International, Inc., Gold River, CA, USA) coupled to a PCXR4 sampling pump (SKC Inc., Eighty Four, PA, USA). To proceed with the analyses, desorption of the VOCs trapped in the sampling adsorption tubes was performed using a UNITY-2 Thermal Desorber (TD) (Markes International, Inc., CA, USA). The sorbent tubes were heated at 290 ºC for 8 minutes while flowing high purity He at a flow rate of 50 mL min^{-1} to desorb the VOCs onto a cold trap at -10 ºC. Afterwards, the cold trap was heated up to 305 ºC at a 40 ºC min^{-1} rate for 5 minutes to desorb the VOCs trapped and to inject them into the chromatographic column. The split ratio used during all the analysis process was 1:10 in order to adjust the resolution of the chromatogram and to avoid column overloading. Then, the gas was driven to the chromatographic column through a transfer line heated at 250 ºC to prevent condensation. VOCs analysis was performed using an Agilent 7820 Gas Chromatography (GC) coupled to an Agilent 5975 Mass Spectrometer (MS) (Agilent Technologies, Inc., CA, USA). The chromatographic column used for VOCs separation was a DB-624 capillary column (60 m x 0.25 mm x 1.4 µm, Agilent Technologies, Inc., CA, USA), using a He gas flow rate of 1 mL min^{-1} as carrier gas. The temperature program for the GC oven was an initial isothermal

stage at 50 ºC for 2 minutes, then a first temperature ramp to 170 ºC at a 3 ºC min^{-1} rate, followed by a second ramp up to 280 ºC at a 8 ºC min^{-1} rate. The GC/MS interface and the transfer line to the MS were maintained at 280 and 235 ºC, respectively, during the whole analysis. Finally, the mass spectra were obtained by electronic ionization at 70 eV and the MS acquired data in scan mode with m/z interval ranging from 35 to 355 amu (atomic mass unit). The total analysis process lasted 54 minutes. The different compounds were identified by matching the mass spectra with the Wiley275 mass spectrum library available in the GC–MS system.

- Total and volatile solids concentrations

Total suspended solids (TSS) and volatile suspended solids (VSS) analyses were performed according to Standard Methods (APHA, 2005). First, samples were filtered through previously weighted (W_1) standard glass microfiber filters of 0.7 μm (GF/F grade, Whatman, USA) and dried at 105 ºC until constant weight (W_2). The ratio between the difference among W_1 and W_2 and the sample volume is the concentration of TSS, that represents the organic and inorganic matter in suspension in the sample. Then, samples were ignited at 550 ºC for about 45 min and weighted (W_3). The difference between W_1 and W_3 divided by the volume of filtered sample represents the concentration of VSS.

- Chemical oxygen demand and TOC analysis

Chemical oxygen demand (COD) was measured using COD kits and a photometer (Lovibond®). Total inorganic carbon (TIC) and total organic carbon (TOC) were analyzed by the high temperature combustion device multi N/C 2100S (Analytik Jena, Germany).

4.3. Microbial analysis

Two molecular biology techniques were used to identify and follow up the changes in microbial population composition in sludge samples along the operation of the UASB reactor: fluorescence *in situ* hybridization (FISH) and Illumina sequencing analysis.

4.3.1. Fluorescence *in situ* hybridization (FISH)

- Sample fixation

This protocol was adapted from Nielsen (2009). Biomass samples were collected from the reactor and granules were smashed by means of a pestle to homogenize the sample. Dilutions were made when required to ease hybridization. For Gram-negative microorganisms 3 volumes of 4 % PFA (1.5 mL) were added to 1 volume of sample (0.5 mL), previously washed (4000 g, 4 min) with 1XPBS ($Na_2HPO_4 \cdot 12H_2O$ 7.2 mM, $NaH_2PO_4 \cdot 2\ H_2O$ 2.8 mM and NaCl 0.13 mM). Washing and centrifugation steps were increased when necessary to decrease the extracellular polymeric substances (EPS) content of the samples. After that, samples were held at 4 ºC for 2-3 h. Cells were then centrifuged (4000 g) to remove fixative and washed twice with 1XPBS. Then, samples were resuspended in one volume (0.5 mL) of 1XPBS per one volume (0.5 mL) of ice-cold ethanol. Fixed cells were spotted onto glass slides or stored at -20 ºC for several months.

- Probe hybridization

Depending on sample concentration, 2-10 µL of fixed sample were applied to each well in the glass slide and dehydrated in ethanol series (3 min each): 50 %, 80 % and 98 % (v/v). The hybridization buffer was prepared in 2 mL microcentrifuge tubes at the time of use (composition is detailed in Table 4.1). Probe solutions were kept in dark and on ice. For the hybridization mixtures, 1 volume of probe working solution

(50 ng μL[1]) was added to 9 volume of hybridization buffer in a 0.5 mL microfuge tube. 10 μL of the hybridization mixture were added to each well on the slide. The remainder hybridization buffer was poured in a 50 mL falcon tube that contained cellulose tissue as a moisture chamber for hybridization. Afterwards, the slide was placed horizontally into the tube and quickly taken to the hybridization oven at 46 ºC for 2.5 h.

Table 4.1. Hybridization buffer composition

Component	Volume to prepare 2 mL (microcentrifuge tub)	Final concentration
5 M NaCl (autoclaved)	360 μL	900 mM
1 M Tris/HCl (autoclaved)	40 μL	20 mM
10 % SDS (not autoclaved)	2 μL	0.01 %
Formamide	0-1600 μL(1)	0-80 %(1)
MilliQ water	up to 2 mL	-

(1) Formamide concentration depends on the probe used.

Washing buffer was prepared and maintained at 48 ºC until its use. The washing buffer composition is described in Table 4.2. After hybridization, the slides were carefully removed from the tube and splashed with warm wash buffer into a beaker. Slides were then quickly placed into the washing buffer tube and taken to the water bath at 48 ºC for 10-15 min. After washing, the slides were gently (to avoid biomass detachment from the slide) rinsed in cold milliQ water. Both sides of the slide are washed to remove any salt which is highly auto-fluorescent. Afterwards, all droplets of water were removed from the wells applying compressed air directly to the slides. To finish, small drops of anti-bleaching reagent (ProLong Diamond Antifade Mountant) were applied to the wells on slides. Slides were covered with a large coverslip for the subsequent microscope observation.

Table 4.2. Washing buffer composition

Component	Volume to prepare 50 mL (falcon tub)	Final concentration
5 M NaCl (autoclaved)	0-9 mL[(1)]	0-900 mM[(1)]
0.5 M EDTA (autoclaved)	0-500 μL[(2)]	0-5 mM[(2)]
1 M Tris/HCl (autoclaved)	1 mL	20 mM
MilliQ water	up to 50 mL	-
10 % SDS (not autoclaved)	50 μL	0.01 %

[(1)] NaCl concentration and [(2)] EDTA concentration depend on formamide concentration in the hybridization buffer.

- Microscope observation and quantification

Relative abundances of the populations of interest were analyzed by FISH technique coupled with confocal laser scanning microscopy (CLSM). A Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystem Heidelberg GmbH; Mannheim, Germany) using a Plan-Apochromatic 63X objective (NA 1.4-0.6, oil) was used to obtain different microscopic fields (z=0) for further quantification. The quantification was performed following a macro for the ImageJ software developed by the microscopy service (UAB). The macro was created to consider the colocalization of the specific probes (DELTA495a and EURY514) over the general probes. Samples without probes were used to stablish thresholds values in each single laser channel. Oligonucleotide probes used in this thesis are detailed in Table 4.3 showing probes names, sequences, optimal formamide concentrations used in the hybridization buffers and their specificity.

Table 4.3. FISH 16S rRNA-targeted oligonucleotide probes, target microorganisms and references used in this thesis

Probe	Sequence (from 5´to 3´)	FA (%)	Specificity	Reference
EUB338 I	GCTGCCTCCCGTAGGAGT	0-50	Most bacteria	Amann et al. (1990)
EUB338 II	GCAGCCACCCGTAGGTGT	0-50	Planctomycetales	Daims et al. (1999)
EUB338 III	GCT GCC ACC CGT AGG TGT	0-50	Verrucomicrobiales	Daims et al. (1999)
NONEUB	ACTCCTACGGGAGGCAGC	Not determined	Control probe complementary to EUB338	Wallner et al. (1993)
DELTA495a	AGTTAGCCGGTGCTTCCT	35	Most Deltaproteobacteria and most Gemmatiomonadetes	Loy et al. (2002); Lücker et al. (2007)
DELTA495a competitor	AGTTAGCCGGTGCTTCTT	35	-	Loy et al. (2002); Lücker et al. (2007)
EURY514	GCGGCGGCTGGCACC	20	Most Euryarchaeota	Jurgens et al. (2000)

4.3.2. 16S Amplicon sequencing analysis (Illumina MiSeq)

Microbial diversity analyses from Chapter 5 were performed using next-generation sequencing. Genomic DNA was extracted by applying the protocol of PowerSoil™ DNA isolation kit (MoBio Laboratories, USA). The quantity and quality of the extracted DNA were evaluated by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and then DNA samples were preserved at -20 °C for further analysis. DNA metabarcoding analysis was performed on an Illumina MiSeq platform by AllGenetics & Biology SL (A Coruña, Spain). For library preparation, a fragment of the bacterial 16S V4-V5 ribosomal RNA gene of around 400 bp was amplified using the primers 515F (5' GTG CCA GCM GCC GCG GTA A 3') and 909R (5' CCG TCA ATT YHT TTR AGT 3') (Herlemann et al., 2011). The coverage of the primers was checked using the test prime tool in Silva's website (www.arb-silva.de), setting the parameters to 1 mismatch for the maximum number of mismatches and 5 bases for the length of 0-mismatch zone at 3′end. As a result, the primer pair presented 85.6 % of coverage for Archaea and 91 % for Bacteria. The reference database used for the taxonomic classification of organisms was Greengenes (DeSantis et al., 2006).

Chapter 5: Exploring the performance limits of a sulfidogenic UASB during the long-term use of crude glycerol as electron donor

A modified version of this chapter has been published as:

Fernández-Palacios, E., Lafuente, J., Mora, M., Gabriel, D., 2019. Exploring the performance limits of a sulfidogenic UASB during the long-term use of crude glycerol as electron donor. Sci. Total Environ. 688, 1184–1192.

The main motivation of this chapter was to operate a UASB reactor for a long-term period using crude glycerol as electron donor to assess the main elimination capacities of the system. Since the reduction of sulfate to sulfide is the most crucial stage to be optimized if elemental sulfur recovery is desired from S-rich liquid effluents, the system was tested under a range of conditions including COD/S-SO_4^{2-} ratios, to obtain information about the limits in which this reactor could perform in terms of sulfate reducing capacity. The specific use of crude glycerol for sulfate reduction was also assessed and a first characterization of the microbial populations taking part in this process was studied through sequencing analysis.

Abstract

SOx contained in flue gases and S-rich liquid effluents can be valorized to recover elemental sulfur in a two-stage bioscrubbing process. The reduction of sulfate to sulfide is the most crucial stage to be optimized. In this chapter, the long-term performance of an up-flow anaerobic sludge blanket (UASB) reactor using crude glycerol as electron donor was assessed. The UASB was operated for 400 days with different sulfate and organic loading rates (SLR and OLR, respectively) and a COD/S-SO_4^{2-} ratio ranging from 3.8 g O_2 g^{-1} S to 5.4 g O_2 g^{-1} S. After inoculation with methanogenic, granular biomass, the competition between sulfate-reducing and methanogenic microorganisms determined to what extent dissolved sulfide and methane were produced. After the complete washout of methanogens, which was revealed by next-generation sequencing analysis, the highest S-EC was reached in the system. The highest average sulfate elimination capacity (S-EC=4.3 kg S $m^{-3}d^{-1}$) was obtained at a COD/S-SO_4^{2-} ratio of 5.4 g O_2 g^{-1} S and an OLR of 24.4 kg O_2 $m^{-3}d^{-1}$ with a sulfate removal efficiency of 94 %. The conversion of influent COD to methane decreased from 12 % to 2.5 % as the SLR increased while a large fraction of acetate (35 % of the initial COD) was accumulated. Our data indicate that crude glycerol can promote sulfidogenesis. However, the disappearance of methanogens in the long-term due to the outcompetition by sulfate reducing bacteria, lead to such large accumulation of acetate.

5.1 Introduction

Combustion of sulfur-containing fuels, such as coal, natural gas, peat, wood and oil, results in SO_2 formation mainly generated in the energetic and industrial sectors (Klimont et al., 2013). These emissions are usually treated through physical-chemical processes that are expensive and generate additional effluents requiring further processing and energy inputs (Srivastava and Jozewicz., 2001; Philip and Deshusses., 2003). As an example, aqueous slurries with high sulfite and sulfate content are generated from wet flue gas desulfurization (WFGD) with sodium hydroxide. The development of environmentally friendly alternatives to valorize not only SO_2 from WFGD but also S-rich liquid effluents is clearly needed. The two-stage bioscrubber concept described in Figure 5.1 is a potential alternative process to recover elemental sulfur from such gaseous effluents (Fernández et al., 2017). The process consists of a first scrubbing stage for SO_x absorption in water at slightly alkaline pH, followed by two-stage biological process to obtain elemental sulfur. The biological process converts, firstly, sulfate to total dissolved sulfide (TDS) using an organic waste as C source and electron donor and, secondly, TDS to elemental sulfur through a partial oxidation performed under oxygen limiting conditions. Partial TDS oxidation can be also performed through autotrophic denitrification.

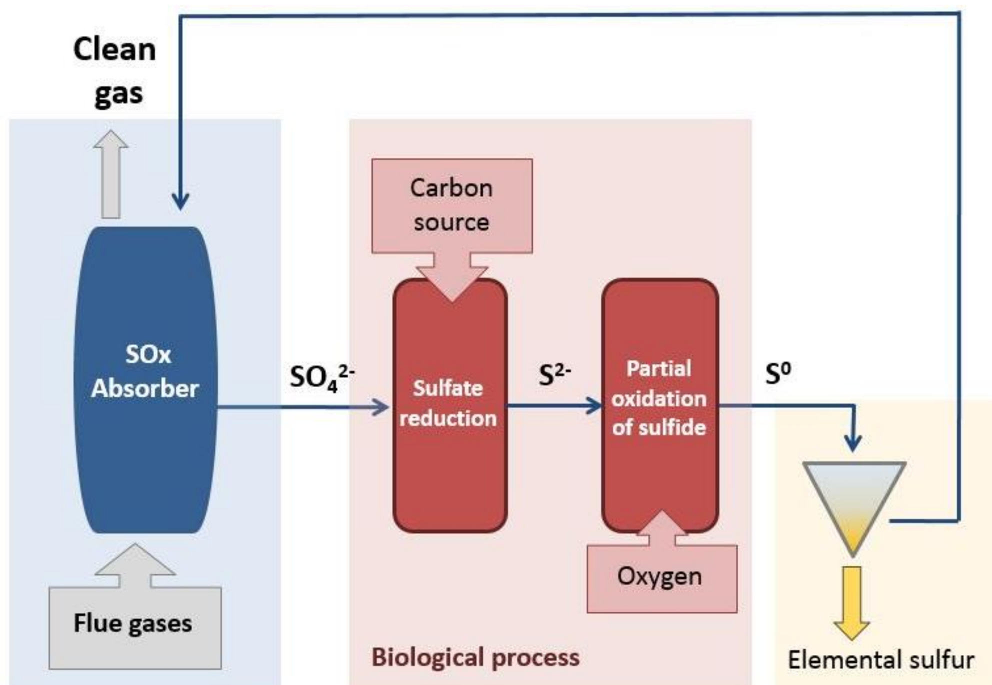


Figure 5.1. Schematic diagram of a two-stage bioscrubber process to recover elemental sulfur from S-rich effluents.

Sulfate to TDS reduction has been studied using different substrates, such as sewage or methanol, and under a range of operating conditions including thermophilic processes (Weijma et al., 2000b; Jiang et al., 2013; Qian et al., 2015). However, the sulfate reduction stage is still the one that requires further economic and technical improvements (Chen et al., 2014). Sulfate reduction, which is catalyzed by sulfate-reducing bacteria (SRB) (Liamleam and Annachhatre, 2007), can be carried out with a large assortment of organic wastes and under different operating conditions. Recently, crude glycerol has been proposed as a competitive substrate to reduce high loads of sulfate to TDS in batch tests as well as during the start-up of an up-flow anaerobic sludge blanket reactor (Mora et al., 2018). Crude glycerol is a waste organic effluent produced in the biodiesel industry with an exceptional COD concentration (≈800 g O_2 L^{-1}) that does not require any additional treatment before its use as carbon source. In most of the recent research, crude glycerol has been used as a suitable substrate for biogas production in anaerobic systems (Nakazawa et al., 2015) or as a

co-substrate in anaerobic digestion to increase biogas production (Nghiem et al., 2014; Athanasoulia et al., 2014). Despite different approaches to reduce sulfate from S-rich streams have been investigated using pure glycerol (Santos et al., 2017), the potential of crude glycerol has been poorly explored in sulfidogenic reactors. In fact, to the best of my knowledge, assessment of the long-term performance of a UASB using crude glycerol as electron donor for sulfate reduction has not been addressed before.

One of the main problems related to the start-up of a reactor for sulfate reduction with organic matter is the competition between SRB and methanogens. Since the inoculum is usually obtained from full-scale anaerobic digesters targeting methane production, the enrichment of sulfate reducing bacteria (SRB) becomes a decisive threat between sulfate reduction and methane production. SRB and methanogens competition for the common intermediates in the anaerobic degradation process, which has been widely reported, results in a variable performance of the reactor. Then, the origin of the inoculum becomes critical as it contains diverse microbial populations leading to differences in initial activity and substrate adaptation (De Vrieze et al., 2015). Some variables that have been studied to assess this competition are COD to SO_4^{2-} ratio (COD/SO_4^{2-} ratio), TOC/ SO_4^{2-} ratio, organic loading rates (OLR), sulfate loading rates (SLR) and the type of electron donor used to reduce sulfate (Pol et al., 1998). Most of them have been studied using different electron donors, such as glucose (O'Reilly and Colleran, 2006), lactate (Zhou et al., 2014), ethanol (Hu et al., 2015) and Volatile Fatty Acid (VFA) mixtures (acetate, propionate and butyrate) (Omil et al., 1996; Omil et al., 1998; Lens et al., 1998a) but, there are no reports on the long-term operation using a substrate with a significant fraction of slowly hydrolysable carbon source such as that contained in crude glycerol. Despite the competition of SRB over methanogens has been widely described, the use of crude glycerol as carbon source implies the production of metabolites through its

fermentation that may lead to microbial diversity changes that have not been yet explored. It remains uncertain if such competition may be beneficial or not to process performance.

Another important parameter in the start-up and long-term operation of UASB reactors for sulfate reduction is biomass granulation. Granular biomass provides a strong structure and good settling properties that contribute to high biomass retention, and stands up against possible shock and high loading rates (Liu and Tay., 2004b). As demonstrated by De Vrieze et al. (2015), selecting an inoculum according to your objective is crucial for a robust operation. In the current chapter, granular sludge from methanogenic anaerobic digesters is used as inoculum in UASB bioreactors for sulfate reduction (Mora et al., 2018) considering that no granular SRB-based reactors are currently operated in the field. Long-term operation may lead to microbial diversity changes that could affect UASB performance during the long-term operation of such sulfidogenic reactors.

To better understand the limits and applicability of sulfate reduction, this chapter aimed at assessing 1) the limits of the process in terms of sulfate reducing capacities and 2) the long-term performance of a UASB for the treatment of synthetic sulfate-rich effluents to produce TDS using crude glycerol as electron donor. This chapter not only provides new information regarding S-rich streams valorization but also assesses the use of crude glycerol specifically for sulfate reduction through the analysis of C sinks to the main bioprocess occurring in the system.

5.2. Materials and methods

5.2.1. Experimental setup

A lab-scale UASB reactor of 2.5 L was used for the long-term operation. The detailed diagram of the reactor and set-up details are described in Section 4.1.1 of Chapter 4. During the operation, inlet pH ranged between 8.4 and 8.6 and temperature was controlled at 35 °C by a thermostatic bath connected to the water jacket of the reactor (Figure 4.1). The composition of the mineral medium was (g L^{1}): K_2HPO_4 (3), NH_4Cl (0.2) dissolved in tap water to add macro- and micronutrients and adjusted to pH=8.8-9.0 with NaOH (2 M). Mineral medium was pumped at a flow rate of 0.5 L h^{-1}, once mixed with the organic influent, from the bottom to the top of the UASB (up-flow velocity of 0.25 m h^{-1}). The flow rate of the organic influent was set at 30 mL h^{-1}. Hence, crude glycerol was diluted to adjust the inlet COD concentration. The hydraulic residence time (HRT), calculated as that corresponding to the reaction volume only (sludge blanket), was 2 h. Biogas produced in the UASB was collected in a 5 L Tedlar bag (FlexFoil, SKC Inc.) to monitor its composition and flow rate. Inlet and outlet flows were also sampled every two/three days to analyze COD, S compounds (sulfate, thiosulfate and TDS) and VFA.

5.2.2 Operating conditions and short-term experiments

Granular sludge obtained from an anaerobic digester treating wastewater in a pulp and paper industry was used to inoculate the reactor to reach an initial volatile suspended solids (VSS) concentration of 28 g VSS L^{-1}. As shown in Table 5.1, the reactor was operated during 400 days at different sulfate inlet concentrations. Inlet sulfate concentrations ranging from 235±17 mg S-SO_4^{2-} L^{-1} to 859±30 mg S-SO_4^{2-} L^{-1} were fed by adding increasing amounts of sodium sulfate to the mineral medium. Different SLR and OLR were tested during the long-term

operation of the UASB in order to assess the sulfate reducing capacity of the system. The operation was divided into 6 different periods according to the initial sulfate inlet concentrations and the COD/S ratio tested (Table 5.1). Period I focused on the UASB start-up to enrich the microbial community with SRB; Period II served to optimize the operation at the same inlet sulfate concentration set in Period I by providing a higher OLR; Periods III and IV were set to study the sulfate reducing activity at a moderate initial sulfate concentration; Period V served to explore the limits of the system by setting the highest sulfate inlet concentration and, finally, Period VI targeted the recovery of the initial UASB stability when the lowest SLR was set. Table 5.1 shows average operating conditions and standard deviations obtained from each operational period. SLR and OLR were calculated considering the reaction volume only.

During period VI, short-term assays were carried out during 60 h to assess the sulfate elimination capacity (S-EC) in the UASB reactor under variable loading rate conditions typically found in industrial activities. The experiment consisted of a stepwise decrease of the sulfate inlet concentration every 12 h (from 450 mg S L^{-1} to 120 mg S L^{-1}). The COD/S was also varied since the OLR remained constant during the short-term experimental assays. At each concentration tested, effluent was collected to measure the concentration of sulfate, TDS and VFA. In addition, sulfate concentration in the influent was also measured every 12 hours.

Table 5.1. Conditions tested in the UASB reactor during the long-term performance.

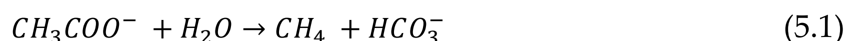
Period	Days	$Sulfate_{inlet}$ (mg S L^{-1})	SLR (kg S-SO_4^{2-} $m^{-3}d^{-1}$)	OLR (kg O_2 $m^{-3}d^{-1}$)	COD/S_{inlet} (g O_2 g^{-1} S)
I	0-99	235±17	3.2±0.4	12.0±2.1	3.8±0.8
II	99-115	235±17	2.9±0.9	15.8±4.6	5.3±0.6
III	115-197	442±47	4.6±0.8	24.4±6.9	5.4±1.0
IV	197-238	442±47	5.0±0.4	27.1±2.5	5.4±0.6
V	238-288	859±30	8.1±0.8	25.4±2.6	3.1±0.2
VI	288-400	442±47	6.0±1.4	25.7±6.9	4.5±1.4

5.2.3. Illumina sequencing analysis

Microbial diversity analysis was performed using next-generation sequencing. Genomic DNA was extracted from samples of the inoculum and on day 190 of the UASB operation by applying the protocol explained in Section 4.3.2 of Chapter 4.

5.2.4. Assessment of COD mass balances

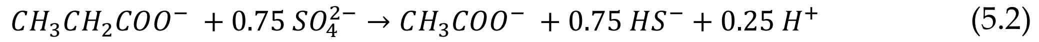
Considering the methane concentration measured in the gas phase and the biogas flowrate produced along the UASB operation, the molar mass flow of methane produced was calculated. The result was used to calculate the acetic acid produced by methanogenic biomass according to the following well-known stoichiometry for the conversion of VFA:



$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad (5.1)$$

The amount of acetic acid consumed for methane production plus the outlet acetic acid flowrate was used to calculate the amount of propionic acid produced

according to the following stoichiometry for the overall sulfate reduction considering propionate as the electron donor.



$$CH_3CH_2COO^- + 0.75\, SO_4^{2-} \rightarrow CH_3COO^- + 0.75\, HS^- + 0.25\, H^+ \quad (5.2)$$

Figure 5.2 summarizes the mechanistic approach used for assessing the carbon sink in the reactor.

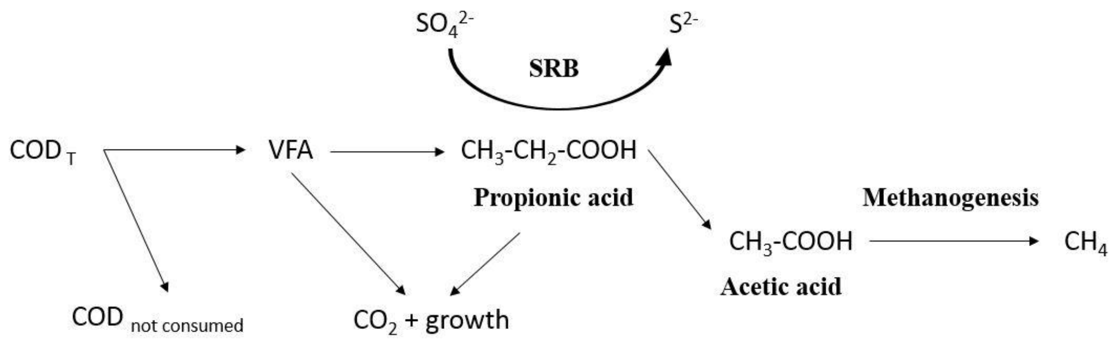


Figure 5.2. Schematic diagram of COD destination considering propionate as the electron donor for sulfate reduction.

5.3. Results

5.3.1. Long-term performance of the UASB and short-term experiments

The UASB performance was evaluated during 400 days of continuous operation in terms of sulfate removal efficiency (S-RE), COD removal efficiency (COD-RE) and sulfate and COD elimination capacities (S-EC and COD-EC, respectively) using crude glycerol as carbon source. Table 5.2 shows the results obtained from the long-term UASB operation as averages and standard deviations of all data acquired in each period. Monitoring results of sulfur species are shown in Figure 5.3 while COD measurements together with the average flowrate of methane and the concentration of each VFA monitored are presented in Figure 5.4.

Table 5.2. Removal efficiencies and elimination capacities obtained during the system operation.

Period	Days	COD/S ($g\ O_2\ g^{-1}\ S$)	S-EC ($kg\ S\ m^{-3}d^{-1}$)	COD-EC ($kg\ O_2\ m^{-3}d^{-1}$)	S-RE (%)	COD-RE (%)
I	0-99	3.8±0.8	2.4±0.6	10.1±2.3	76.8±14.4	86.1±8.6
II	99-115	5.3±0.6	2.4±0.4	12.6±1.0	96.5±2.7	89.3±3.3
III	115-197	5.4±1.0	4.3±0.8	9.4±4.9	94.0±4.1	38.1±13.2
IV	197-238	5.4±0.6	3.9±0.6	9.8±4.2	79.4±11.6	35.1±11.7
V	238-288	3.1±0.2	3.4±0.5	7.2±4.2	41.7±6.0	31.2±4.4
VI	288-400	4.5±1.4	2.6±0.8	6.7±3.7	47.9±17.3	24.7±8.3

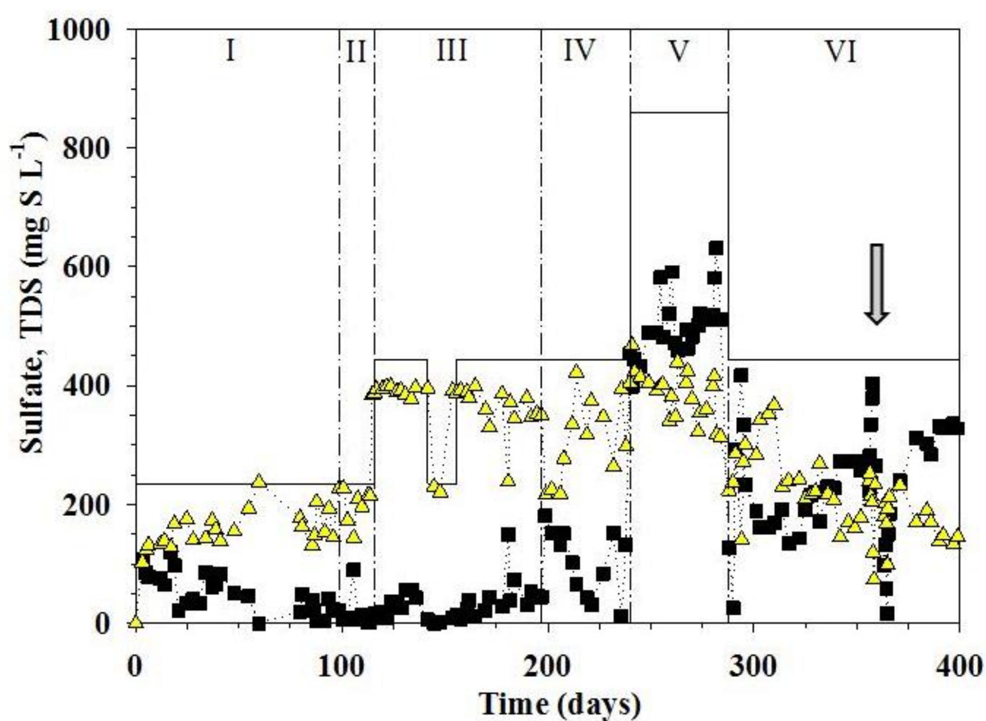


Figure 5.3. UASB performance during the long-term operation. Sulfate concentration in the influent (solid line) and in the effluent (■) and total dissolved sulfide concentration in the effluent (△). Dotted vertical lines represent periods I to VI (see Tables 5.1 and 5.2). Arrow represents the time when short-term experiments were carried out.

As shown in Table 5.1, the UASB operation was divided into six periods. During the UASB start-up, sulfate inlet concentration and OLR were maintained at 235±17 mg S-SO_4^{2-} L^{-1} and 12.0±2.1 kg O_2 m^{-3} d^{-1}, respectively. As can be observed in Figure 5.3 sulfate reduction started almost immediately after inoculation and increased steadily during period I. During period II (days 99-115) the OLR was stepwise increased to avoid organic matter limitation for the complete reduction of sulfate. An S-RE up to 99 % with an almost complete removal of the COD (Figure 5.4A) was obtained at the end of this period. Afterwards, the SLR and OLR were increased in Period III, day 115 to 197, by doubling the inlet sulfate and COD concentrations in order to reach a higher sulfate reduction capacity in the system while maintaining the COD/S ratio slightly above 5 g O_2 g^{-1} S, which was found to provide the best results in terms of sulfate and COD removal efficiencies during period II. Even finding almost a complete sulfate removal (S-RE up to 94 %) during period III, there was a progressive VFA accumulation coupled to a decrease of the COD-RE (Figure 5.4B).

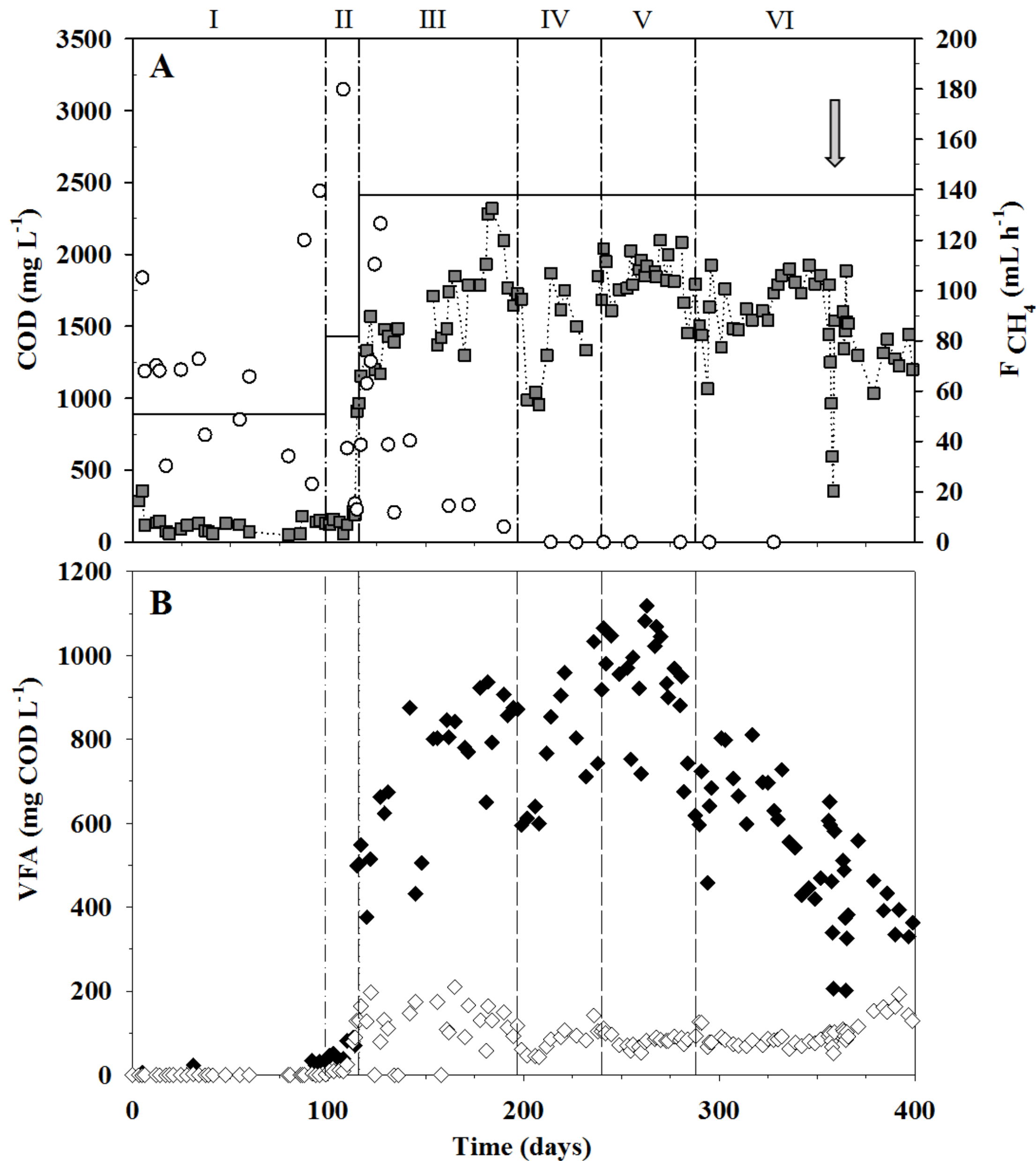


Figure 5.4. Performance of UASB. A) COD in the influent (solid line) and in the effluent (■) and flow of methane in the gas phase (○). B) VFA concentration: acetic acid (◆) and propionic acid (◇). Dotted vertical lines represent periods I to VI (see Tables 5.1 and 5.2). Arrow represents the time when short-term experiments were carried out.

Despite S and C loads were not changed, a fourth period was defined because crude glycerol (glycerol 1) was replaced by a new batch from the supplier at the

beginning of period IV. The new crude glycerol (glycerol 2) contained 35 % less water, an average COD of 900 g O_2 L^{-1} (640 g $C_3H_8O_3$ L^{-1}) and a lower BOD_5/COD ratio (Table 5.3).

Table 5.3. Physical-chemical analysis of glycerol 1 and glycerol 2.

Parameters analyzed	Glycerol 1	Glycerol 2
Organic Material	34.0 %	67.0 %
Water	56.0 %	18.0 %
Soluble salts	5.0 %	11.0 %
Elemental sulfur	2.3 %	4.4 %
COD	500 mg O_2 L^{-1}	800 mg O_2 L^{-1}
BOD_5	345 mg O_2 L^{-1}	400 mg O_2 L^{-1}
Total solids	374 g kg^{-1}	734 g kg^{-1}
Volatile solids	295 g kg^{-1}	637 g kg^{-1}
Kjeldahl nitrogen	5100 mg L^{-1}	7700 mg L^{-1}
pH	5.9	6.1

Lower averages S-REs were found with glycerol 2 (Table 5.2) despite some progressive acclimation of functional bacteria to this crude glycerol towards the end of period IV (S-RE above 85 %) could be observed. Consequently, the SLR was increased in Period V (days 238-288) to verify the maximum treatment capacity of the reactor. In Period V the lowest COD/S ratio was tested despite the system was already overloaded. VFAs accumulated until reaching their maximum concentrations (Figure 5.4B) as described in Section 5.3.2. During

Period VI the UASB operated during 112 days under the conditions tested during period III-IV to recover the initial stability of the system.

In addition, short-term experiments were performed. For that purpose, different sulfate inlet concentrations were tested at the end of period VI (days 360-370) to verify system robustness to face quickly variable inlet loads during the operation. Figure 5.5 shows sulfate and sulfide concentration profiles as well as the corresponding S-RE and S-EC obtained during the short-term assays. As can be observed, the sulfate RE was almost doubled for the lowest sulfate concentration tested (120 mg $S\text{-}SO_4^{2-}$ L^{-1}) compared to the initial situation before the short-term experiment.

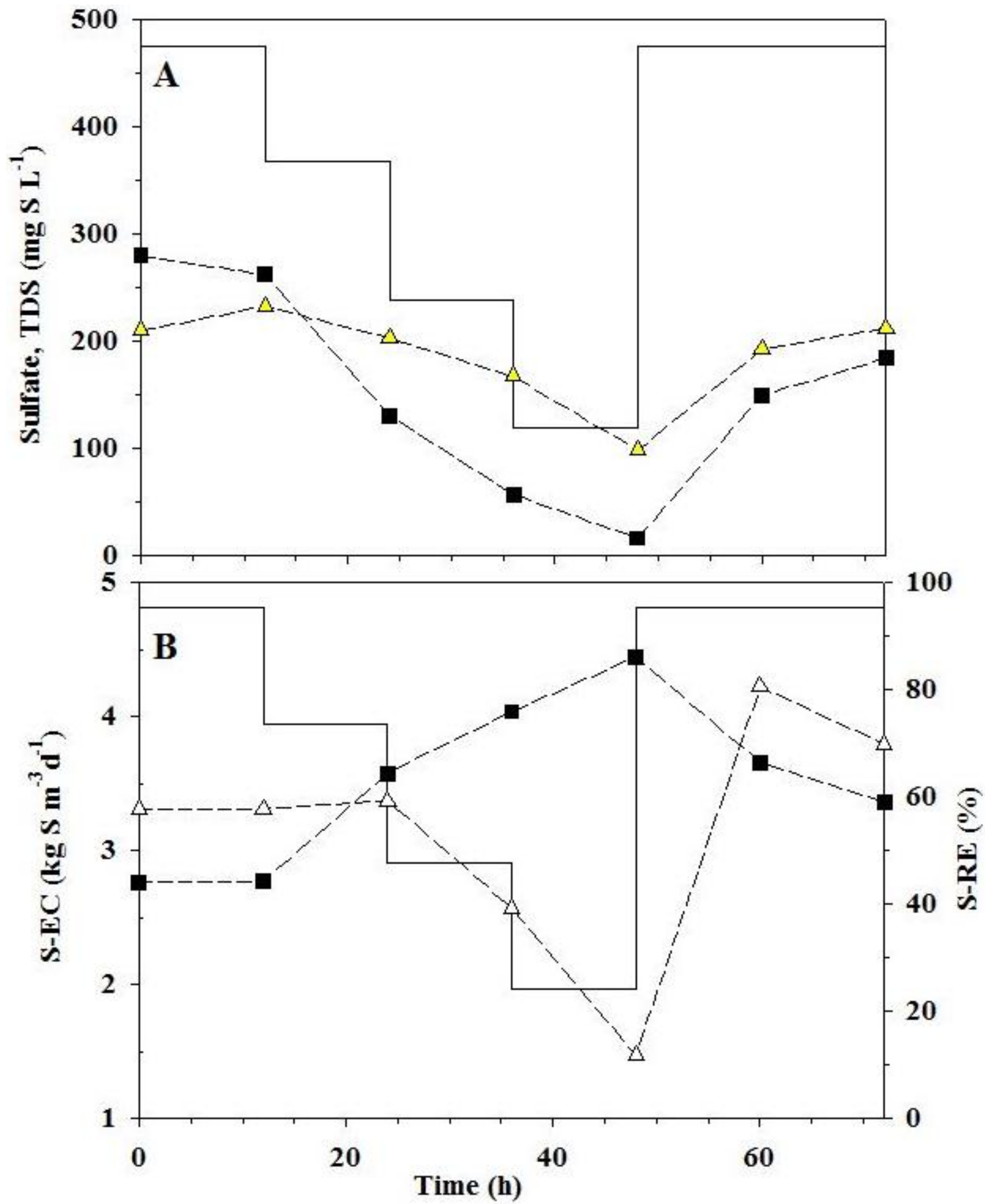


Figure 5.5. S profiles and removal efficiencies obtained from short-term assays performed in the UASB. (A) Sulfate concentration in the influent (solid line) and in the effluent (■) and total dissolved sulfide concentration in the effluent (△). (B) Sulfate removal efficiency (■) and sulfate elimination capacity (Δ).

5.3.2. Organic matter sink: sulfate reduction and biogas and VFA production

Even if traces of other VFA were measured from the biodegradation of crude glycerol, only acetate and propionate were predominant and therefore considered for further analysis (Figure 5.4B). During periods I and II, inlet COD was completely consumed and no VFA were detected in the effluent while some CH_4 was produced and recovered as part of the gas phase. From period III onwards, when an average OLR of 25 kg O_2 m^{-3} d^{-1} was fed (Table 5.1), the effluent contained mainly acetate. As can be observed in Figure 5.4A, this increase in acetate coincided with a decrease in CH_4 production, which ceased 75 days after the beginning of period III. During period V, the maximum concentration of acetate in the reactor was reached (1000 mg acetate L^{-1}), which progressively decreased until the end of the operation when acetate concentrations below 340 mg L^{-1} were detected.

The conversion of COD resulting from each operating period was also assessed in terms of CH_4 composition in biogas, VFA concentrations in the effluent (acetate and propionate), and COD used for sulfate reduction (Table 5.4). The COD balance was calculated based on measurements of inlet and outlet COD and VFAs and corresponding methane production. According to the methane composition in biogas, the biogas flowrate and the TDS and residual COD in the effluent, COD conversion proportions along the different periods were obtained according to processes stoichiometry (see equations 5.1 and 5.2). Table 5.4 shows that along Periods I and II around 11 % of the inlet COD was directed to methane production while almost no VFA accumulated in the reactor. However, between 33 and 41 % of the influent COD ended up in acetic acid from Period III until the end of the operation while between 3 and 6% of the inlet COD was converted to propionic acid from Period III onwards. Concomitantly, the COD fraction converted to methane had the opposite behavior and was around 0 % from Period III onwards, which was taken into account as a way of reporting the

percentage of electrons utilized by methanogens. The potential use of COD for sulfate reduction was more stable along the operation even if a progressive deterioration could be detected that accounted for a 26.5 % less of organic matter calculated for this purpose comparing the last and the first periods. The rest of COD was assumed to be used for growth and CO_2 formation, although it could not be accurately quantified.

Table 5.4. COD sinks during the operation of the system.

COD sink	Period I	Period II	Period III	Period IV	Period V	Period VI
Methanogenesis (%)	10.7	11.0	2.3	0.0	0.0	0.0
Sulfate reduction (%)	40.4	29.5	23.3	16.8	19.7	13.9
CO_2 and growth (%)	48.5	52.0	33.7	41.9	36.3	47.7
Outlet acetic acid (%)	0.4	5.0	35.0	38.0	41.0	33.0
Outlet propionic acid (%)	0.0	2.5	5.7	3.3	3.0	5.4

5.3.3. Illumina sequencing analysis and bacterial community assessment

The scope of the microbial analysis was not to describe the evolution of the microbial diversity along the UASB operation but to provide further data to explain the switch from methane production to non-methane production conditions from a microbial perspective. Thus, the bacterial community through Illumina analysis of the 16S rRNA gene was applied to compare the methanogenic granular sludge used as inoculum with the biomass developed after 190 days of the UASB operation when no methane production was observed. Results obtained from the microbial analysis are presented in Figure 5.6. *Deltaproteobacteria* and *Methanomicrobia* were the main classes detected in the inoculum with a relative abundance of 20 % and 16 % respectively

(Figure 5.6). *Clostridia* was the third class in order of abundance (13.5 %). After operating the UASB for 190 days, *Deltaproteobacteria* increased their relative abundance to 49 % in the sludge bed sample, clearly the most abundant class of the total reads; while *Methanomicrobia* decreased to 0 % without detecting any other methanogenic microorganism. *Clostridia* was the second class in order of abundance (12.2 %), followed by *Gammaproteobacteria* and *Bacteroidia* (11 and 7.3 % of total reads, respectively).

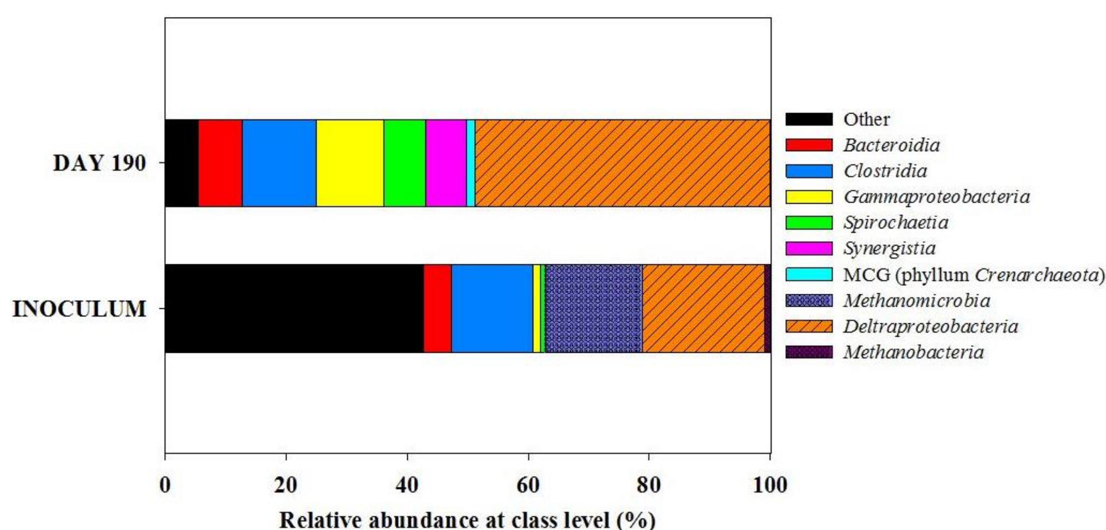


Figure 5.6. Microbial diversity in the UASB reactor at class level.

As the operation proceeded, in the biomass community of day 190 (Tables 5.5 and 5.6), *Desulfovibrio* was the most abundant OTU at genus level, with a 35.3 % of total retrieved sequences. In the case of *Proteobacteria* that were not SRB, the highest relative proportion of microorganisms belonged to the *Enterobacteriaceae* family (11 %).

Table 5.5. The most abundant OTUs found in the inoculum with relative abundance higher than 1 %.

Phylum	Class	Order	Family	Genus	Relative Abundance %
Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	19.63
Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*	15.88
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophobacteraceae*	*Syntrophobacter*	9.64
Proteobacteria	Unassigned	Unassigned	Unassigned	Unassigned	6.90
Firmicutes	*Clostridia*	Unassigned	Unassigned	Unassigned	6.57
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	Unassigned	Unassigned	4.33
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	3.32
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Smithella*	3.00
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	2.71
Chloroflexi	*Anaerolineae*	*Anaerolineales*	Unassigned	Unassigned	1.99
Firmicutes	Unassigned	Unassigned	Unassigned	Unassigned	1.80
Bacteroidetes	*Sphingobacteriia*	Unassigned	Unassigned	Unassigned	1.38
Proteobacteria	*Deltaproteobacteria*	Unassigned	Unassigned	Unassigned	1.10
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobulbus*	1.09
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*	1.06
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	1.06
Other	Other	Other	Other	Other	18.55

Table 5.6. The most abundant OTUs found in sample of day 190 of the UASB operation with relative abundance higher than 1 %.

Phylum	Class	Order	Family	Genus	Relative Abundance %
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	35.30
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	Other	11.12
Firmicutes	*Clostridia*	*Clostridiales*	Other	Other	8.58
Proteobacteria	*Deltaproteobacteria*	Other	Other	Other	8.29
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Dysgonomonas*	6.89
Spirochaetes	*Spirochaetes*	*Sphaerochaetales*	*Sphaerochaetaceae*	*Treponema*	5.49
Synergistetes	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	Other	2.99
Synergistetes	*Synergistia*	*Synergistales*	Other	Other	2.53
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	Other	Other	1.84
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfatirhabdium*	1.81
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Oscillospira*	1.67
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophobacteraceae*	*Syntrophobacter*	1.63
Spirochaetes	*Spirochaetes*	*Sphaerochaetales*	*Sphaerochaetaceae*	*Sphaerochaeta*	1.54
Crenarchaeota	*MCG*	*Other*	Other	Other	1.38
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	Other	1.29
Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*	1.10
Other	Other	Other	Other	Other	6.54

5.4. Discussion

5.4.1 Start-up of a sulfate reducing UASB reactor

In practice, start-up of full-scale UASB reactors for sulfate reduction is handicapped because of the lack of reactors from where inocula with a high density of SRB can be withdrawn. Few works have reported the start-up of sulfidogenic reactors with inocula that have not been adapted to sulfidogenic conditions (Omil et al., 1998; García-Solares et at., 2014). Inoculation with methanogenic sludge from widespread, full-scale mesophilic anaerobic digesters is the most common alternative and, probably, the only alternative in practice at full-scale. The evolution of the UASB performance observed in Figure 5.3 shows that stable sulfate removal efficiencies higher than 80 % were achieved just one month after the continuous operation of the UASB reactor initially inoculated with granular sludge from an anaerobic digester treating wastewater in a pulp and paper industry. The inoculum was not pre-adapted, but sulfate reduction started almost from the beginning of the operation since sulfate was present in the wastewater from the pulp and paper industry and, consequently, sulfate-reducing bacteria. This is in agreement with Roest et al. (2005), who stated that anaerobic digesters sludge from paper mill industries are suitable for providing an appropriate process culture to promote sulfidogenesis.

Compared to previous works, such short and efficient start-up was remarkable considering the source of the inoculum used. As an example, Gonçalves et al. (2005) needed over 6 months to bioactivate an UASB to obtain anaerobic sulfidogenic sludge able to degrade 400 mg SO_4^{2-} L^{-1} using molasses as carbon source. Bertolino et al. (2015) also needed over 200 days to enrich granular sludge from an UASB treating domestic wastewater during the treatment of 0.67 g S-SO_4^{2-} L^{-1} influent with pure glycerol. In our work, the granular sludge used as inoculum was mainly methanogenic, which was confirmed through

Illumina sequencing. *Methanosaeta* and *Methanobacterium* were the most abundant methanogens at genus level in the inoculum, what was expected as they are the most characteristic archaeal sequences found in anaerobic digesters (Leclerc et al., 2004), while a reduced amount of SRB were found. Figure 5.4A shows that the maximum flow of methane was produced during period I and II due to the influence of the inoculum. During period I (OLR of 12.0±2.1 kg O_2 m^{-3} d^{-1}) and II (OLR of 15.8±4.6 kg O_2 m^{-3} d^{-1}), the average organic matter consumption was 86 % and 89 % respectively. COD concentrations were below 100 mg O_2 L^{-1} in the effluent, which probably corresponded to the less biodegradable matter contained in crude glycerol considering that the anaerobic biodegradability of crude glycerol due to presence of such inhibitory impurities has been reported to be between 65-85 % (Viana et al., 2012). Furthermore, 10 % of the oxidized organic matter was used for methane production during period I (Table 5.4). Similarly, during period II, 11 % of the transferred electrons were utilized for methane production. Despite such methanogenic activity, Figure 5.3 shows that a stable operation in terms of sulfate reduction was reached by the end of period II with almost complete removal of sulfate and COD (S-RE of 96.5 % and COD-RE of 89.3 %). Despite methane production, results confirmed that organic substrates were available for sulfate reduction and that microbial communities underwent a fast and gradual acclimation to their environment. Results obtained in this chapter demonstrates that using methanogenic granular sludge from a paper and pulp industry leads to a fast start-up of sulfidogenic UASBs when moderate inlet sulfate concentrations of 235±17 mg S-SO_4^{2-} L^{-1} are treated using crude glycerol as electron donor at C/S ratio of 3.8 g O_2 g^{-1} S.

5.4.2 Shifts in the organic matter sink

The sink of the organic matter can shift drastically due to the evolution of the microbial populations, which influences the performance of the UASB reactor.

During Period III, a high S-RE was reached after few days of operation which allowed obtaining a maximum S-EC of 6.6 kg S-SO_4^{2-} m^{-3} d^{-1} (273 g S-SO_4^{2-} m^{-3} h^{-1}) from this period (Period III). Compared to previous periods, the COD-RE dropped drastically (Figure 5.4A) and an accumulation of VFA was observed (Figure 5.4B), which indicated that a steady, almost complete sulfate reduction, could be reached at a SLR of 4.6 kg S-SO_4^{2-} m^{-3} d^{-1} and COD/S ratios lower than 5 g C g^{-1} S. As previously described by Pol et al. (1998), when a sulfate-rich wastewater is fed into an anaerobic reactor, organic matter will be removed both via methanogenesis and sulfate reduction and when methanogenesis becomes suppressed then a gradual decrease in the organic matter conversion (COD removal) is observed, which was corroborated herein with crude glycerol instead. During this third period (OLR of 24.4 kg COD m^{-3} d^{-1}), the average organic mater consumption was 38 % while only 2.3 % of the COD removed ended in methane production when the COD/S was increased to 5.4 g O_2 g^{-1} S (Tables 5.2 and 5.4). Taking into account that the reported cellular yield for acidogenic bacteria (0.14-0.17 g VSS/g COD) is five times higher than that of acetogenic bacteria (0.025-0.051 g VSS/g COD) or methanogenic archaea (0.01-0.054 g VSS/g COD) (Pavlostathis and Giraldo-Gomez, 1991), glycerol will most probably be readily available for acidogenic bacteria, and the limiting step will be the methanogenesis.

During the anaerobic digestion of glycerol, some organic acids (acetic, propionic, butyric, valeric and others), produced by fermentative acidogenic bacteria, cannot be consumed by methanogenic archaea at the same rate at which they are produced (Viana et al., 2012). The accumulation of VFA indicated that the slowly growing methanogens could not sufficiently and rapidly metabolize the intermediate products from VFA producers (acidogenic and acetogenic populations). Since acetate is mainly converted by methanogens, and no methanogens were found in the sludge sample from day 190 (Figure 5.6),

increasing concentrations of acetic acid were found in the reactor between period III and V. This is in agreement with the production of methane measured from the gas phase (Figure 5.4A) and with some authors statements (Harada et al., 1994; Omil et al., 1998), who pointed out that the predominance of SRB over methanogens in sulfate-rich streams is only achieved after long-term operation (more than 100 days) in UASB reactors. As reported with other electron donors (Raskin and Rittmann, 1996; Dar et al., 2008), SRB also outcompeted methanogens using crude glycerol.

5.4.3 Long-term UASB performance and microbial diversity changes

High sulfate reduction efficiencies together with VFA accumulation were also observed by Bertolino et al. (2012). From period III onwards, the acetate concentration remained in the 400-1100 mg O_2 L^{-1} range. Although the S-RE was significant in Period III (80 %), it progressively decreased to below 80 % (even below 50 % in periods V and VI). Despite some SRB are able to oxidize acetate to CO_2 (Widdel and Pfennig, 1982; Szewzyk and Pfennig, 1987; Muyzer and Stams, 2008), only incomplete oxidizers were detected in the 190-day sample (Table 5.6). Consequently, promoting acetate-oxidizing SRB may be an alternative to increase the sulfate reduction, producing concomitantly a less C loaded effluent. Most of the COD used for methane production lead to acetate and propionate accumulation from Period III onwards (Table 5.4) together with an evolution of the microbial diversity (Figure 5.6). *Deltaproteobacteria* was the most abundant class after 190 days of operation. Many genera such as *Desulfovibrio, Desulfobacter* and *Desulfuromonas* belong to this class and play a fundamental role in the sulfur cycle. Oude Elferink et al. (1994) reported that *Desulfovibrio* had higher affinity for sulfate and higher growth rate than other SRB genera such as *Desulfobulbus* and *Desulfobacter*. Interestingly, *Desulfatirhabdium* sp. accounted for 2 % of total operational taxonomic units at genus level which has been described as butyrate-

oxidizing bacteria (Balk et al., 2008). Only 4 % of reads were not identified at class level, compared to the 31 % of reads not identified in the inoculum sample. This result indicated that the microbial community specialized in more specific functions and that populations were selected according to operating conditions.

As acclimation proceeded under the high TDS concentration reached during the operation of the reactor, methanogens were completely washed out. In the presence of sulfate, SRB usually outcompete methanogens, which only dominate in a low-sulfate environment (Oude Elferink et al., 1994). In general, sulfate-reducing bacteria can grow with a much wider substrate range than methanogens (Muyzer and Stams, 2008). Consequently, methanogenic communities require syntrophic associations, which are not essential in sulfate reducing environments (Janssen et al., 2009). Several SRB are able to use glycerol as an electron donor and some *Desulfovibrio* species have been reported to grow with glycerol (Stams et al., 1985; Kremer and Hansen, 1987; Esnault et al., 1988). As reported by Hu et al. (2015) and Lens et al. (1998b) the complete or incomplete oxidation of organic substrates accomplished by some species of SRB will depend on the COD/SO_4^{2-} ratio in the influent. Then, with the results obtained in this chapter, it is reasonable to conclude that SRB always performed incomplete oxidation at the ratios tested. The disappearance of methanogens and the concomitant accumulation of acetate in the system suggested that methanogens were probably the only microorganisms consuming acetate at observable rates.

It remains an open question how acetate oxidation can be stimulated in order to improve the reactor performance. As an example, Kimura et al. (2006) reported a strong association between two acidophiles, a sulfate reducing bacterium and a non-sulfate reducing bacterium which catalyzed dissimilatory sulfate reduction, using glycerol as electron donor, at pH 3.8–4.2. In pure culture, the sulfate reducing bacterium oxidized glycerol incompletely, producing stoichiometric amounts of acetic acid. However, in mixed culture with a non-

sulfate reducing acidophile, acetic acid was present only in small concentrations and its occurrence was transient. Despite this example and some other promising attempts that have been made (Lens et al., 1998b), further development of strategies for the bioaugmentation of acetotrophic SRB are warranted to increase the sulfidogenic capacity of the process.

Also, despite VFA accumulation has been regarded as a sign of process failure in anaerobic digestion, VFA accumulation can be seen as an opportunity in sulfate-reducing UASBs since VFA have important biotechnological potential; these carboxylates can be used as substrates to produce biofuels and bioplastics, or in other bioprocesses. The loss of organic matter from the UASB reactor is economically undesirable since the reducing power supplied with glycerol is only partly used. In addition, further resources must be used to treat the excess of COD from the anaerobic reactor. In the sulfur recovery process depicted in Figure 5.1, a reduction-oxidation bioprocess is proposed. Then, the COD in the effluent could be treated in the CSTR reactor for the partial oxidation of sulfide to elemental sulfur although an extra consumption of oxygen to treat COD would be required. Consequently, optimization in the use of the electron donor needs to be warranted.

5.4.4 Potential process limitations

Inhibitory substances are often found to be the main cause of anaerobic reactor disturbance and failure as they cause an adverse shift in the microbial population or inhibition of bacterial growth (Chen et al., 2008). Inhibition of anaerobic digestion is usually diagnosed by a decrease of the steady-state rate of methane gas production and accumulation of organic acids (Kroeker et al., 1979), which was found in the long-term operation of the UASB. Organic acid and methane forming microorganisms differ widely in terms of physiology, growth kinetics,

and sensitivity to environmental conditions (Pohland and Ghosh, 1971). At pHs below 7.0, most carboxyl groups are undissociated, thus they pass freely through the membrane and can inhibit the growth of many bacteria. Uncharged molecules such as acetic acid may be inhibitory because they diffuse across the cell membrane and act as an uncoupler (Ghose and Wiken, 1955), whereas acetate ion is not permeant. Inhibition concentrations of $4.68 \cdot 10^{-3}$ mg free acetic acid L^{-1} (pH=7.5) have been reported to block acetoclastic methanogenesis (Fukuzaki et al., 1990). Despite the buffer used, the pH in the reactor varied from 8.4-8.7 at the inlet to 6.7-7.5 at the outlet due to VFAs accumulation, and particularly acetic acid. Considering the pk_a of acetic acid (4.76) and the concentrations of acetate found in the early stages of period III (around 375 mg acetate L^{-1}), concentrations above 0.23 mg free acetic acid L^{-1} found in the UASB could have led to a significant inhibition of methanogenesis. In addition, Koster et al. (1986) reported that free H_2S concentrations leading to 50 % inhibition of methanogenesis were 250 mg S L^{-1} in the pH range 6.4–7.2 and 90 mg S L^{-1} at pH=7.8–8.0. Therefore, indicating that methanogens were also inhibited by sulfide accumulation in the early stages of Period III. Overall, TDS and acetate accumulation lead to a fast decrease of the methanogenic activity in Period III. No methane production from period IV until the end of the operation indicated that methanogenic communities were more susceptible to dissolved sulfide concentration than SRB as was also observed in Jing et al. (2013).

Despite the high concentrations of acetate found in the reactor during period III, IV and V (400-1100 mg O_2 L^{-1}), sulfate reduction proceeded at high S-RE during period III, indicating that SRB were not affected by acetic acid. Similarly to methanogens, free H_2S may inhibit SRB. However, inhibitory free H_2S concentration in literature are often contradictory and confusing probably due to the difference in anaerobic inocula used, the susceptibility of anaerobes and the experimental methods and conditions tested in each study, and particularly the

pH in the bioreactor. Anaerobic treatment of sulfate-rich wastewater proceeds successfully at COD/SO_4^{2-} ratios lower than 10 g O_2 g^{-1} SO_4^{2-} when precautions are taken to prevent sulfide toxicity (Pol et al., 1998). The TDS during the operation reached 460 mg S L^{-1} by day 240 (Period V). Reis et al. (1992) found that more than 547 mg H_2S L^{-1} can completely inhibit SRB activity at pH 6.2, whereas at pH 9.0, dissolved H_2S is mainly in the form of HS^-, which does not penetrate into cells easily (Mora-Naranjo et al., 2003) and therefore would not have a strong inhibitory effect over SRB. As Reis et al. (1992) observed, sulfate uptake decreased when sulfide concentration in the medium increased, and increased again when it was removed from the medium, which pointed out that sulfide is an inhibitor factor for SRB. A pH range of 6.7-6.8 at the outlet of the reactor pointed out at a reduction of the potential maximum SRB rates due to inhibition of SRB by hydrogen sulfide.

5.4.5 Overall performance of the sulfidogenic UASB

Sulfidogenesis was achieved through adaptation of methanogenic granular sludge, using electrons derived from substrate towards sulfate reduction. While adapting methanogenic granular sludge to sulfate reduction is one of the most common and widespread procedures to engineer microbial sulfate reduction (García-Solares et al., 2014), the stability of the system during long-term operations is still a cause of concern. During period V, SLR was maintained during 50 days but the high sulfate inlet concentration affected the sulfate-reduction efficiency. The system was overloaded and its maximum capacity, 6.5 kg S m^{-3} d^{-1}, was reached after 165 days of operation at a SLR of 6.7 kg S-SO_4^{2-} m^{-3} d^{-1} and a COD/S of 5.6 g O_2 g^{-1} S. Overall, the performance of the UASB is comparable to that obtained by Bijmans et al. (2008) (9.7 kg S m^{-3} d^{-1}) using formate, which is more biodegradable than crude glycerol. Higher S-ECs were found compared to Boshoff et al. (2004) (0.6 kg SO_4 m^{-3} d^{-1}), although they

used tannery effluent as carbon source, which is less biodegradable than crude glycerol.

After a long-term operation of 360 days, short-term SLR assays (Figure 5.5) were performed to study the capability of the UASB to reduce sulfate at such a low HRT (2 h) and under dynamic conditions with non limiting COD availability. The UASB adapted well to transient load changes and, more interestingly, recovered to the initial load exhibiting a 25 % higher S-RE compared to that before the short-term experiment. However, it remains to be investigated why such temporary load decrease resulted apparently beneficial for the UASB performance considering that the same sulfide concentration was found before and after the stepwise decrease of the inlet sulfate concentration. Based on a sulfur balance, a larger C/S ratio during the short-term experiment (up to 19.1 g COD g^{-1} S) could have led to an increase in the production of organosulfur compounds. Overall, a sulfur balance of 85-95 % along the UASB operation was obtained based on inlet and outlet sulfate ($S-SO_4^{2-}$) concentrations and produced TDS. Such imbalance was attributed to other organosulfur compounds such as dimethyl sulfide (DMS) or dimethyl disulfide (DMDS) amongst others that were qualitatively detected in the effluent of the UASB (see Table 5.7). Organosulfur compounds, also called organic sulfur compounds, are a subclass of organic substances that contain sulfur and that are known for their varied occurrence and unusual properties. They are present in the bodies of all living creatures in the form of certain essential amino acids (such as cysteine and methionine, which are components of proteins) and of enzymes, coenzymes, vitamins, and hormones. Vairavamurthy and Mopper, (1987) reported that H_2S formed from bacterial sulfate reduction can become incorporated into organic matter during early diagenesis. However, little is known about the exact chemical mechanisms by which this sulfur reacts with sedimentary organic matter. Some cyclic disulfides

and polysulfides can be formed by reaction of elemental sulfur with unsaturated compounds as fatty acids (Zhu et al., 2019)

Table 5.7. Organic volatile compounds with sulfur found during the operation of the reactor.

Compound	
Carbon sulfide	S=C=S
Carbonyl sulfide	O=C=S
Dimethyl sulfide	H_3C-S-CH_3
Dimethyl disulfide	H_3C-S-S-CH_3
Dimethyl trisulfide	H_3-S-S-S-CH_3

5.5. Conclusions

Long-term operation of a sulfidogenic UASB reactor can be successfully achieved using crude glycerol as carbon source at low up-flow velocities. During the long-term operation a maximum S-EC of 6.6 kg S-SO_4^{2-} m^{-3} d^{-1} (273 g S-SO_4^{2-} m^{-3} h^{-1}) was obtained. Compared to previous works, a remarkable and efficient start-up was obtained considering the source of the inoculum used. Despite methane production during the first operational periods, results confirmed that organic substrates were available for sulfate reduction and that microbial communities underwent a fast and gradual acclimation to their environment. It was demonstrated that at OLR above 24 kg O_2 m^{-3} d^{-1} and SLR of 4.6 kg S-SO_4^{2-} m^{-3} d^{-1} VFA were accumulated. Consequently, COD-RE dropped drastically, which indicated that a steady, almost complete sulfate reduction could be reached at a SLR of 4.6 kg S-SO_4^{2-} m^{-3} d^{-1} and COD/S ratios lower than 5 g C g^{-1} S. VFA accumulation was related to the disappearance of methanogenic activity and when methane production decreased, glycerol was converted mainly to acetic acid and propionic acid. It was not only the COD/S-SO_4^{2-} ratio, but a sum and combination of factors along the operation that determined the

competition between SRB and methanogenic archaea. *Desulfovibrio* was the most abundant OTU at genus level, with a 35.3 % of the total retrieved sequences. However, more research is needed to understand to which extent is this competition beneficial or, if losing completely the presence of methanogens at such a low up-flow velocity would imply losing S-EC due to other problems. Potentially, diffusional limitations and bed stratification may appear due to the lack of gas bubbles moving upward.

Chapter 6: Microbial evolution and interpretation of the microbial composition in a sulfidogenic UASB reactor

Considering the results obtained in Chapter 5 and with many questions to answer, the main aim of this chapter consisted on exploring the microbial evolution of a sulfidogenic UASB previously studied from an operational point of view. The chapter discusses thoroughly the UASB performance at constant loading rate to confirm tendencies already observed. Moreover, detailed microbiological analyses through sequencing and FISH techniques are performed to obtain information on how the different microbial populations evolve along the long-term performance. The interpretation of the microbial evolution together with physical and chemical parameters is one of the fundamental aspects to gain more knowledge about the process and complement missing information from previous chapters.

Abstract

In this chapter, long-term performance of the UASB reactor used along Chapter 5 and described in Section 4.1.1. was assessed using crude glycerol, as organic carbon source and electron donor but under constant loading rate in this case. The reactor was inoculated with granular sludge obtained from a pulp and paper industry. Apart from the regular analysis of ionic species (sulfate, thiosulfate and sulfide), volatile fatty acids (VFA) and TOC, Illumina analysis of the 16S rRNA gene and fluorescent in situ hybridization (FISH) were used to study the dynamics of the bacterial community and its evolution along the whole operation. FISH analysis was applied as a culture-independent molecular approach using specific alexa fluor labelled oligonucleotide probes. The relative abundance was calculated using EUB338 probes, to detect general eubacteria, and DELTA495a, to detect major species of *Deltaproteobacteria* sulfate reducers. The reactor was sampled along the operation to monitor its diversity and the evolution of targeted species to gain insight into the performance of the sulfidogenic UASB.

6.1 Introduction

Over the last decades, research on microbial communities evolution in engineered biosystems has gained interest since it is of high importance to get them controlled and continuously enhanced. For a long time, biological reactors have been considered as a "black box" where microbiological phenomena taking place were not elucidated (Sanz and Köchling, 2007). This indifference was mainly due to the lack of analytical techniques able to identify those microorganisms playing a role in biodegradation processes. Conventional methods for microbial identification have been classically based on isolation of pure cultures and their consecutive characterization, which is unfeasible in engineered ecosystems as most of the biodiversity is nonculturable (Moter and Göbel, 2000). Only around 1 % of the microorganisms present in the environment can be cultivated using conventional methods such as standard cultivation and plating techniques, which highlights their limitations (Vartoukian et al., 2010). During the 1990s a new set of molecular techniques transformed microbial ecology research (Sanz and Köchling, 2007), overcoming the scarcity of information provided by traditional methodologies. As a result, the most broadly used techniques nowadays are fluorescent in situ hybridization (FISH), cloning or sequencing of 16S rRNA and denaturing gradient gel electrophoresis (DGGE).

Cloning provides very precise taxonomical information, but requires qualified researchers and advanced technologies, which are not always available. DGGE technique quickly provides information from microbial samples thanks to the charasteristic band patterns of each specific sample. Moreover, DGGE enables further genetic analysis of the sample, if required, by sequencing any of the particular appearing bands (Sanz and Köchling, 2007). However, this method also presents drawbacks. Muyzer et al. (1993) advised, as a general rule, that any targeted DNA that is less than 1 % of the total target pool is unlikely to be detected by DGGE. As such, DGGE analyses would only represent predominant

organisms or 'phylotypes' in a community (Green et al., 2015). More important are practical limitations. This method requires well-separated bands that can be extracted from the gel afterwards to be sequenced and identified. When analyzing highly diverse environments, discrete fingerprint bands may not always be discernible, which converts this technique into heavy-handed and rather time consuming.

There is a clear trend among the literature to combine molecular techniques as well as microbiological methods in engineered ecosystems (Teske et al., 1998; Engelen et al., 1998; Brinkhoff et al., 1998). This is important to reduce potential biases and limitations of the different techniques, and hence to obtain a more realistic picture of microbial community structure and function. Therefore, FISH and 16S sequencing have been used along this chapter to get a better understating of the processes taking place in the reactor.

Sequencing of the gene that codes for the 16S rRNA is one of the most widely applied technique for microbial identification. The rRNA molecule has regions which are extremely well conserved among all organisms that share that type of rRNA. At the same time, it has regions which are highly variable and the degree of variation in these regions can vary from one taxon to another. These specific characteristics of the rRNA molecule allow comparing organisms within a single domain and also enable strains, within the same genus or even species, to be differentiated and the microbial diversity to be phylogenetically classified (Woese, 1987). Furthermore, with the current technology, samples can be easily sequenced, and the gene sequences obtained are sufficiently long for the data generated to be statistically relevant. This fact has led to a knowledge expansion in the area of phylogeny (construction of phylogenetic trees) and quantitative schematic representations of the evolutionary diversity (Olsen et al., 1994).

On the other hand, FISH is a fast and powerful technique that allows detecting in situ and quantifying microorganisms present in a wide variety of ecosystems,

including wastewater or activated sludge based systems (Garrido-Cardenas et al., 2017); although this quantification could be either complex or tedious and subjective. FISH is based on the existence of known and specific rRNA sequences of an organism, which allows complementary sequences, known as probes, being designed. These are short sequences of DNA (15-30 nucleotides) labeled with a fluorescent dye which recognize complementary 16S rRNA sequences in previously fixed cells (permeable to the probe). For that reason, some previous knowledge of the expected microorganisms in the sample is often required to successfully apply this identification technique. The lack of probes targeting the desired bacterial taxon or group becomes one of the main limitations of this technique (Dezotti et al., 2017). Although, in theory, it is possible to design the most appropriate probe according to specific needs, it may be impossible to develop a probe that targets certain groups of microorganisms sharing metabolic properties, such as sulfate-reduction capability for example (Sanz and Köchling, 2007).

A better understanding of the biological processes taking place in the UASB reactor operated along this thesis is anticipated to be gained in the present chapter. Chapter 5 highlighted the potential of crude glycerol utilization as a possible carbon source to reduce sulfate in an anaerobic bioreactor (UASB reactor) treating sulfate laden wastes. It also led to several conclusions in terms of reactor design, operation of the reactor and exposure to inhibitors. Nevertheless, almost no information about the links between the microbial community structure and the bioreactor performance is currently available. Therefore, the purpose of this chapter was to: i) evaluate the microbial stratification in the sulfidogenic UASB reactor under low up-flow velocity regime (≈0.25 m h^{-1}), ii) examine the temporal dynamics of the microbial populations during constant TOC/S ratio, and iii) correlate physical-chemical parameters with microbiological changes to explain better the dynamics of the process.

6.2. Materials and methods

6.2.1 Reactor set-up and operation (UASB long-term continuous operation)

A new start-up of the UASB reactor described in Section 4.1.1 of Chapter 4 was set, followed by a long-term operation period of 550 days. The experimental setup and the different conditions used along this chapter were already explained in Section 5.2.1. The composition of the mineral medium was (g L^{-1}): K_2HPO_4 (3), NH_4Cl (0.2) and Na_2SO_4 (1.15) dissolved in tap water to add macro- and micronutrients and adjusted to pH=8.8-9.0 with NaOH (2 M). The reactor was operated at a constant sulfate inlet concentration of 252.8±20.8 mg S L^{-1}, therefore the SLR was 5.1±0.7 kg S-SO_4^{2-} m^{-3} d^{-1}. A constant OLR of 7.6±1.6 kg C m^{-3} d^{-1} was set in order to obtain a steady-state operation and a constant TOC/S ratio of 1.5±0.3 g C g^{-1} S, thus minimizing the effect of changing conditions. Inlet and outlet flows were sampled every two/three days to perform chemical analyses (see Section 4.2.1). The inoculum, 1 L of granular sludge, was obtained from the same anaerobic digester treating wastewater in a pulp and paper industry as the one used to inoculate the UASB reactor in Chapter 5. The hydraulic residence time (HRT), calculated as that corresponding to the reaction volume only (sludge blanket), was ranging between 1 h and 1.8 h. Moreover, inoculum and biomass samples collected along the reactor operation were analyzed to observe the evolution of the different microbial populations through Illumina sequencing and FISH analysis.

6.2.2. Illumina sequencing analysis

Identification of the microbial population was performed using Illumina platform, on different samples, along the operation of the reactor (Table 6.1), at different reactor´s heights (Figure 6.1) and including the inoculum. Different UASB heights were sampled at different operation times because the sludge was

not static. Therefore, obtaining sludge from the same sampling points was not always possible. Further discussion about the stability of the sludge bed will be provided in Chapter 7. Genomic DNA of all samples was extracted by applying the protocol of PowerSoil™ DNA isolation kit (MoBio Laboratories, USA) following the supplier's instructions. The quantity and quality of the extracted DNA were assessed by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and then DNA samples were preserved at -20 ºC for further analysis.

Table 6.1. Biomass sampling days during the long-term operation of the reactor.

Day of operation	UASB height
85	UASB 6
149	UASB 1,2,3
173	UASB 1,2,3
230	UASB 1,4,6
294	UASB 1,2,6
538	UASB 6

For samples collected along the operation (Table 6.1), sequencing analyses were performed by "Genomic and Bioinformatics service" at the UAB University. Amplicon sequencing that targets the V3-V4 hypervariable regions (HVRs) of the 16S rRNA gene on Illumina MiSeq platform were carried out using specific primers (Table 6.2). The primer pair 341F (Bakt_341F)/805R(Bakt_805R) used in this protocol was selected from Klindworth et al. (2013) as the most promising bacterial primer pair. With the intention of having a more detailed information about archaeal communities, the 519wF/1017R primer pair suggested by RTLGenomics (Texas, USA) and Dykstra and Pavlostathis (2017) was selected. The database used for the classification of organisms is based on the Greengenes database (http://greengenes.lbl.gov/).

Table 6.2. Sequencing primers used in this study.

Primer	Sequences (5′- 3′)
341F	CCTACGGGNGGCWGCAG
805R	GACTACHVGGGTATCTAATCC
519wF	CAGCMGCCGCGGTAA
1017R	GGCCATGCACCWCCTCTC

GC-clamp (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) is attached to the 5′ end of the gene-specific sequences.

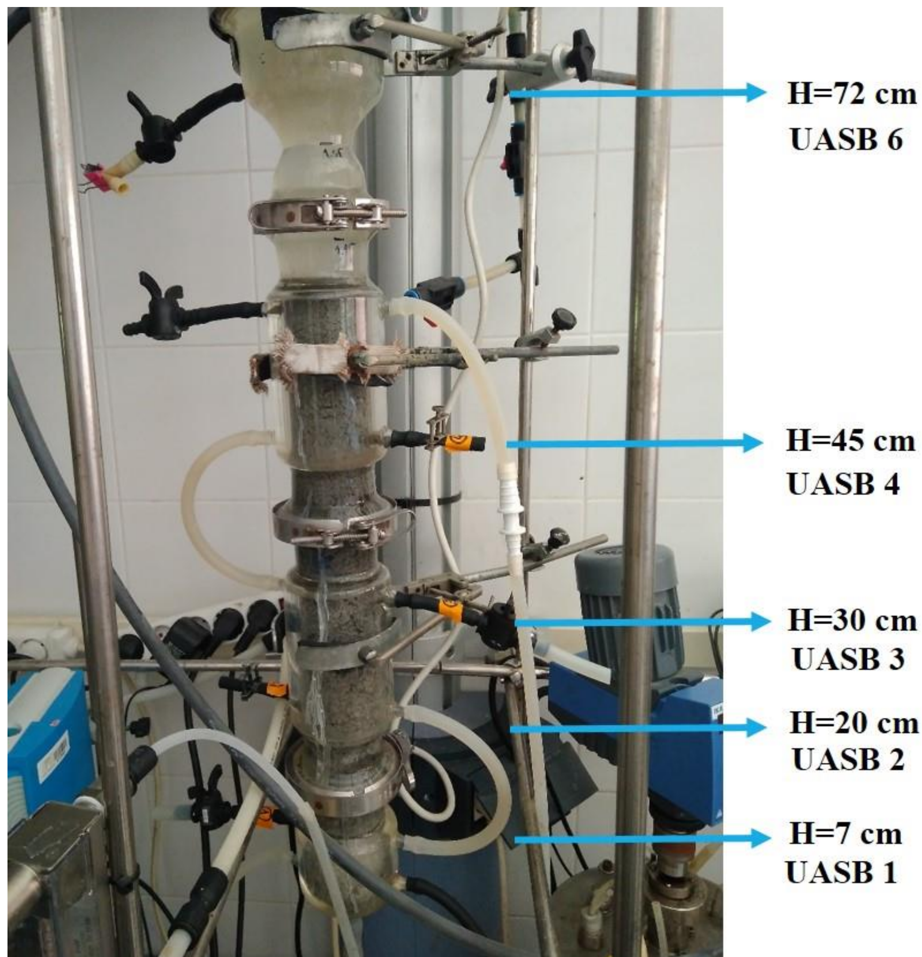


Figure 6.1. UASB reactor with sampling points at different heights (H).

The coverage of the primers was checked using the test prime tool in Silva's website (www.arb-silva.de), setting the following parameters as recommended by the testprime tutorial: 1 mismatch for the maximum number of mismatches and 5 bases for the length of 0-mismatch zone at 3′end. As a result, the primer pair 341F/805R presented 66.6 % of coverage for Archaea and 92.4 % for Bacteria.

Specifically, for *Methanobacteria* the coverage was 92.8 % and 85.5 % for *Methanomicrobia*. Regarding primers 519wF and 1017R, the coverage was 88.4 % for Archaea in particular, 94.6 % for *Methanobacteria* and 93.6 % for *Methanomicrobia.*

Apart from the samples on Table 6.1, a sample of the inoculum was collected to perform also Illumina sequencing analysis. This time, sequencing was performed using a MiSeq System by an external service (Scsie UV, Valencia, Spain). The high throughput sequencing was carried out by amplifying the V3-V4 hyper variable region of 16S RNA gene of the extracted DNA with the universal primers 341F (5'- CCT ACG GGN GGC WGC AG-3') and 806R (5'- GGA CTA CHV GGG TAT CTA AT-3').

6.2.3. Fluorescence in situ hybridization (FISH)

FISH was performed the same days that samples were collected for Illumina sequencing analysis (Table 6.1). For the selection of the probes, raw data from the sequencing analysis of Chapter 5 was used. These sequences were used to look for appropriate probes in the database of rRNA-targeted oligonucleotide probes and primers probeBase (Greuter et al., 2016). The selected probes are presented in Table 4.3 of the general materials and methods section (Chapter 4). In silico, predictions of the proper probes and hybridization stringency are still not sufficiently accurate, and thus optimization of probe specificity and sensitivity was performed experimentally. The general bacteria probe (EUBmix) was an equal mixture of probes EUB338I, EUB338II and EUB338III. For probes requiring different stringency, a successive hybridization procedure was followed (Wagner et al., 1994). The whole protocol is explained in detail in Section 4.3.1 of Chapter 4. To deal with the problem of autofluorescence, different samples were used to perform preliminary tests. Autofluorescence typically (but not always) has a broad emission spectrum. The choice of fluorochromes is important if low

fluorescent signals occur (Nielsen et al., 2009). In our case, auto fluorescent cells were initially checked. The emission spectrum of samples exhibiting auto fluorescence was checked with a spectral analysis (λ scan function) at 405 nm in a range between 425 and 785 nm in a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystem Heidelberg GmbH; Mannheim, Germany) to determine their maximum emission. Afterwards, fluorochromes were selected and are presented in Table 6.3 for each of the different probes. For the nonsense probe (NONEUB338), the same fluorescing fluorochrome as the one used with the general probe (mix EUB) was chosen.

Table 6.3. FISH probes used in this thesis with their corresponding fluorochrome.

Probe	FA (%)	Fluorochrome
EUB mix	0-50	Alexa-488
NONEUB	Not determined	Alexa-488
DELTA495a	35	Alexa-647
EURY514	20	Alexa-594

6.3. Results

6.3.1. Long-term performance of the UASB at constant loading rate

A new UASB performance, at constant sulfate loading rate, was evaluated during 550 days of continuous operation in terms of sulfate removal efficiency (S-RE), TOC removal efficiency (TOC-RE) and sulfate and TOC elimination capacities (S-EC and TOC-EC, respectively). Table 6.4 shows the results obtained from the long-term UASB operation as averages and standard deviations of all data acquired. The operation was divided into 3 different periods according to the stability of the performance. Period I (day 0-250) corresponds to the start-up of the reactor together with the period in which the reactor was performing

positively obtaining excellent values of removal efficiencies; Periods II and III correspond to a progressive decline in the removal efficiencies leading to an unstable and failured operation.

Table 6.4. Removal efficiencies and elimination capacities obtained during the system operation.

Period	Days	TOC/S (g C g^{-1} S)	S-EC (kg S m^{-3}d^{-1})	TOC-EC (kg C m^{-3}d^{-1})	S-RE (%)	TOC-RE (%)
I	0-250	1.4±0.3	4.4±1.3	5.3±2.1	79.8±16.8	70.0±26.8
II	250-400	1.7±0.3	2.5±0.7	1.4±1.1	52.0±14.5	16.4±10.7
III	400-550	1.4±0.3	1.6±0.4	1.3±1.1	32.6±7.5	18.2±12.8

Results of the monitoring of sulfur species is presented in Figure 6.2, while TOC profile and the concentration of each VFA monitored together with the average flowrate of methane can be observed in Figure 6.3.

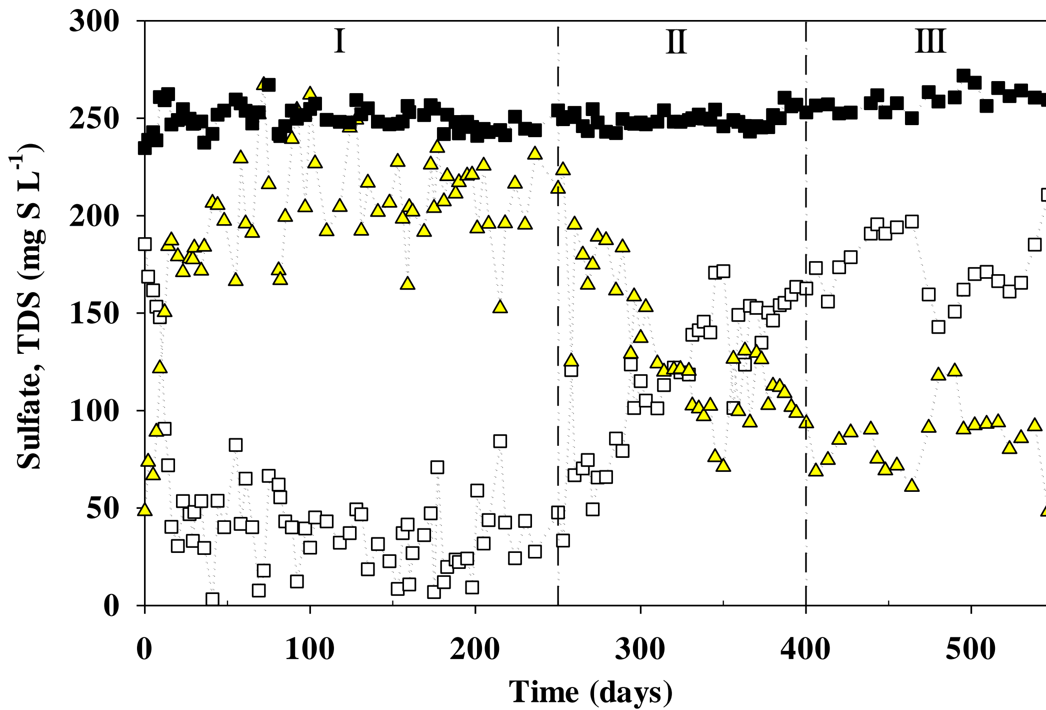


Figure 6.2. UASB performance during the long-term operation at constant sulfate loading rate. Sulfate concentration in the influent (■) and in the effluent (□), total dissolved sulfide concentration in the effluent (△). Lines represent the different periods considered.

TOC/S ratio was constant during the whole operation with the intention of working with stable conditions in terms of carbon and sulfate inlet loading rates, thus allowing the reactor and the evolution of the populations to evolve without changing scenarios. As shown in Table 6.4, a clear difference can be seen between each operating period in terms of efficiencies, even if inlet conditions were constant along the whole operation. Period I represents the start-up of the reactor together with a promising performance; Period II is a transition period between the good performance and the complete failure of the operation confirmed in period III. As can be observed in Figure 6.2, sulfate reduction started almost immediately after the start-up of the reactor, something that occurred also in the previous operation (Chapter 5). Outlet sulfate decreased steadily until reaching an almost steady state along the first 250 days of operation. From day 0 until day 250 (period I), the average sulfate removal efficiency was 79.8±16.8 % while from day 251 until day 400 (period II) the efficiency was 52.0±14.5 %, and in period III it was 32.6±7.5 % indicating a huge sulfate efficiency loss. This efficiency decrease in the long run was also observed in Chapter 5, ending up with a decrease in the efficiency of the whole process, in both cases. This common trend and the accumulation of several problems detected in the long-term operations will be further discussed in Chapter 7, trying to focus on the causes for these failures.

TOC was almost completely consumed during the first 135 days of operation obtaining values below 43.4 mg C L^{-1} in the effluent (Figure 6.3A). However, a reduction was observed in the TOC removal efficiency that decreased from 70 % in period I to 16.4 % in period II. Sulfate elimination capacity during period I was 4.4 kg S m^{-3} d^{-1}, almost the same value (4.3 kg S m^{-3} d^{-1}) that was obtained during period III in the previous UASB operation (Chapter 5), but with a lower concentration of sulfate in the influent. However, in periods II and III, sulfate elimination capacity decreased to 2.5 kg S m^{-3} d^{-1} and 1.6 kg S m^{-3} d^{-1}, respectively.

This significant decrease of the TOC-RE was coupled to a progressive VFA accumulation, especially acetate (Figure 6.3).

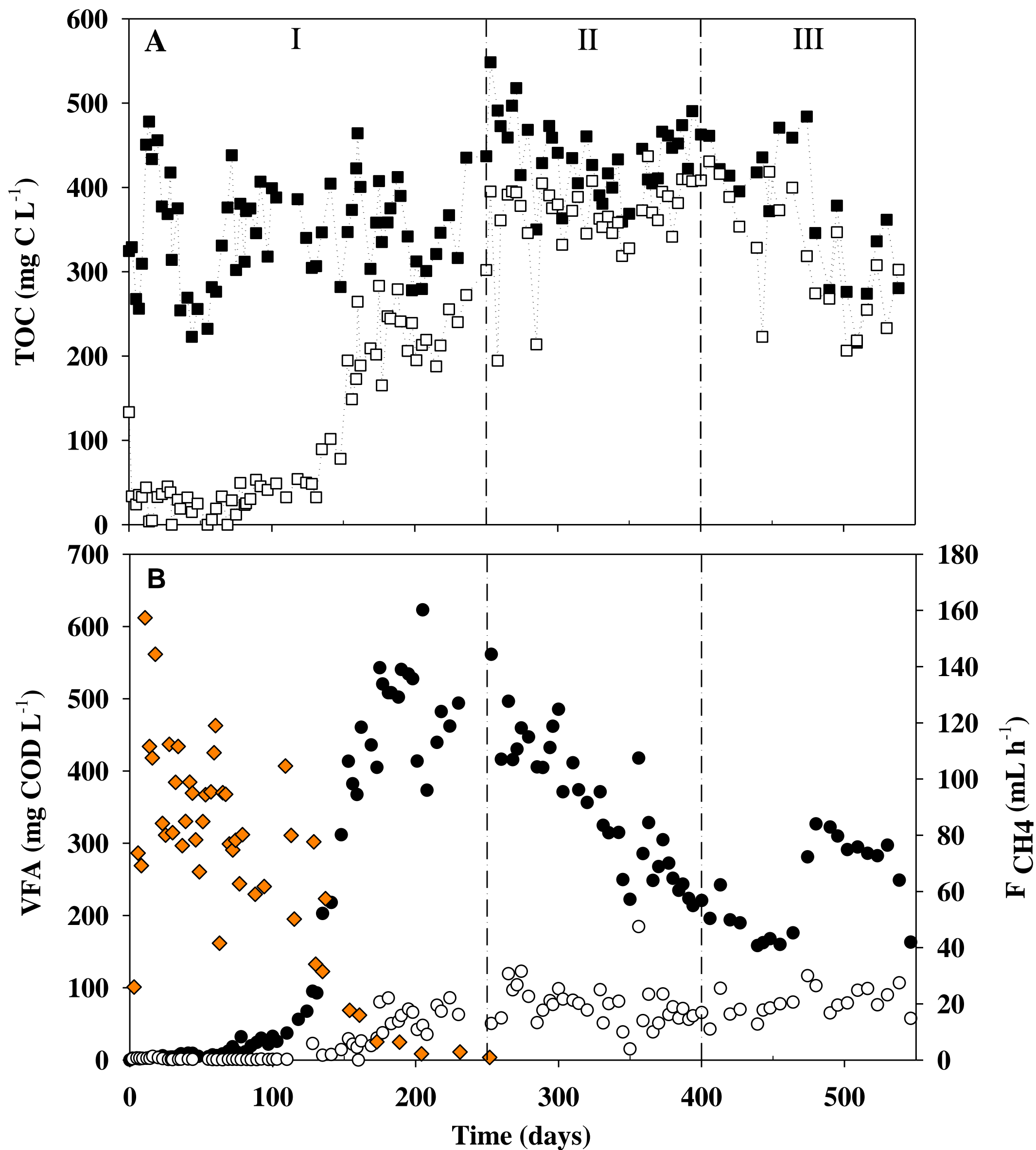


Figure 6.3. Performance of UASB. A) TOC in the influent (■) and in the effluent (□). B) VFA concentration: acetic acid (●) and propionic acid (○) and flow of methane in the gas phase (◆). Lines represent the different periods considered.

At the same time, the increase in acetate concentration coincided with a significant decrease in CH_4 production after 135 days of the start-up of the operation. This behavior is comparable to that noticed in Chapter 5. Even if sulfate concentration in the influent was not as high in this operation

(252 mg S L^{-1}) as the one in Chapter 5 on day 150 (442 mg S L^{-1}), the flow of methane had almost ceased completely by that time in both cases. Sulfide concentration by that day was 100 mg S L^{-1}, lower in this operation, compared to the one in Chapter 5 (303 mg S L^{-1}). Considering that the sulfate load in the influent was also lower in this case, a similar behavior could be observed in both operations.

6.3.2. Microbial evolution: 16S rRNA sequencing

When verifying and validating the size of the library, after the amplicon PCR steps for the 519wF/1017R primer pair, non-specific amplifications were detected (Figure 6.4). Different bands, apart from those with the expected size, were observed in the gel. Therefore, the primer pair 519wF/1017R, that was selected to gain better insight of archaeal communities, was not considered for the sequencing since the raw data could not be processed neither reads classified. As a consequence, Section 6.3.2 provides results obtained with the general primer pair 341F/805R.

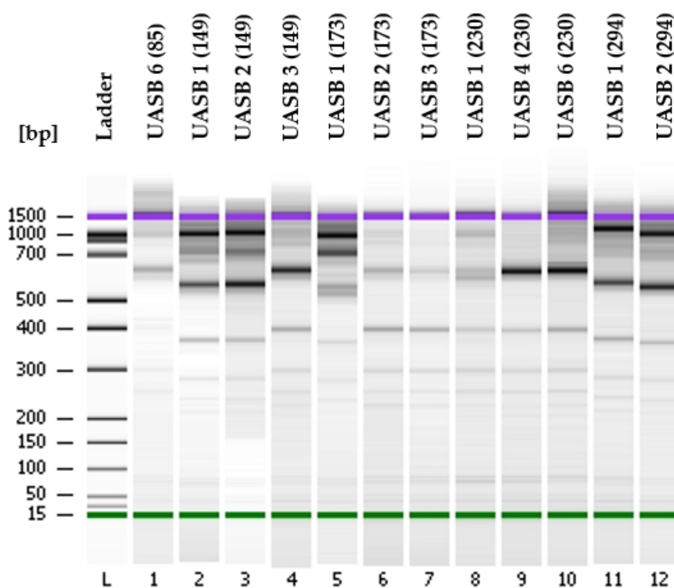


Figure 6.4. Archaeal PCR gel profile of the different UASB samples (see Table 6.1). Sample UASB 6 (294) and UASB 6 (538) are not shown.

- Long- term comparison from UASB 1 and UASB 6

According to the results obtained from the different reactor´s heights and profiles, only results from UASB 1 are considered for this section when samples from different reactor heights were available (days 149, 173, 230 and 294). In the case of samples from days 85 and 538, samples from UASB 6 are presented. The operation of the UASB in which Illumina analyses were held is presented in Figure 6.5 together with the biomass sampling events (arrows).

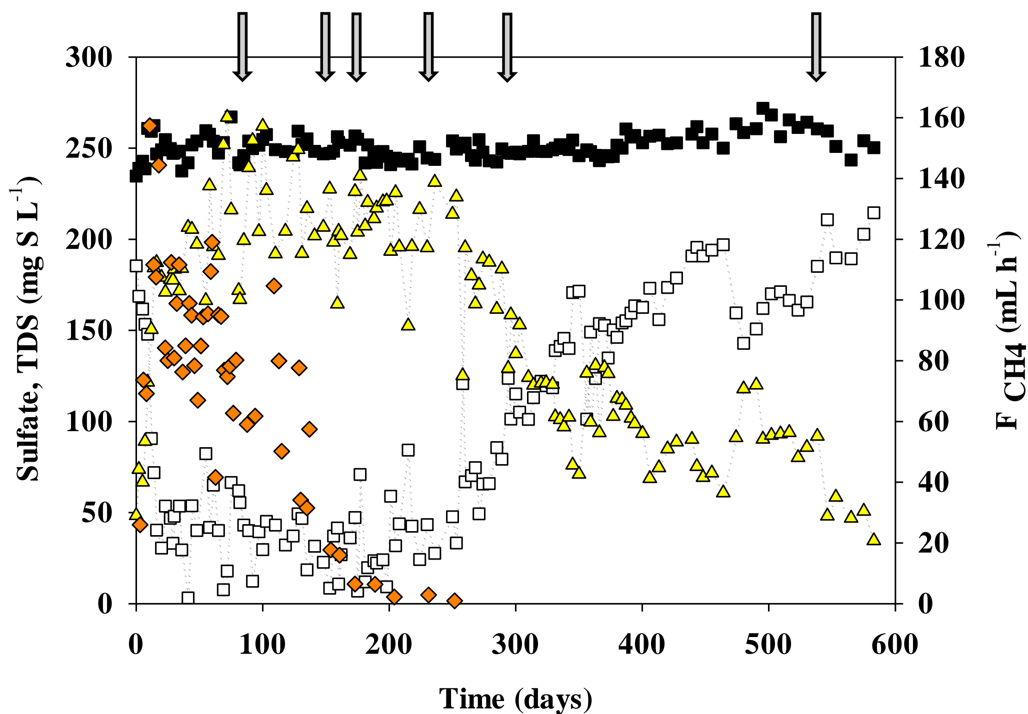


Figure 6.5. UASB performance during the long-term operation at constant sulfate loading rate. Sulfate concentration in the influent (■) and in the effluent (□), total dissolved sulfide concentration in the effluent (△) and flow of methane in the gas phase (◆). Arrows represent the time when samples for sequencing were collected.

As can be observed from Figure 6.5, samples were collected along the whole operation coinciding with different situations from which Illumina information could be useful to infer conclusions in relation with the performance. On day 85, when the first sample was collected, there was a significant amount of gas

produced (80.2 mL h^{-1}) and increasing sulfate reduction efficiencies were being achieved (82.4 %). Figure 6.6 presents the relative abundances (%) at genus level of all the different samples along the operation of the reactor.

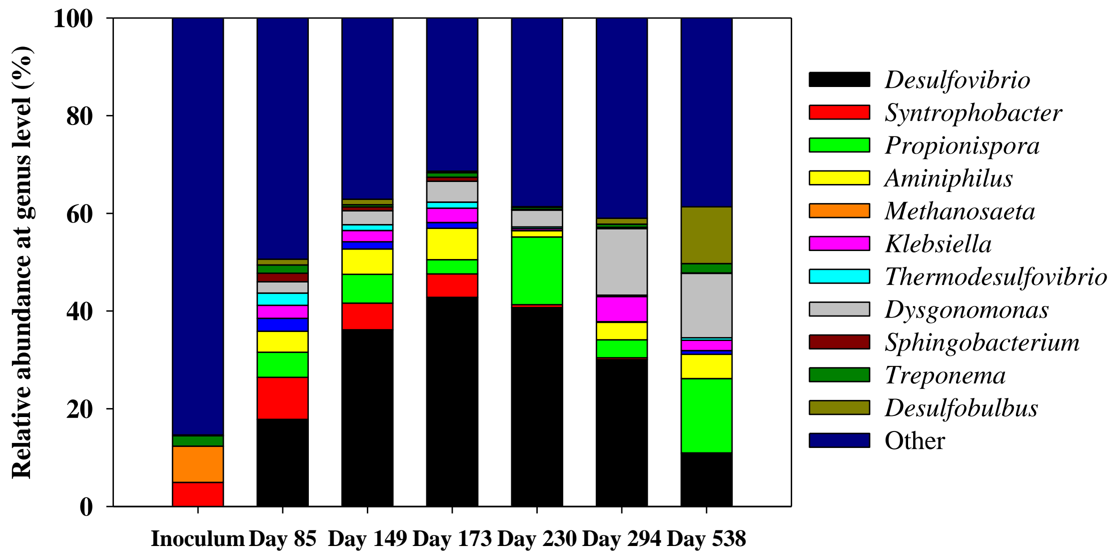


Figure 6.6. Relative abundances (%) at genus level of all the different samples along the operation of the reactor.

As can be observed, on day 85, *Desulfovibrio* was the most abundant genus detected, with a relative abundance of 17.8 %. Considering that the inoculum came from an anaerobic digester, where only a 0.02 % of relative abundance of genus *Desulfovibrio* could be detected, it is a remarkable aspect. In less than 100 days this genus was able to increase its relative abundance noticeably. *Syntrophobacter* and *Propionispora* were the following most abundant genus detected in this sample with a relative abundance of 8.6 % and a 5.1 %, respectively. On day 149 both TOC-RE and S-RE were the highest of the different sampling events, 90.8 % and 72.3 % respectively. Methane production was almost depleted by that time. On the other hand, acetate was starting its accumulation in the system and its concentration was 292.3 mg L^{-1}. On that day, *Desulfovibrio* was the most abundant genus detected, with a relative abundance of 36.2 %. *Propionispora*, *Syntrophobacter* and *Aminiphilus* were the next ones in order of relative abundance with a 5.9, 5.4 and 5.2 %, respectively. On day 173, acetate

concentration was 379.9 mg L^{-1} and TOC removal efficiency had decreased to 43.7 %. According to sequencing results, on that day, *Desulfovibrio* was the most abundant genus detected with a 42.8 %, the highest value among all the samples considered.

On day 230, acetate accumulation was reaching its highest value, registered on day 253 (526.6 mg L^{-1}). From day 230 onwards, no methane was produced. The relative abundance of *Desulfovibrio* by that day was 40.7 %. *Propionispora* was the next genus with higher abundance, 13.9 %. By day 294, TOC-RE had decreased significantly, reaching a value of 17.4 % whereas S-RE was 50 %. Acetate concentration measured that day was 405.6 mg L^{-1}. *Desulfovibrio* decreased its relative abundance to 30 % and *Dysgonomonas* became the next genus in order of abundance with a 13.6 %. On day 538, UASB performance was failing, obtaining a S-RE of 28.9 %. According to the results gathered by Illumina, *Propionispora* was the most abundant genus with a relative abundance of 15.2 % 13.2 %, followed by *Dysgonomonas* (13.2 %) and *Desulfobulbus* (11.6 %). *Desulfovibrio* decreased its relative abundance until 10.8 %. Having a look at the domain *Archaea*, only *Methanosaeta* genus was detected with a relative abundance higher that 1 %. Therefore, this genus is the only one shown in Figure 6.6. *Methanosaeta* was the most abundant group in the inoculum, but its relative abundance decreased along time, being almost undetectable in samples from day 230 onwards, indicating that these populations were being washed-out from the system.

To get a better comparison between two groups of interest in our case, Table 6.5 presents the results of the relative abundances (%) of all the methanogens detected and considered as a group, together with genus *Desulfovibrio*. This genus was selected as it was the main one of all the sulfate-reducers detected during the whole operation. As can be observed from Table 6.5, there was a clear decrease and a remarkable increase in the relative abundance of methanogens and sulfate reducers (*Desulfovibrio)* respectively. In

the long-term a decrease in the relative abundance of *Desulfovibrio* can be also detected.

Table 6.5. Relative abundances (%) of methanogens and sulfate reducers (genus *Desulfovibrio*) on the different samples along the operation of the reactor.

	Methanogens	**SRB (Genus *Desulfovibrio*)**
Inoculum	10.8	0.0
Day 85	2.9	17.8
Day 149	1.5	36.2
Day 173	1.4	42.8
Day 230	0.0	40.7
Day 294	0.2	30.1
Day 538	1.2	10.8

Methanogens are a diverse group of microorganisms and even if substrates that they utilize are very limited, Figure 6.7 presents the major classes detected to get a better insight among this group. This information could be helpful in obtaining an explanation to parameters observed in the long-term operation such as acetate accumulation. *Methanosaeta* was the most predominant genus in the inoculum, with a relative abundance of 7.5 % and was still present until day 173 but not in the sample from day 230. *Methanobacteria* class was the next one in order of abundance detected in the inoculum sample with a 2 %. Hydrogenotrophic methanogens, such as the order *Methanomicrobiales*, was only detected in the inoculum sample and with a relative abundance of 0.7 %. Figure 6.7 provides a visual overview on how methanogens were only predominant in the inoculum and how *Desulfovibrio* could colonize quickly the UASB reactor under the operating conditions.

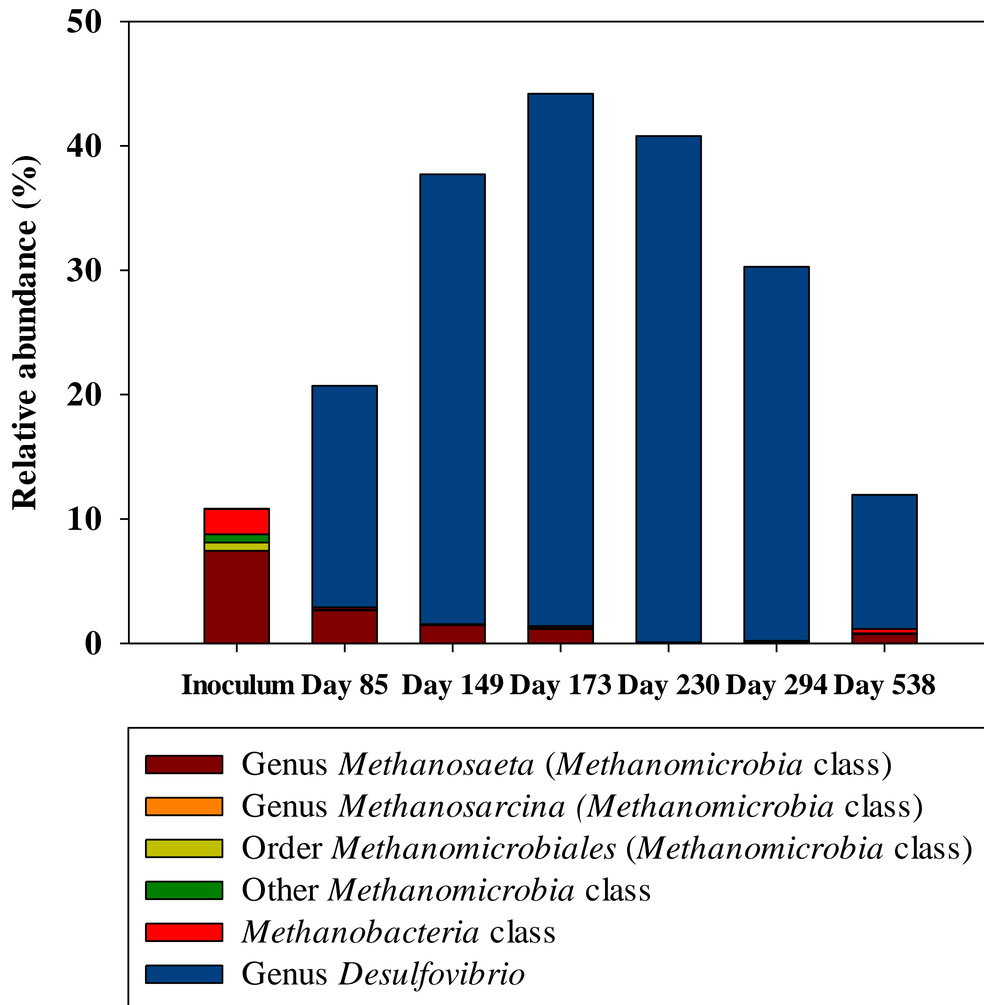


Figure 6.7. Relative abundances (%) of all the methanogens detected in all the samples together with the main sulfate reducer (genus *Desulfovibrio*).

- Different reactor´s heights and profiles

Illumina sequencing analysis were performed through the operation of the reactor to gain more knowledge about how the microbial community evolved with a constant loading sulfate and organic rate and a stable operation in terms of inlet conditions. With that purpose, different samples (Table 6.2) were analyzed throughout the operation and at different reactor´s heights. From all the samples analyzed, only day 173 and day 230 will be considered in this section, as they were considered the more representative ones in terms of profiles along the different heights of the UASB reactor. Figure 6.8 shows the most abundant

genus on day 173 at different reactor´s heights (UASB 1, UASB 2 and UASB 3). It reveals that the most abundant genus on that day was *Desulfovibrio*, and that there was a considerable decrease from UASB 1 and UASB 2 to UASB 3. The relative abundances in samples UASB 1 and UASB 2 were 42.8 % and 49.8 % respectively, whereas in UASB 3 it was only 19.5 %. Another difference that can be observed from Figure 6.8, is the increase in the OTUs detected and assigned to the genus *Syntrophobacter*. The relative abundance of this genus was 6.4 % in UASB 1 and 9.9 % in UASB 3. *Sphingobacterium* genus also increased its relative abundance from 0.8 % in UASB 1 to 5 % in UASB 3.

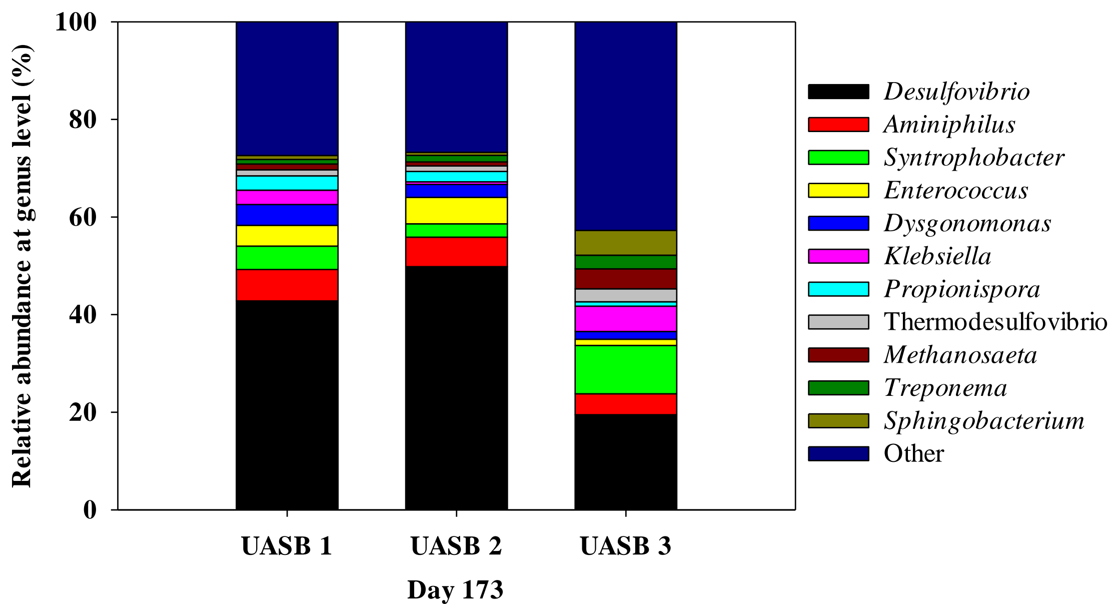


Figure 6.8. Microbial diversity along the sludge bed heights at genus level. Samples were collected on day 173 of the reactor operation.

Figure 6.9 shows the measured concentration for the different sulfur species and VFA obtained from the different reactor´s heights on day 173. The already mentioned decrease in *Desulfovibrio*´s relative abundance along the different heights, can be supported by the different sulfate reduction velocities calculated for these heights. As can be observed in Figure 6.9, almost all the sulfide was produced in the lowest part of the reactor (until UASB 2). From UASB 3 to the outlet, there was no appreciable sulfide production, what can be related with the

fact that in UASB 3 the relative abundance of the mayor sulfate reducer detected (*Desulfovibrio*) decreased significantly as well. From the inlet point until UASB 1, 440.9 mg S L^{-1} h^{-1} were produced, whereas from UASB 1 until UASB 2 only 147.7 mg S L^{-1} h^{-1} and from UASB 2 until UASB 3, 34.4 mg S L^{-1} h^{-1}. Figure 6.9B also shows the accumulation of acetate from UASB 3 to the outlet of the reactor. This trend could indicate that the more active part of the reactor is the lowest part, both in terms of sulfate reduction and degrading compounds to other VFAs.

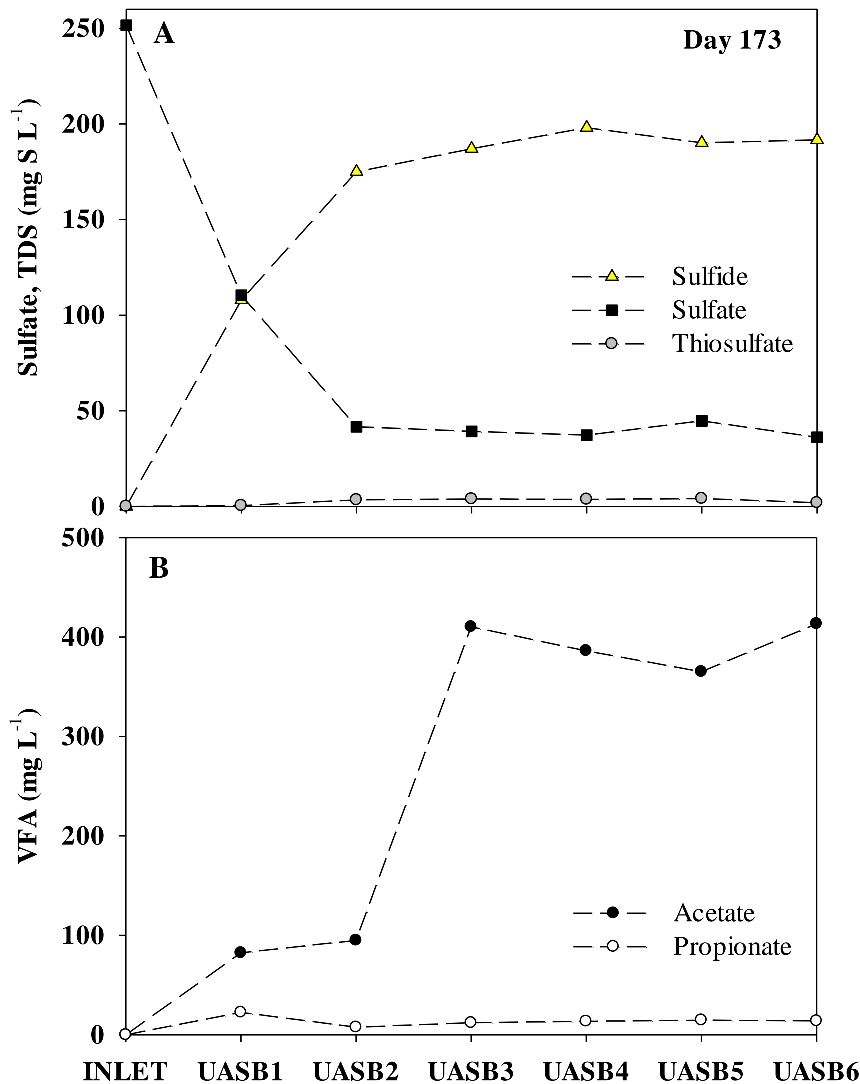


Figure 6.9. Experimental profiles obtained from the different reactor heights on day 173. (A) Sulfur species concentrations measured along the reactor heights. (B) VFA concentrations.

Figure 6.10 shows the microbial diversity of the most abundant genus detected in samples collected on day 230 of the operation (UASB 1, UASB 4 and UASB 6). No significant differences between the relative abundances of genus *Desulfovibrio* can be seen from the samples of the different heights if compared to the situation already described on day 173. The relative abundances of this genus for UASB 1, UASB 2 and UASB 3 were 40.7 %, 36.2 % and 36.1 % respectively.

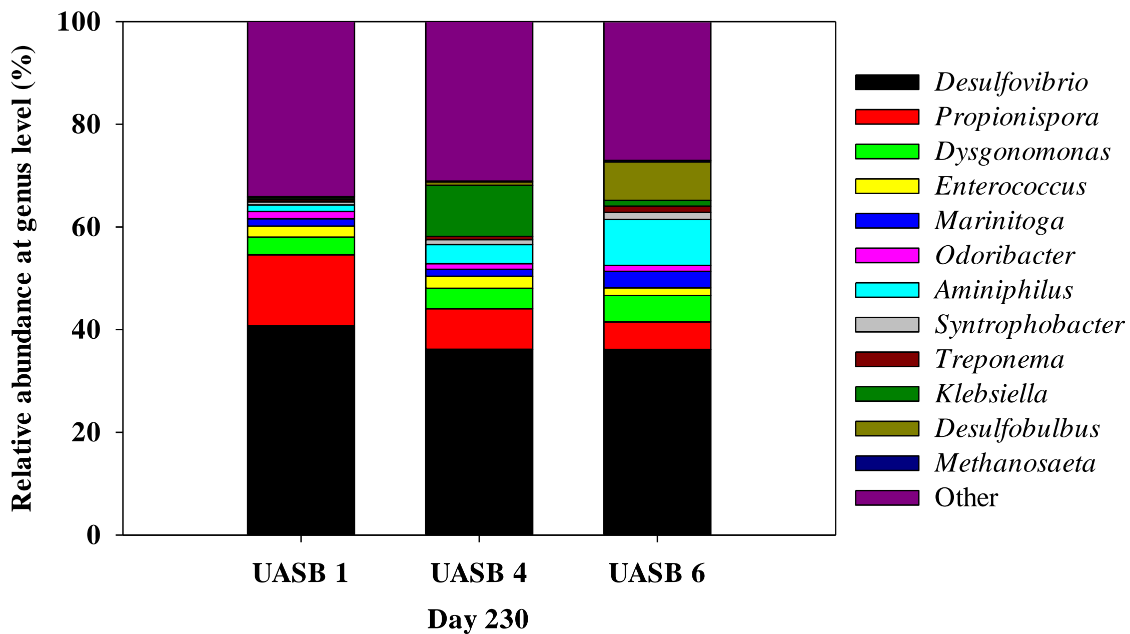


Figure 6.10. Microbial diversity along the sludge bed heights at genus level. Samples were collected on day 230 of the reactor operation.

Propionispora decreased its relative abundance from the bottom to the top of the reactor: 13.9 % in UASB 1; 1.8 % in UASB 4; and 5.4 % in UASB 6. On the other hand, *Aminiphilus* presented the opposite behavior: 1.3 % in UASB 1; 3.7 % in UASB 4; and 9 % in UASB 6. This same trend was also observed for the genus *Desulfobulbus*. Its relative abundances for the different heights were: 0.1 % in UASB 1; 0.6 % in UASB 4 and 7.5 % in UASB 6. Curiously, *Klebsiella* presented a higher abundance in UASB 4 (10 %) compared to UASB 1 and UASB 6, 0.3 and 1.1 %, respectively.

Figure 6.11 shows the concentration of the different sulfur species measured along the different heights. What stands out from this figure is that, to reach the same final concentration of sulfide in the effluent, 191.7 mg S L^{-1} and 195.4 mg S L^{-1} on day 173 and 230 respectively the profiles are quite different. Whereas on day 173 almost the highest concentration has been already reached by UASB 2, a different progression is observed on day 230, where this concentration was not reach until the upper part of the reactor, meaning that sulfate reduction was also taking part in UASB 5-6 (upper part of the reactor). On day 230, from the inlet point until UASB 1, 337.5 mg S L^{-1} h^{-1} were produced; from UASB 1 until UASB 2, 93.5 mg S L^{-1} h^{-1}; from UASB 2 until UASB 3 only 8.8 mg S L^{-1-} h^{-1} and then a huge sulfide production was detected again from UASB 3 to UASB 4, 116.8 mg S L^{-1} h^{-1}.

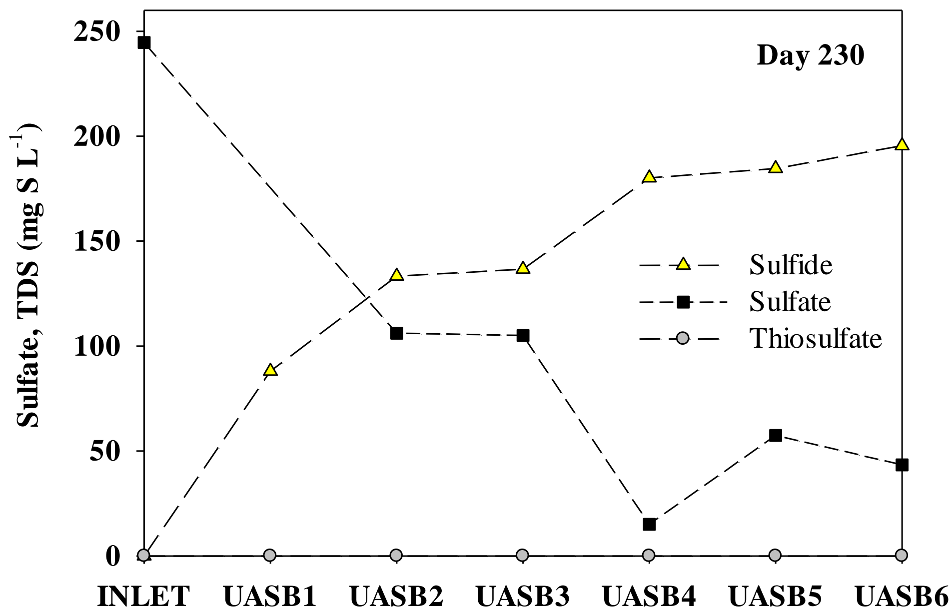


Figure 6.11. Sulfur species concentrations measured along the reactor heights on day 230.

What is also interesting and can be seen in Figure 6.12 is how glycerol is degraded into other compounds. This figure shows the concentration of glycerol along the different reactor´s heights on day 173 and on day 230 to compare the behavior between both operation dates. On day 173 glycerol could not be

detected from UASB 2 onwards (meaning until the outlet of the reactor). On the contrary, on day 230 glycerol could be detected (48.6 mg L^{-1}) even in the effluent of the reactor, meaning that a gradual drop in the fermenting capacity was also taking place in the reactor.

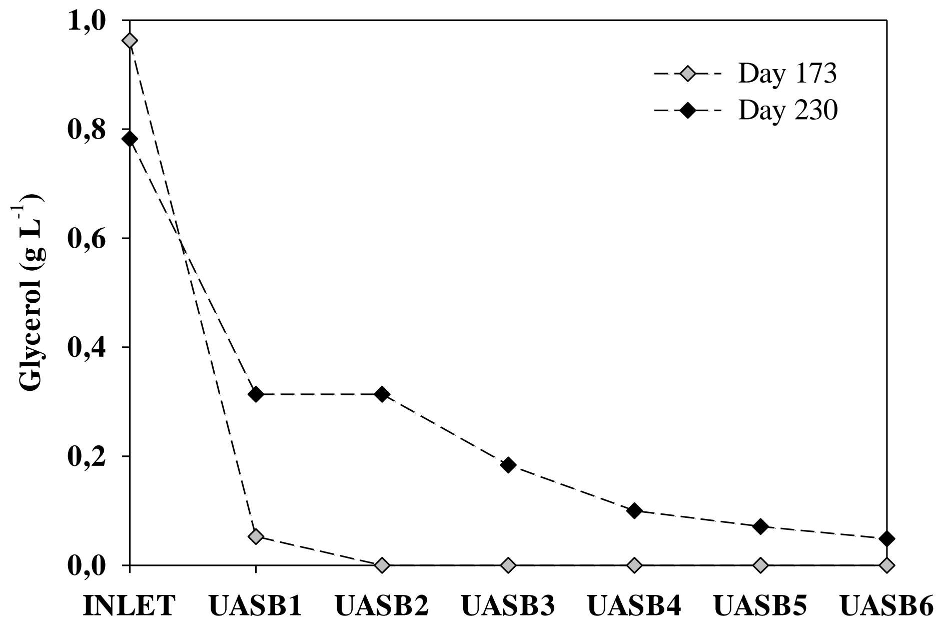


Figure 6.12. Glycerol concentration measured along the different reactor´s heights on days 173 and 230.

6.3.2. Fluorescence in situ hybridization (FISH)

FISH was used to evaluate the evolution in sulfate-reducers and methanogens in the granular sludge along the long-term operation of the UASB. First of all, Table 6.6 provides the percentage of relative abundance assigned to the family *Peptococcaceae* through Illumina sequencing. The family *Peptococcaceae* belongs to *Clostridia* class and the probe DELTA495a used for the identification of sulfate-reducers did not include this group (Table 4.3). Therefore, Table 6.6 shows the putative bacteria that were not being considered for FISH as sulfate-reducers even if they were present in the samples. As can be observed from this table, the relative abundance of this family is lower than 1 % in all the samples analyzed,

what indicates that DELTA495a was covering sulfate-reducer populations almost completely.

Table 6.6. Relative abundances (%) of the sequences assigned to the family *Peptococcaceae* on the different samples collected from the UASB operation along the operation of the reactor and at different reactor´s heights.

Day	UASB Height	% Relative abundance *Peptococcaceae* family
85	UASB 6	0.7
149	UASB 1	0.1
	UASB 2	0.2
	UASB 3	0.3
173	UASB 1	0.2
	UASB 2	0.3
	UASB 3	0.3
230	UASB 1	0.1
	UASB 4	0.1
	UASB 6	0.1
294	UASB 1	0.0
	UASB 2	0.0
	UASB 6	0.2

From all the samples collected for FISH analysis (Table 6.1), results for day 173 of the long-term operation will be presented. That day not only Illumina but also results from the profiles at different heights of the UASB reactor were available. All the quantifications for FISH were done according to the procedure explained in Section 4.3.1. However, quantification for the probe ERY514 was not possible as the general EUBmix probe did not cover *Archaea.* Therefore, a qualitative analysis of the images was performed just to confirm the presence or absence of these populations during the performance of the reactor. As shown in Table 4.3 the specificity of the probe ERY514 is restricted to the phylum *Euryarchaeota.* Therefore, Table 6.7 presents the relative abundances (obtained through

Illumina) of this phylum for all the samples collected along the operation including the different heights (UASB 1, UASB 2 and UASB 3). As can be observed, the relative abundances were always below 6 %, but there was a tendency to increase in the upper part of the reactor compared to the bottom. This tendency was also observed with FISH images (Figure 6.13B, 6.14B and 6.15B) for samples on day 173.

Table 6.7. Relative abundances (%) of the sequences assigned to phylum *Euryarchaeotay* on the different samples collected from the UASB operation along the operation of the reactor and at different reactor´s heights.

	Day 85	Day 149	Day 173	Day 230	Day 294	Day 538
UASB 6	2.9			0.4	0.6	
UASB 4				0.2		
UASB 3		5.9	4.6			
UASB 2		1.3	1.0		0.2	
UASB 1		1.5	1.3	0.1	0.2	1.2

Table 6.8 presents the relative abundances of the sequences assigned to class *Deltaproteobacteria* whereas Table 6.9 provides the % of sequences assigned to genus *Desulfovibrio* from the total amount of class *Deltaproteobacteria.* Herein, the opposite tendency could be observed. Hypothesizing that *Desulfovibrio* was the main SRB reducing sulfate, Table 6.9 clearly shows a decrease of the populations in the upper part of the reactor and as the performance evolved. Therefore, a possible explanation for the increase of the % of relative abundance detected for *Euryarchaeota* is related with the fact that they do not have to compete with so many *Desulfovibrio* in the upper part of the reactor. Even so, in the long-term these populations are completely washed-out as already mentioned.

Table 6.8. Relative abundances (%) of the sequences assigned to class *Deltaproteobacteria* on the different samples collected from the UASB operation along the operation of the reactor and at different reactor´s heights.

	Day 85	Day 149	Day 173	Day 230	Day 294	Day 538
UASB 6	32			46.2	39.4	
UASB 4				38.6		
UASB 3		35.8	33.5			
UASB 2		42.2	54.7		37.3	
UASB 1		44.2	49.2	42.9	32	23.4

Table 6.9. Relative abundances (%) of the sequences assigned to genus *Desulfovibrio* (considering only class *Deltaproteobacteria*) on the different samples collected from the UASB operation along the operation of the reactor and at different reactor´s heights.

	Day 85	Day 149	Day 173	Day 230	Day 294	Day 538
UASB 6	55.7			78.3	87.2	
UASB 4				93.6		
UASB 3		48.1	58.1			
UASB 2		84.2	91.0		74	
UASB 1		81.7	86.9	95.0	94.0	46.1

Figures 6.13, 6.14 and 6.15 provide a selection of images from day 173 of the UASB operation at different heights, UASB 1, UASB 2 and UASB 3 respectively. On day 173, 33 ± 12 % of the population was quantified as SRB (*Deltaproteobacteria* class) for sample UASB 1, 42 ± 18 % for UASB 2 and 30 ± 20 % for UASB 3. According to Illumina results, the relative abundance of the sequences assigned to *Deltaproteobacteria* class were: 49.2 % for UASB 1; 54.7 % for UASB 2 and 33.5 % for UASB 3. From these sequences the ones assigned to the genus *Desulfovibrio* were: 42.8 % for UASB 1; 49.8 % for UASB 2 and 19.5 % for UASB 3, which represents almost the majority, especially in UASB 1 and UASB 2. With both techniques the amount of sulfate reducers detected in UASB 2 was the highest of

the different heights. These results are in accordance with the profiles presented in Figure 6.9, where the highest sulfate reduction rate was reached by UASB 2. From that point until the outlet of the reactor almost no more sulfide was produced.

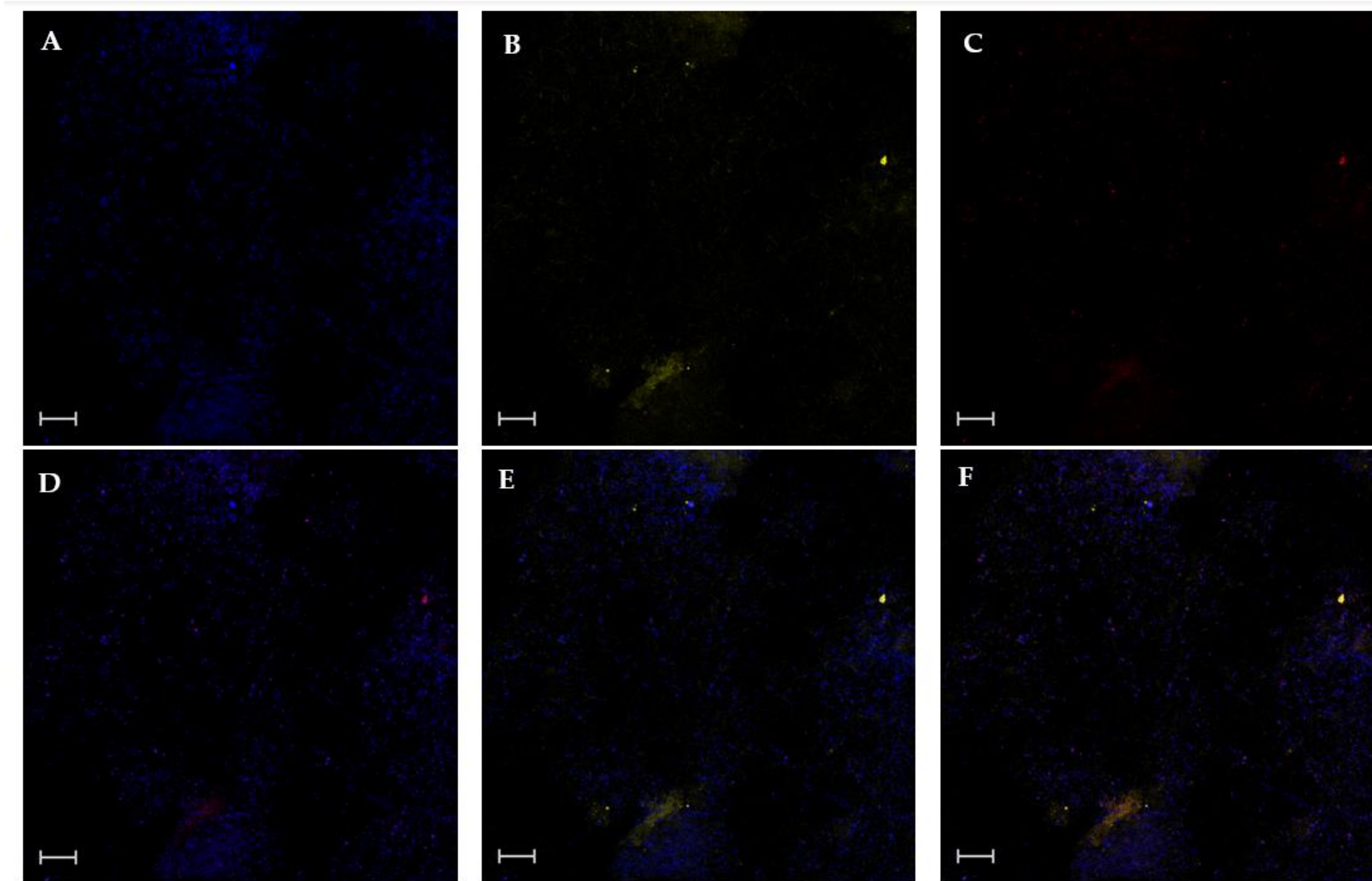


Figure 6.13. CLSM images of FISH for sample UASB 1 on day 173. A) All bacteria detected (blue); B) *Euryarchaeota* detected (yellow); C) *Deltaproteobacteria* detected (red); D) Merged images A and C; E) Merged images A and B; F) Merged images A, B and C. Scale Bar = 20 µm.

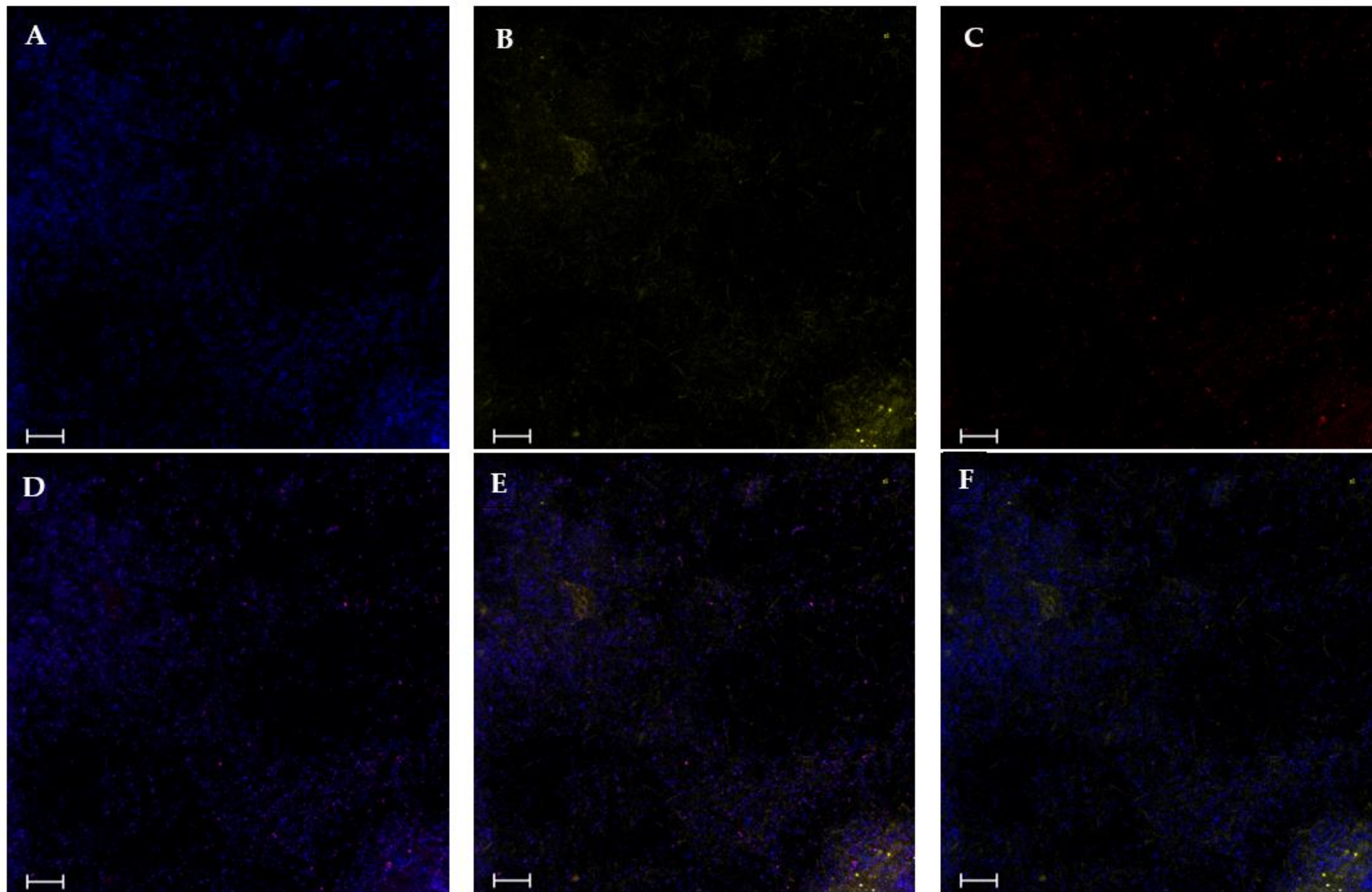


Figure 6.14. CLSM images of FISH for sample UASB 2 on day 173. A) All bacteria detected (blue); B) *Euryarchaeota* detected (yellow); C) *Deltaproteobacteria* detected (red); D) Merged images A and C; E) Merged images A and B; F) Merged images A, B and C. Scale Bar = 20 μm.

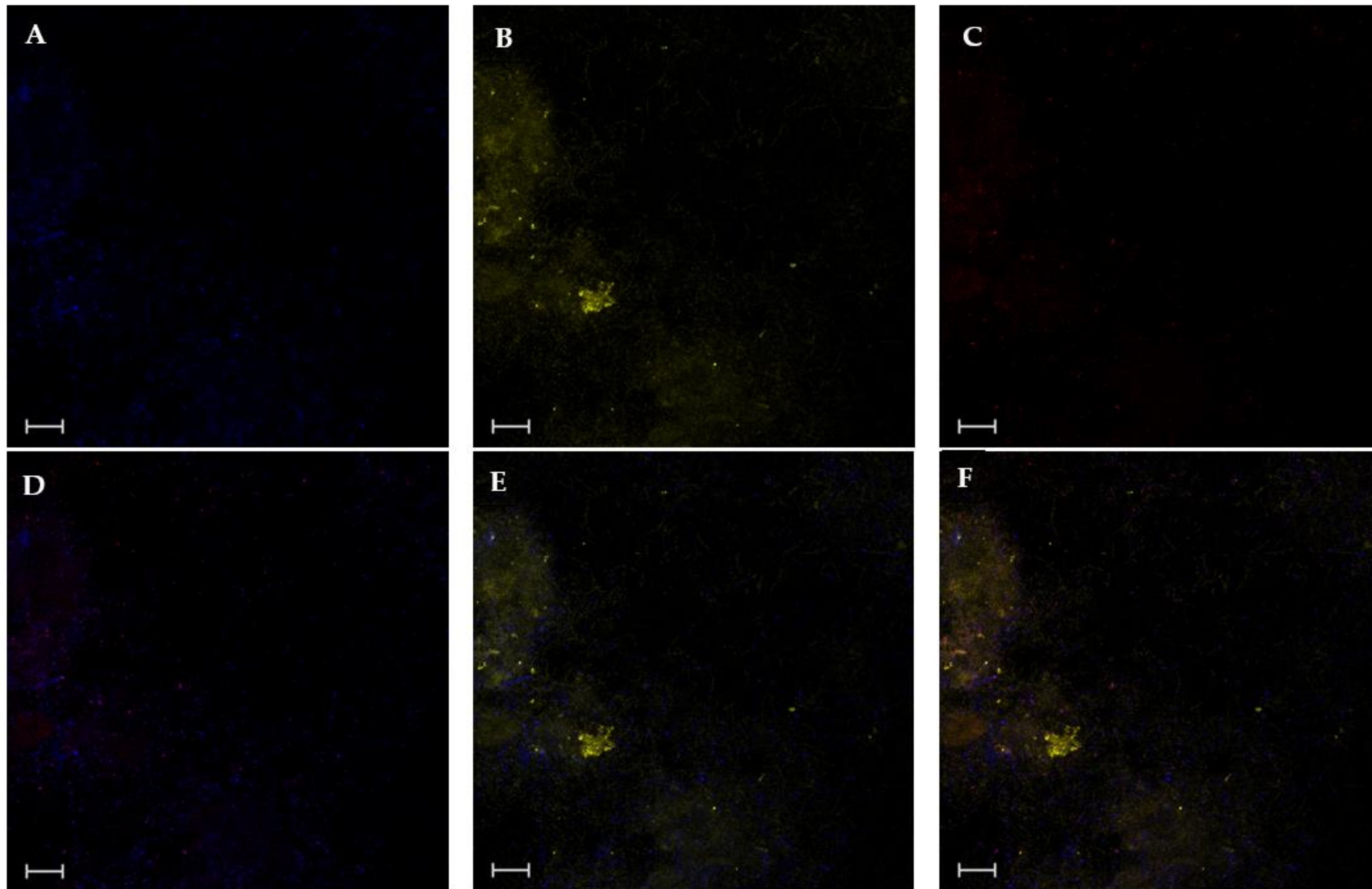


Figure 6.15. CLSM images of FISH for sample UASB 3 on day 173. A) All bacteria detected (blue); B) *Euryarchaeota* detected (yellow); C) *Deltaproteobacteria* detected (red); D) Merged images A and C; E) Merged images A and B; F) Merged images A, B and C. Scale Bar = 20 μm.

6.4 Discussion

6.4.1 Long-term performance and evolution in microbial populations

Taken together, results presented along this chapter provide the evolution of the operation both in terms of physical-chemical parameters and microbial evolution. Section 6.3.1 provides the results of the evolution of the operation according to removal efficiencies and the different parameters measured along this performance. In the previous UASB performance (Chapter 5) methane production stopped after 200 days. Similarly, both UASB performances (Chapter 5 and the one presented herein) showed a methane production rate decrease the first 100 days. This fact indicates that methane production was not only influenced by the TOC/S ratio since methanogens were affected under all conditions tested. This result is confirmed in this chapter through Illumina sequencing analysis. Table 6.5 provides the evidence that confirms the wash-out of methanogens since they could not be detected from day 173 onwards. The most striking result to emerge from Table 6.5 is the increase in relative abundance of *Desulfovibrio*, which goes from 0 % in the inoculum until its maximum, 42.8 % on day 173. Equivalently, Figure 6.6 shows the disappearance of the genus *Methanosaeta* (the major methanogen detected) that was only present in the inoculum. In our case, the interest was mainly focused on methanogens and sulfate reducers and how they evolved during the long-term performance. For that reason, Figure 6.7 presents only the relative abundance (%) of the detected methanogens together with the genus *Desulfovibrio*, as this one became the major sulfate reducer along the operation. All known methanogens belonged to the *Euryarchaeota* phylum; within this phylum, the classes *Methanobacteria, Methanococci, Methanofastidiosa, Methanomassillicocci, Methanomicrobia* and *Methanopyri* are methanogenic. However, recently there has been genomic evidence that within *Bathyarchaeota* and the novel phylum *Verstraetearchaeota,* methane production also occurs (Vanwonterghem et al., 2016). All these

graphical materials together suggest that sulfate-reducers could outcompete methanogens during the performance. Therefore, methanogens were washed-out from our system whereas sulfate-reducers (*Desulfovibrio* genus) became the major group detected. This would mean that both populations were not able to grow in this UASB under the conditions tested. In general, sulfate reducers always predominate in carbon source utilization and electron flow transmission, and suppress the activity of methanogens (Shin et al., 1997; Jing et al., 2013). The population structure determined by Illumina sequencing could be linked to the functional changes observed along the operation in the reactor, in this case the rate of methane production. However, the in situ metabolic functions of the microorganisms in the UASB were not characterized. The competition of sulfate-reducing and methanogenic populations, in anaerobic reactors and in the presence of nonlimiting sulfate concentrations have been studied previously (Isa et al., 1986; Nanninga and Gottschal, 1986; Halkjaer Nielsen, 1987; Yoda et al., 1987). Most of these studies were performed with granular biomass or attached-growth reactors, so factors such as microbial adhesion and colonization or mass transfer limitations become a crucial factor affecting the competition between these populations (Raskin and Rittmann, 1996). This chapter did not consider so many parameters but demonstrated that at a TOC/S ratio between 1.4-1.7 g C g^{-1} S, SRB had a competitive advantage over methanogens and that, after 200 days of operation, methanogens were always washed-out from the system.

The significant decrease in CH_4 production after 135 days of the start-up of the operation, that has been previously discussed, coincided with an increase in acetate concentration. This acetate accumulation and low methane production was also observed by Jing et al. (2013). As can be observed in Figures 6.6 and 6.7, *Methanosaeta* was the most abundant genus among the phylum *Archaea*. *Methanosaeta* together with the genus *Methanosarcina* have been described as

acetoclastic methanogens that use acetic acid as carbon source to produce methane directly (Shigematsu et al., 2003). The almost complete disappearance of these groups from day 85 (when the relative abundance of *Methanosatea* was 2.7 %) onwards was related to the decrease in methane production rate. At the same time this fact can explain the increase in acetate concentrations. After the wash-out of these populations, the system began to accumulate this metabolite as they were the ones in charge of using it to produce methane. Furthermore, hydrogenotrophic methanogens, such as the order *Methanomicrobiales*, can produce methane indirectly from acetate, which is converted into H_2 and CO_2 and further to methane (Fotidis et al., 2014). However, this order was only detected in the inoculum sample and with a relative abundance of 0.7 %, which also supports the conclusion that there were no populations able of using this acetate, that ended up accumulating in the system.

Glycerol was also detected in the effluent from day 225 (31.9 mg L^{-1}) onwards, reaching concentrations of 361.8 mg L^{-1} on day 550. These results would suggest that crude glycerol could not even be completely hydrolyzed or converted to other easily biodegradable compounds. From day 230 onwards, the relative abundance of the OTUs assigned to the genus *Desulfovibrio* decreased significantly until the end of the operation. That may be related with the decay on the performance of the reactor and the almost complete loss of sulfate removal efficiencies. Overall, these results suggested, that not only sulfate reducers were being affected but the whole system was not performing properly.

6.4.2 Different reactor heights and profiles

Samples for 16S sequencing were not collected in triplicates, so no statistical analysis could be performed. Nevertheless, as reported by Bautista-de los Santos et al. (2016), bias and variability inherent to the PCR amplification and

sequencing steps is significant enough to hide differences between bacterial communities from replicate samples. PCR amplification and sequencing errors have been considered an inconvenient for 16S rRNA gene amplicon (Quince et al., 2009; Delforno et al., 2017). Nevertheless, this approach offers a broad overview for a large microbial community characterization and allows detecting rare species in complex communities. 16S sequencing is neither a quantitative technique (Sanz and Köchling, 2007) so, no clear relation can be made between the relative abundances of the microorganisms involved, the metabolic activities and sulfide production in this case. But the comparison between the profile measurements provided us with information about how the reactor was performing.

When having a look at the different samples along the reactor´s heights presented in Figure 6.8, the most abundant genus found in all samples were the same and the main results have already been presented. However, an increase in the OTUs detected and assigned to the genus *Syntrophobacter* can be observed if UASB 1 is compared to UASB 3. The first species named species of *Syntrophobacter* (*S. wolinii*) was discovered in 1980 by Boone and Bryant. (1980). Nevertheless, in our case, classification at species level was not possible. Even so, all members of this genus anaerobically degrade propionate to acetate in the presence of methanogens. *Syntrophobacter* is often found in sludge from anaerobic waste treatment facilities and is useful for further degrading organic compounds from propionate and lactate to acetate. If that was the main reaction happening, this fact would also support the accumulation of acetate.

If considering the total amount of sulfide produced on day 173, by UASB 2 the 91.3 % had been already produced whereas on day 230 only a 68.2 % had been produced at the same reactor´s height. Those results suggested that the reactor was losing sulfate reducing capacity in the first part of the sludge bed. Even so, the concentration of sulfide measured in the outlet of the reactor was 226.2 mg L^{-1}

and 195.4 mg L^{-1} on day 173 and 230, respectively. That would mean that there was not much difference (30.8 mg L^{-1}) on the total amount of sulfide produced. Still, the sulfate reducing activity was not equally being developed in terms of reactor heights. FISH results also supported the fact that UASB 2 was "more active" in terms of sulfate reduction. Direct visual feedback of the analyzed samples with the microscope can be a key advantage of FISH over other methods, for example those based on PCR. The rule ''count what you see'', is less prone to biases than quantitative PCR approaches (Nielsen et al., 2009). However, when working with different fluorochrome, many practical problems can be encountered, that can lead to misleading information. As an example, 25 % laser power for laser 488 was used during this chapter, whereas for laser 561 and 633, 45 % and 25 % was used. With these settings, the amount of fluorescent visually observed for *Euryarchaeota* was much higher than that for *Delt aproteobacteria* what can made one think that more population of the first group are present in the sample. Therefore, many efforts are needed to optimize the protocol applied to new samples. Bouvier and del Giorgio. (2003) published a detailed review presenting many of the factors influencing the sensitivity of FISH, effect of fluorochrome type, and stringency conditions. Kramer and Singleton. (1992) and Fukui et al. (1996) concluded that cells with low activity might have rRNA at a sufficient concentration to yield a fluorescent signal detectable with FISH. This is another advantage compared to sequencing methods that favour those organisms that are more abundant in the samples due to PCR. Furthermore, PCR-based approaches do not actually quantify microbial cells, but measure copy numbers of marker genes. The number of this gene copies per genome can vary among microbes as can also vary the number of genomes per cell (Ludwig and Schleifer, 2000).

As it has already been mentioned, a combination of different molecular biological techniques is the best way to obtain an accurate picture of what is

happening during the operation of a reactor. Therefore, many authors have reported the use of conventional microbiological methods in combination with kinetic modelling, 16S rRNA gene analyses, FISH, DGGE or other techniques to get insight into the microbial community of different systems (Roest et al., 2005; Dar et al., 2007a; Silva et al., 2011; Portune et al., 2014).

6.5 Conclusions

Overall, results obtained in this chapter demonstrated that long-term operation of a sulfidogenic UASB reactor under constant loading rate can be achieved and lead to highly dynamic conditions. Microbial communities specialized in more specific functions and SRB populations were selected according to operating conditions. The non-acetate degrader *Desulfovibrio* was found to be the most abundant SRB genus detected and the increase in acetate concentration was related to the wash-out of methanogens. Physical and chemical parameters and Illumina data correlated well to explain methanogenesis dynamics. However, microbial diversity dynamics did not correlate well with the decrease in sulfate and TOC removal efficiencies. Causes are still unclear. Probably it is not essential to know the phylogenetic position at species level of an individual microorganism for the design and operation of a reactor for wastewater treatment. However, a general overview of the evolution of microbial populations along the operation could help us to relate key factors on the operation with changes in these populations. As an example, in this chapter, the combination of different methods (FISH and Illumina) together with operational data allowed the establishment of a link between the population structure and function of the anaerobic communities in the UASB reactor under certain conditions tested.

Chapter 7: Operational issues during the long-term performance of a sulfidogenic UASB: from success to failure

The motivation of this chapter was to gain knowledge and, if possible, infer the causes of the failure of UASB performance described in previous chapters. In this case, the study was mainly focused on analyzing the main parameters affecting both operations so as to give a conclusion that could be applicable to other sulfidogenic UASB similar to the one used along this thesis. Therefore, a procedure could be available on how to operate a sulfidogenic UASB reactor fed with crude glycerol in a long-term stable mode, which is, based on the existing knowledge, still unavailable. Moreover, many different techniques, both physical-chemical and biological were additionally applied in order to characterize the biomass and the biofilm formed along the long-term operation. This fact is quite interesting, as many different approaches from different methodologies were included to gain more knowledge in an effort of understanding the process failure observed.

Abstract

The performance´s success of an UASB reactor is a function of the inoculum, substrate, reactor configuration, temperature, pH and finally, its operation. Many authors have pointed out the special importance of understanding the microbial community composition to stabilize the performance of anaerobic reactors. In this sense, many efforts have been made to operate steadily anaerobic reactors and obtain high methane yield. However, long-term performances at lab-scale to test the degradation of different organic wastes such as crude glycerol, carry out still unresolved issues. In this chapter, the long-term performance of an UASB presented in Chapters 5 and 6 was reconsidered in terms of the operational issues that led in both cases to a failure of the system. A loss of granular structure and the growth of an unidentified non-SRB, non-methanogenic biofilm was observed during both performances. To properly assess the activity of this biofilm, namely slime, activity tests in serum bottle were performed. Samples of the slime were also characterized through different physical-chemical techniques. Data obtained from the different techniques applied along the chapter indicate that granules play an important role in maintaining the stability and removal efficiencies of the reactor. Bulky microbial aggregates as the slime may reduce their specific gravity leading to their flotation. Mass transfer rate and limitation due to the gelatinous

and sticky nature of the slime may be also a crucial aspect leading to a failured operation.

7.1 Introduction

Up-flow anaerobic sludge bed (UASB) reactors represent a proven, mature and sustainable technology for the treatment of a wide variety of industrial or domestic wastewaters (Lettinga, 1995). They are by far the most robust and well-established wastewater treatment process with more than 1000 reactors installed worldwide (Tiwari et al., 2006). Long-term operations of UASB reactors have been discussed by many researchers and some common shortcomings have been highlighted, including start-up aspects, biomass granulation, temperature limitation and effluent quality (Chong et al., 2012). All these parameters stay in strong interactions, what means that a stable operation depends on many factors affecting each other. The success of an UASB reactor´s performance is therefore a function of the type of inoculum and substrate, reactor configuration, environment (temperature, pH) and, finally, the operation mode. It is also important to assure long-term operations to analyze and be aware of the many problems arising when all the abovementioned parameters deal with each other for long periods of time. However, long-term performances at lab-scale to test the degradation of different organic wastes such as starch, methanol, alcohols, acetate or crude glycerol, carry out still unresolved issues. As an example, Lu et al. (2015b), pointed out the limited knowledge during the long-term operation of a lab-scale starch-fed UASB reactor and the need to push forward in terms of achieving continuous stability, if industrial applications are desired.

Many industrial wastewaters contain high concentrations of sulfate, such as those from the fermentation, edible oil or pulp and paper industries (O'Flaherty et al., 1999a). In general, wastewaters containing a COD/Sulfate ratio higher than 10 g O_2 g^{-1} SO_4^{2-} do not pose problems for methanogenic treatment (Rinzema and Lettinga, 1988), which is usually the main objective. Sulfate reduction during the anaerobic treatment of wastewaters is generally undesirable because of reduction in methane yield and problems of corrosion and toxicity caused by H_2S. The

production of hydrogen sulfide (H_2S) by sulfate-reducing bacteria (SRB) can be toxic towards the various trophic groups of bacteria involved in the process (Oude Elferink et al., 1994). This toxicity can cause severe process disturbance and, in extreme cases, complete process failure. Reduction of sulfate to sulfide reduces the amount of organic matter available for its conversion to methane and therefore, the quantity of methane produced (Parkin et al., 1990). Many authors have pointed out the special importance of understanding microbial community composition to stabilize the performance of anaerobic reactors. Hence, the importance of understanding such communities as well as the interactions within species, for the effective operation and improvement of the reactor's performance could be crucial. *Methanosaeta* spp. and *Methanosarcina* spp. have been identified as important acetolactic methanogens for the initial stages and later development of granular sludge (Schmidt and Ahring, 1995).

In the case of setting sulfidogenic conditions, as those set during UASB reactors´ performances shown in this thesis, most studies have focused on minimizing the reaction volume. In most of these studies, UASB or expanded granular sludge bed (EGSB) reactors are used to maximize at the same time the sulfate loading rate (Dries et al., 1998) and to focus on the C/S ratios (Zhou et al., 2014), without paying attention to other important parameters playing an important role. However, one of the main problems encountered in sulfidogenic UASB reactors with mixed cultures is the granulation step, which has been described as the key factor to operate successfully UASB reactors (Schmidt and Ahring, 1995). But, although sulfate reduction has many similar characteristics as those found in anaerobic fermentation processes, little literature is available on the immobilization and granulation of SRB in sulfidogenic systems (Reilly and Colleran, 2006). Moreover, little is known about the time required for SRB to form a biofilm (Visser, 1995). This is mainly due to the fact that most of the studies are

focused on the control and prevention of sulfate reduction in anaerobic granulation processes (Hao et al., 2013).

The main purpose of our process was to maintain granules, minimizing the proliferation of methanogens and addressing the use of the carbon source to reduce sulfate. The low up-flow velocity used during the long-term operations of the UASB reactor along this thesis (0.25 m h^{-1}) is one of the operational conditions that could affect negatively biofilm growth. Almost every surface can be colonized by bacteria, forming biofilms. After adhesion, the cells embed themselves in a layer of extracellular polymeric substances, known as EPS, which are highly hydrated biopolymers of microbial origin. Almost all microorganisms on earth live in biofilm-like microbial aggregates rather than as single organisms as this mode of life provide many strong ecological advantages (Flemming, 2008). However, bacterial adhesion cannot be explained only as a physical–chemical process, as there are many complex aspects behind this process (Hulshoff Pol et al., 2004). To maintain the matrix structure and stability of anerobic granules, EPS are considered as the major important materials (Liu et al., 2004a). EPS comprises different classes of organic macromolecules such as polysaccharides, proteins, nucleic acids, (phospho) lipids, and other polymeric compounds, which have been found to occur in the intercellular spaces of microbial aggregates (Wingender et al., 1999). Also, granulation is usually produced under stressing conditions, such as the shear stress generated by setting up-flow velocities over 0.5 m h^{-1}.

During the long-term operation of the sulfidogenic UASB studied in this thesis, failured performances were encountered. One of the unresolved questions was the formation of a huge biofilm from day 300 onwards. This biofilm was not only covering the reactor walls but also surrounded the granules. The appearance of this biofilm seemed to be important for our performances, as the more biofilm/EPS was accumulated, the worst the operation was in terms of sulfate

and COD removal efficiencies. To gain more knowledge and be able to describe what was this substance that was being accumulated in our reactor, several analyses were performed. The use of different physical-chemical and biological techniques such as FTIR, SEM or 16S rRNA helped us to understand the properties of this biofilm/slime substance. Some authors have pointed out that higher organic loads can be treated in granular-based biosystems when a better understanding of the process performance is gained, resulting also in start-up time reduction and more sustainable operation (Liu et al., 2003; Lew et al., 2011). Therefore, this chapter pursues enhancing our insights into the long-term disturbances and mechanisms affecting the UASB operation to provide preliminary basis and, if possible, create a guidance for practical improvements to achieve long-term stable operations thinking in a future industrial application.

7.2 Materials and methods

7.2.1 Sampling of the slime substance

Figure 7.1 and 7.2 illustrate the aspect of the granular sludge both when the reactor was inoculated and after UASB long-term performances. As can be observed in these figures, after approximately 300 days of operation, a huge biofilm was covering the reactor wall and the aspect of the granules had change completely (compared to the inoculum) obtaining a white color and becoming a cotton-like fluffy sludge. Samples of this slime were taken from both UASB reactors´ operations. Different analyses were performed on the substance to know if it contained EPS or could be indeed some kind of EPS coming from microbial aggregates formation. From now on, this substance will be named slime along the chapter.

Figure 7.1. UASB described in Chapter 5. A) Granular sludge (inoculum); B) Slime attached to the upper part of the reactor (day 490); C) Day 300 of the operation; D) Day and 400 of the operation.

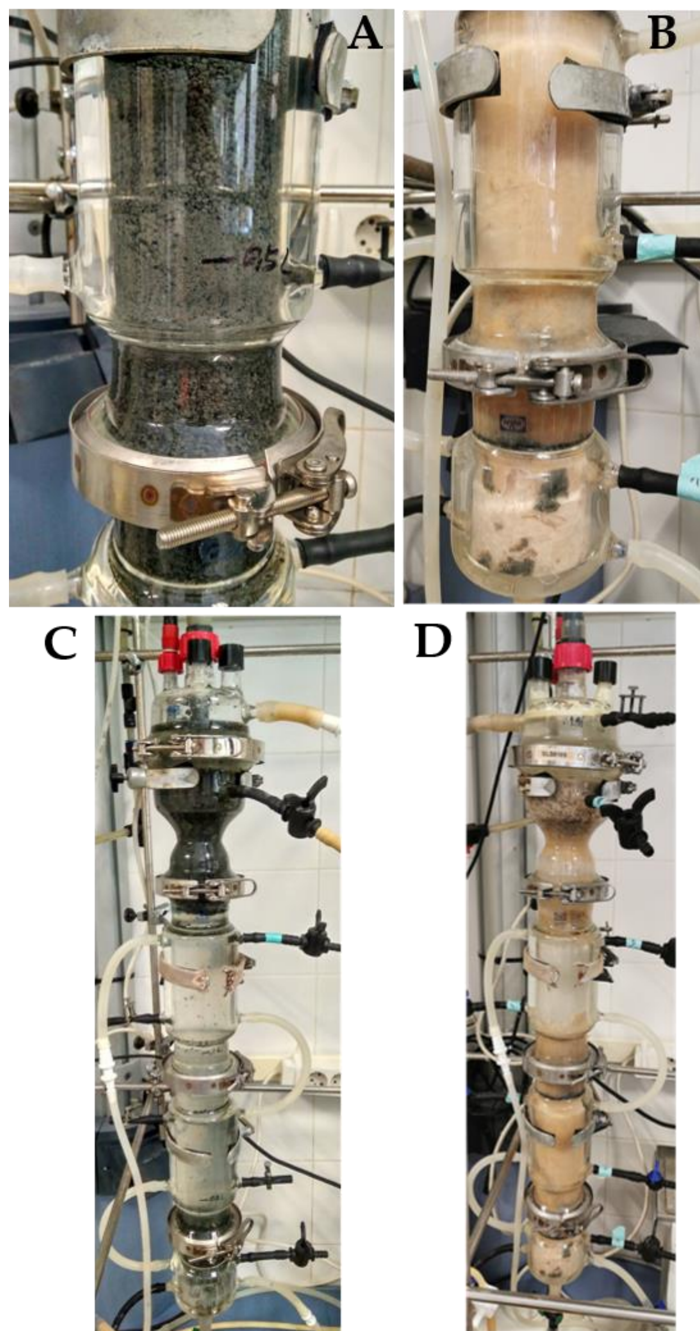


Figure 7.2. UASB described in Chapter 6. A and C) Granular sludge the day the reactor was inoculated; B and D) Slime attached to the reactor the last day of the operation (day 630).

To clarify, the operation described in Chapter 5 will be called operation 1; and the one described in Chapter 6 will be referred to as operation 2 along this chapter. Different analyses were performed to the slime collected from both

operations to try to elucidate whether it could be biomass or other mixture of organic/inorganic matter coming from the impurities of crude glycerol, generated due to the interaction among intermediate compounds or produced biologically. As many different samples were collected along both operations and many different analyses were performed, Table 7.1 presents a summary of all of them with the names that will be used along the chapter for each of the samples. Slime will be called SLM to simplify whether granular sludge will be called GRS.

Table 7.1. Biomass sampling days during the long-term operation of the UASB reactor.

Sample name	Description	SEM	Elemental analysis	FTIR	FAMEs	16 S rRNA	Bottle test
SLM_OP1_490 (1,2,3,4)*	On day 490 of operation 1, samples of the slime were collected, including samples at different reactor´s heights (UASB1-2 and UASB 2-3)	X			X		
SLM_OP2_540 (1,5)	On day 540 of operation 2, samples of the slime were collected, including samples at different reactor´s heights (UASB 1 and UASB 5)	X	X				
SLM1_OP2_413 (6)	Duplicates of the slime collected from UASB 6 on day 413 of operation 2			X			
SLM2_OP2_413 (6)				X			
GRS_OP2_413 (6)	Granules collected from UASB 6 on day 413 of operation 2			X			
SLM_OP2_358 (1)	Slime collected from UASB 1 on day 358 of operation 2					X	X
SLM_OP2_538 (6)	Slime collected from UASB 6 on day 538 of operation 2					X	
GRS_OP2_538 (6)	Granules collected from UASB 6 on day 538 of operation 2					X	

*Numbers in brackets indicate UASB heights

7.2.2. Chemical structure analysis by Fourier transform infrared spectroscopy (FTIR)

On day 413 of operation 2, biomass samples from the upper part of the reactor (UASB 6) were collected. Two samples of the slime (SLM1 and SLM2) collected as duplicates together with one sample from the anaerobic granules (GRS) were analyzed (Table 7.1). All samples were washed several times with demi-water and afterwards they were lyophilized and sent to Delft University of Technology where FTIR spectra were measured. The FTIR spectra of the lyophilized samples were recorded as described in Lin et al. (2018). The absorbance of the samples and background were measured as 16 scans each. The signal was set as transmittance (%), but absorbance was calculated as follows:

$$A = 2 - \log 10\ (T) \tag{7.1}$$

After FTIR measurements, the Blyscan™ Sulfated Glycosaminoglycan Assay (biocolor) was used for the analysis of sulfated proteoglycans and glycosaminoglycans, (sGAG). The Blyscan Assay is a quantitative method that contains a blue dye which turns bright pink when it binds to sulfated glycosaminoglycans (Figure 7.3). It is 1,9-dimethylmethylene blue and it is employed under conditions that provide a specific label for the sulfated polysaccharide component of proteoglycans or the protein free sulfated glycosaminoglycan chains.

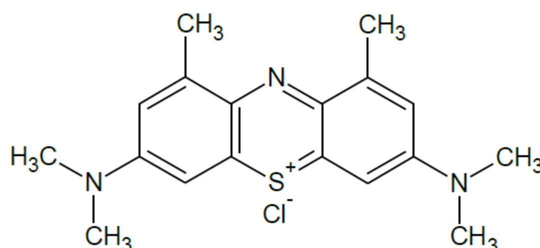


Figure 7.3. Molecular Structure of Blyscan Dye.

The absorbance values of the reagent blank, reference standards and test samples were measured against water. The reagent blank's absorbance value was subtracted from the standards and test samples absorbance readings. Afterwards, the sGAG reference standard absorbance means was plotted against their known concentrations to produce a straight line (Absorbance vs Concentration), that was used as the calibration curve. Test sample concentrations could be either read off the graph, or calculated from the slope. The assay could also be adopted to determine the O- and N-sulfated glycosaminoglycan ratio within our samples. Its detection limit is 0.25 μg. Prior to measurement, an extraction and dialysis procedure was applied to the samples. The extraction was done at 80 ºC with NaOH (0.1 M) for 30 min. This was done in order to solubilize the EPS, and then sample were centrifuged, and the supernatant collected. The supernatant was dialyzed against demi-water overnight to remove salts. The retentate was frozen at -80 ºC and subsequently freeze-dried. The whole sample was solubilized, and the extraction yield was 100 %.

7.2.3 Scanning electron microscopy

Samples described in Table 7.1 were taken for SEM studies. Granules were previously separated from the slime by washing them with distilled water several times. Samples were fixed with a solution of 2.5 % (vol/vol) glutaraldehyde (electron microscopy grade; Merck, Darmstadt Germany), and processed according to conventional electron microscopy methods as previously described (Julián et al., 2010). Samples were treated with osmium tetraoxide, dehydrated with ethanol and dried with CO_2 in a Bal-Tec CPD030 critical-point dryer (BalTec). Then, samples were coated with few nanometers of Au-C (E5000 Sputter Coater), to increase signal detection, and visualized on a Scanning

Electron Microscope (SEM, Zeiss EVO ® MA 10; Microscopy Service of the Universitat Autònoma de Barcelona).

7.2.4 FAMEs

Samples of sludge at different heights of the UASB reactor were collected (Table 7.1) to perform fatty acid methyl ester (FAMEs) analysis. Samples were centrifuged at 4000 g during 10 min several times. In each centrifugation cycle, supernatant was discarded, and the slime was manually transferred to a new falcon tube where distilled water was added, and a new centrifugation cycle was started. Granules were settled as a pellet in every centrifugation step and were discarded as well. This procedure was performed in order to successfully separate the granules from the slime. Samples were frozen at -80 ºC overnight and lyophilized. After lyophilization, samples were homogenized with a mortar and a pestle. Table 7.2 shows the weight of the different samples after the lyophilization step. These analyses were outsourced to "Centres Científics i Tecnològics" of the University of Barcelona.

Table 7.2. Samples collected from the reactor and its weight after lyophilization.

Sample	Dry weight (mg)
Biofilm attached to the walls of the reactor	47.4
UASB 1-2	148.4
UASB 2-3	292.2
UASB 3-4	468.7

FAMEs analysis was performed using a Gas Chromatograph (GC) coupled to a Mass Spectrometer (MS) (Shimadzu QP2010). The chromatographic column used for the analysis was a BPX-70 capillary column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies, Inc.), with a He gas flow rate of 1 mL min^{-1} as the carrier

gas. The injector temperature was set at 260 ºC. After the injection of the sample (split ratio 1:50), the initial oven temperature (60 ºC) was held for 1 min and then ramped at 6 ºC min^{-1} to 260 ºC, which was held for 10 min. Finally, the MS acquired the data in scan mode with m/z interval ranging from 50 to 650 uma. In each analysis, a FAME standard was also analyzed, surrogate ($C_{19:0}$) in our case, which was used as an internal standard to identify and quantify the FAMEs in the samples.

For a further quantification of free fatty acids presented in the different samples, the extracts used for FAMEs analyses were derivatized with 150 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) during 1 h at 90 ºC. Afterwards, samples were dried and dissolved in 800 µl of hexane: dichloromethane 1:2. The chromatographic column used in this case, was a Sapiens X5-MS capillary column (30 m x 0.25 mm x 0.25 µm), with a He gas flow rate of 1 mL min^{-1} as the carrier gas. The injector temperature was set at 320 ºC. After the injection of the sample (split ratio 1:50), the initial oven temperature (50 ºC) was held for 1 min and then ramped at 6 ºC min^{-1} to 320 ºC, which was held for 10 min.

7.2.5. Elemental analysis

A semi-quantitative analysis of the elements existing in the slime at different sludge bed heights (UASB 1 and UASB 5) was performed on day 540 of operation 2 (Table 7.1). Three samples of each of the reactor´s heights were cleaned with demi-water and centrifuged at 4000 rpm during 5-10 min (3 times). Afterwards, samples were lyophilized and homogenized. C, H, N and S were determined from the elemental analysis of lyophilized samples with a Flash EA 2000 CHNS instrument (Thermo Fisher Scientific) connected to a microbalance (MX5, Mettler Toledo). The totality of this analysis was outsourced to the Servei

d'Analisis of the Universitat Autònoma de Barcelona. The limit of quantification of the different elements: C, H, N and S was 0.1 %.

7.2.6. 16S rRNA gene amplification of the slime

Identification of microbial populations was performed using Illumina platform on different samples (Table 7.1). Before DNA extraction procedure, granules were manually separated from the slime, to keep only the slime and discard granules. Afterwards, samples were cleaned with 1XPBS and centrifuged at 14000 rpm during 5 min (3 times). For community characterization of the sample taken on day 358, DNA was extracted using the FAST DNA® spin kit for soil (MP Biomedicals), following the manufacturer's protocol. DNA extracts were stored at -20 ºC until their use. The same methodology and kit were used to extract DNA from the biomass collected from bottle experiments described in Section 7.2.7.

On day 538, a manual separation between granules and slime was performed to try to elucidate microbial differences. Afterwards, genomic DNA was extracted from both samples using the PowerSoil™ DNA isolation kit (MoBio Laboratories, USA). The quantity and quality of the extracted DNA were assessed by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and then DNA samples were preserved at -20 ºC for further analysis. Amplicon sequencing 16S rRNA genes of all samples was performed by "Genomic and Bioinformatics service" on Illumina MiSeq platform at the Universitat Autònoma de Barcelona amplifying the V3-V4 hyper variable region with the universal primers by Illumina (5'-CCTACGGGNGGCWGCAG-3') and (5'-GACTACHVGGGTATCTAATCC-3') selected from Klindworth et al. (2013).

7.2.7. Bottle test with slime substance

A range of tests with sulfate, crude glycerol and acetate were performed in serum bottles with the slime obtained on day 358 from the UASB described in Chapter 6 (Table 7.1). The slime was previously washed with demi-water. Average concentrations used to perform the different test are presented in Table 7.3. Average glycerol inlet concentration in the UASB was 828.84 mg L^{-1} (9 mM). Therefore, that was the concentration for the test to simulate the conditions, even if measured concentrations (Table 7.3) were lower than expected. The same crude glycerol used to feed the reactor was used to feed the bottles.

Table 7.3. Average conditions set in the bottle test with slime.

Sulfate (mg L^{-1})	Sulfate (mM)	Acetate (mg L^{-1})	Acetate (mM)	Glycerol (mg L^{-1})	Glycerol (mM)
217.9±10.8	6.8±0.3	586.2±55.4	7.1±0.7	637.4±31	6.9±0.3

All characterization tests were performed using 120 mL bottles containing 50 mL of liquid. Acetate and crude glycerol deoxygenated stock solutions were prepared, autoclaved and preserved to be used as required along the experiments. The bottles were incubated at 37 ºC under static conditions. Product and substrate profiles were in all cases assessed using the methodology described in Section 4.2.2 of Chapter 4.

Experiments were conducted in mineral medium (MM) containing (per liter of MM): KH_2PO_4, 0.408 g; $Na_2HPO_4{\cdot}2H_2O$, 0.534 g; NH_4Cl, 0.3 g; NaCl, 0.3 g; $MgCl_2{\cdot}6H_2O$, 0.1 g; Na_2SO_4, 2.2 g; yeast extract 0.5 g and resazurin 0.5 mg. The MM was supplemented, per liter, with 61.8 μg H_3BO_3, 61.25 μg $MnCl_2$, 943.5 μg $FeCl_2$, 64.5 μg $CoCl_2$, 12.86 μg $NiCl_2$, 67.7 μg $ZnCl_2$, 13.35 μg $CuCl_2$, 17.3 μg Na_2SeO_3, 29.4 μg Na_2WO_4 and 20.5 μg Na_2MoO_4. The MM was prepared, boiled and subsequently cooled on ice under a continuous nitrogen flow. Bottles were filled with MM and instantly capped with rubber stopper and aluminum

cap. The final pH of the medium was 7.0-7.2. The gas phase was exchanged with N_2 resulting in a final pressure of 1.5-1.8 atm. The serum bottles were then autoclaved and stored at room temperature until further use. Before inoculation, the MM was augmented with the following volumes of stock solutions: 1 % v/v of 11 g L^{-1} $CaCl_2 \cdot 2\ H_2O$, 1 % of a vitamin solution containing per liter: biotin 20 mg, nicotinamide 200 mg, paminobenzoic- acid 100 mg, thiamine (vitamin B1) 200 mg, panthotenic acid 100 mg, pyridoxamine 500 mg, cyanocobalamine (vitamin B12) 100 mg, riboflavine 100 mg. The MM was reduced by introducing a 5 % v/v of a sterilized reducing stock solution containing 4.8 g L^{-1} $Na_2S \cdot 9\ H_2O$ and 80 g L^{-1} $NaHCO_3$. Test bottles were inoculated with 5 mL of slime whereas abiotic controls consisted of bottles containing MM with the corresponding compounds (acetate and crude glycerol) without slime and were used to exclude abiotic transformations. Microcosms that depleted the initial dose of sulfate were reamended with the same dose presented in Table 7.3. Stock solutions of Na_2SO_4 were used to feed the bottles.

The dilution-to-extinction technique in liquid medium was used to enrich the degrading cultures. Sequential dilutions series were generated through serial 1 in 10 dilutions starting from an active culture and up to 10^{10} dilution (Figure 7.4). Dilutions were incubated at static conditions at 37 ºC and monitored for substrate consumption evaluation.

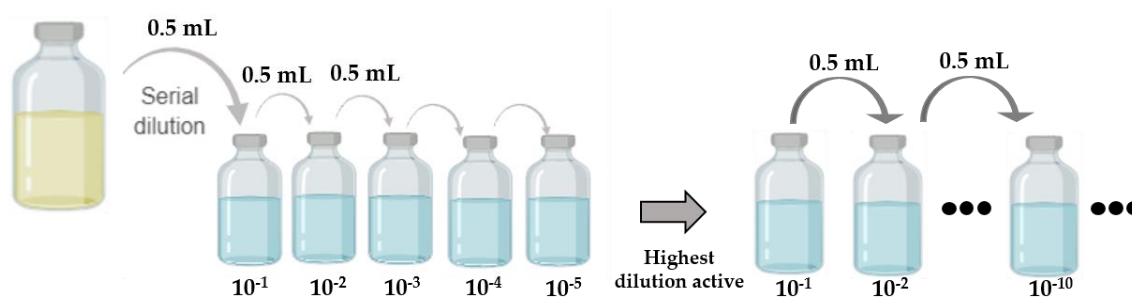


Figure 7.4. Dilution-to-extinction technique.

7.3 Results

7.3.1. Comparison of long-term operations and limits of the system

A comparison of both UASB operations (operation 1 and 2) previously presented in Chapters 5 and 6 is used herein to discuss the general tendencies observed in both cases. Figure 7.5 shows the concentration of acetate and propionate measured in both performances. What can be clearly seen in this figure is the gradual increase in the concentration of acetate from day 150 onwards. The same tendency is observed in both operations, indicating that the main reason for this accumulation was the wash-out of methanogens, which were the ones using acetate at observable rates. This was already discussed and confirmed through 16S rRNA sequencing in Chapter 6. A clear accumulation of VFAs (> 300 mg L^{-1}), which subsequently caused the UASB performance to worsen was also observed by Lu et al. (2015b) when working at HRT of 3 h. Acetate accumulation implies the loss of large amounts of COD that could be used for sulfate reduction. Thus, enhancement of acetate uptake by sulfate reducers is key for the improvement of the use of the carbon source. As previous results have shown that CH_4 production is completely blocked, acetate should be used to reduce sulfate instead of to produce methane.

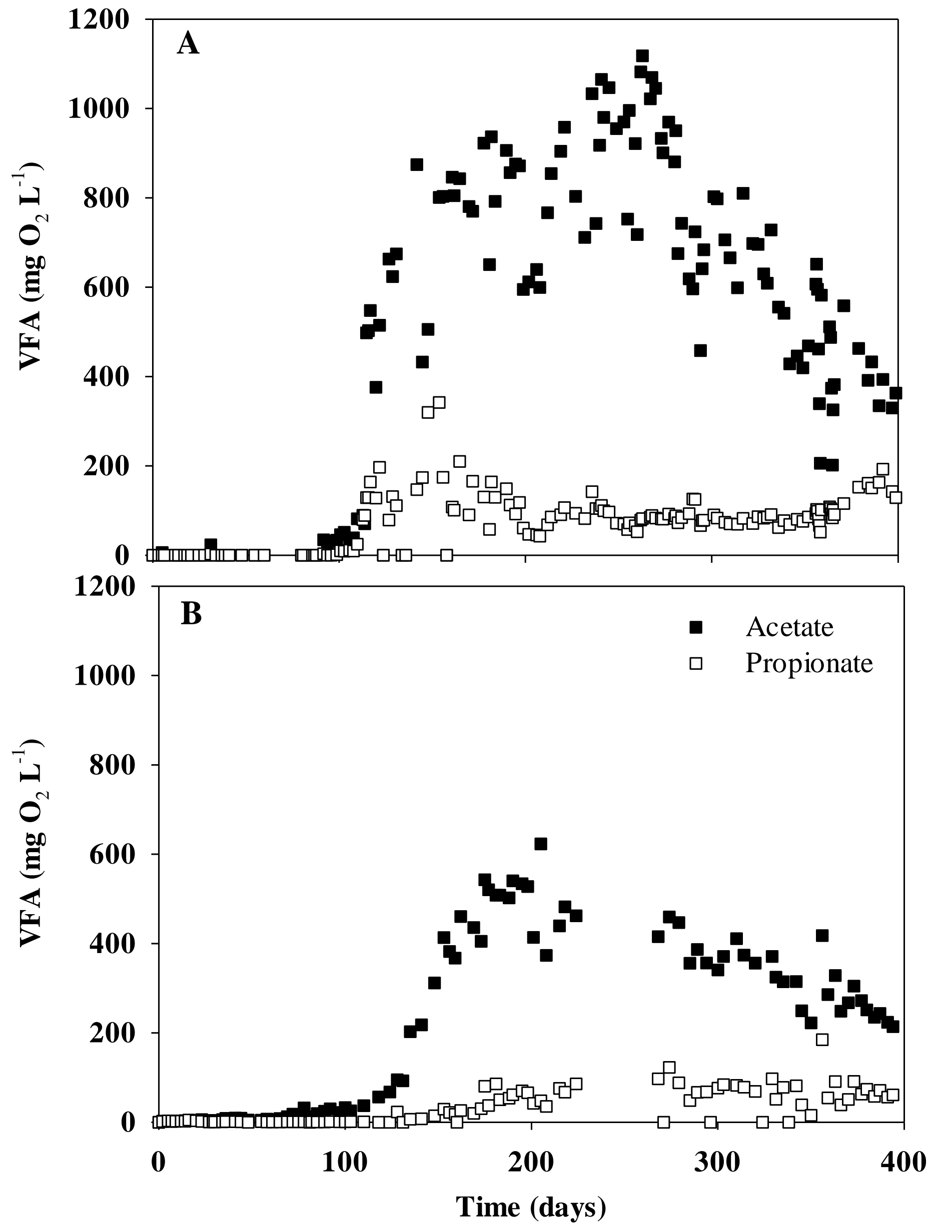


Figure 7.5. VFA concentration (acetic acid and propionic acid). A) Operation 1. B) Operation 2.

Figure 7.6 compares the removal efficiencies obtained in both operations in terms of sulfate and organic matter. In Figure 7.6B a clear decrease in TOC removal efficiency from day 100 onwards can be seen, even if by that time sulfate removal efficiencies were still over 80 % until day 300 when a progressive failure occurred. These results and the same trend can be observed in Figure 7.6A, where COD removal efficiency started decreasing first, followed by a diminishment of the sulfate removal efficiency. The conditions in the UASB by day 350 (Figure 7.6B) allowed SRB to overcompete methanogens and TOC and sulfate removal efficiencies were 11 % and 30 % respectively, indicating a progressive decline of removal efficiencies and a deterioration in the operation considering our initial goals and objectives. In the long-term, for both cases, a clear decrease in sulfate and organic matter removal efficiencies are seen, leading to a failure of the operation.

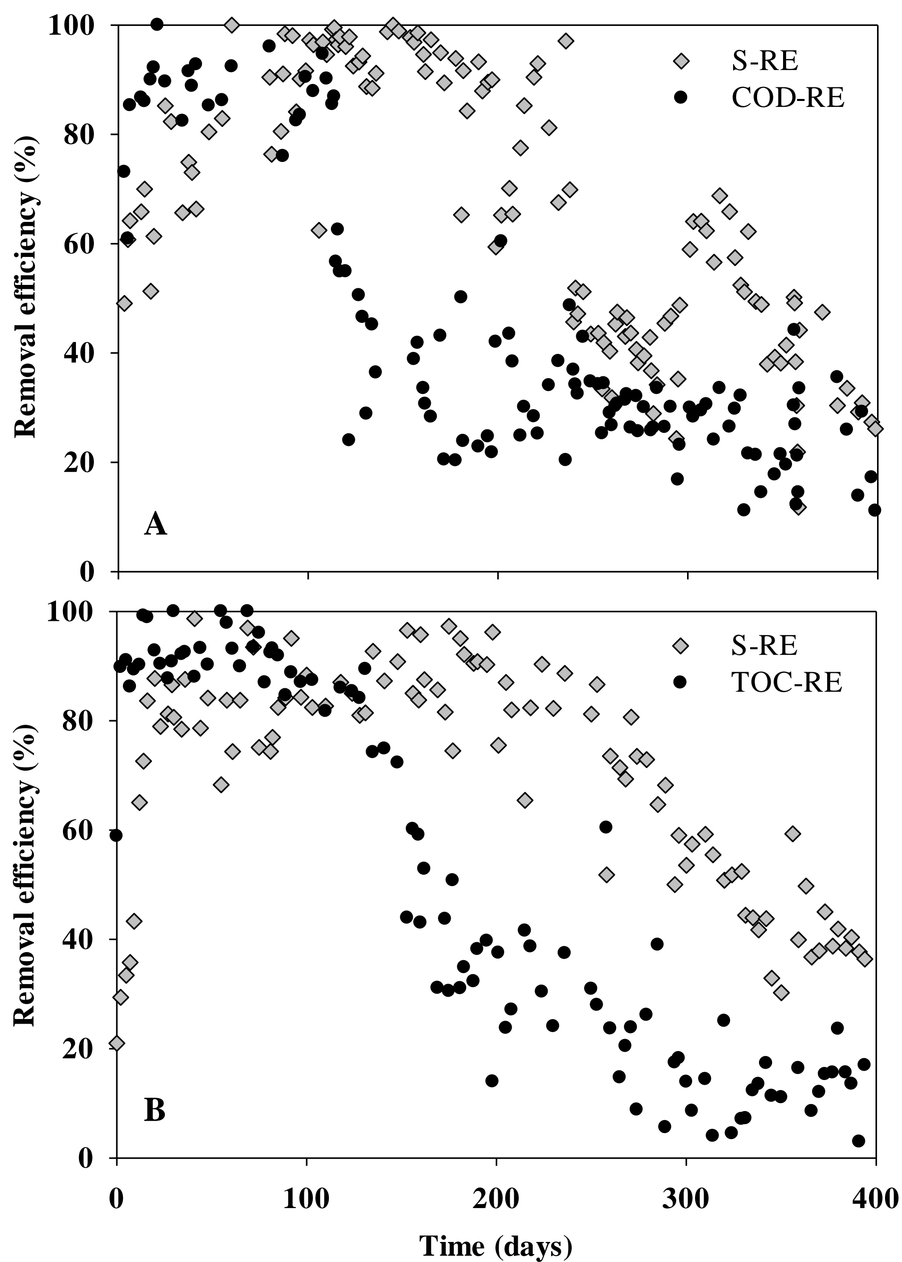


Figure 7.6. Experimental profiles corresponding to TOC, COD and sulfate removal efficiencies obtained in the sulfidogenic UASB reactor. A) Operation 1. B) Operation 2.

7.3.2 Physical-chemical characterization of the slime

SEM

Slime samples obtained from both operations 1 and 2 were collected for SEM analysis (Table 7.1). A selection of all the images obtained is presented in Figures 7.7 and 7.8. The growth of biofilm and a different variety of microorganisms in the slime were observed. Interestingly, in some images, big strings much larger than the microorganisms can be seen around them. It is not clear whether these could be fibers coming from the crude glycerol or huge microorganisms.

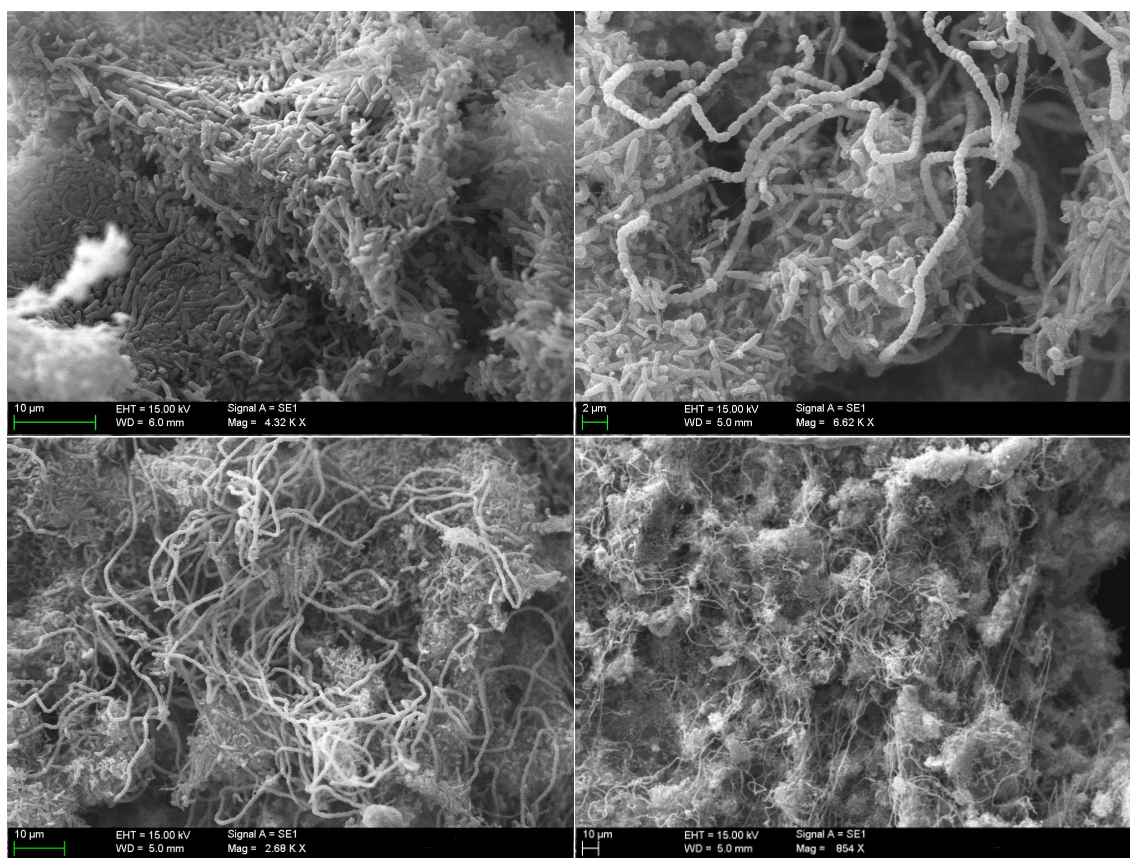


Figure 7.7. SEM pictures of the slime from operation 1.

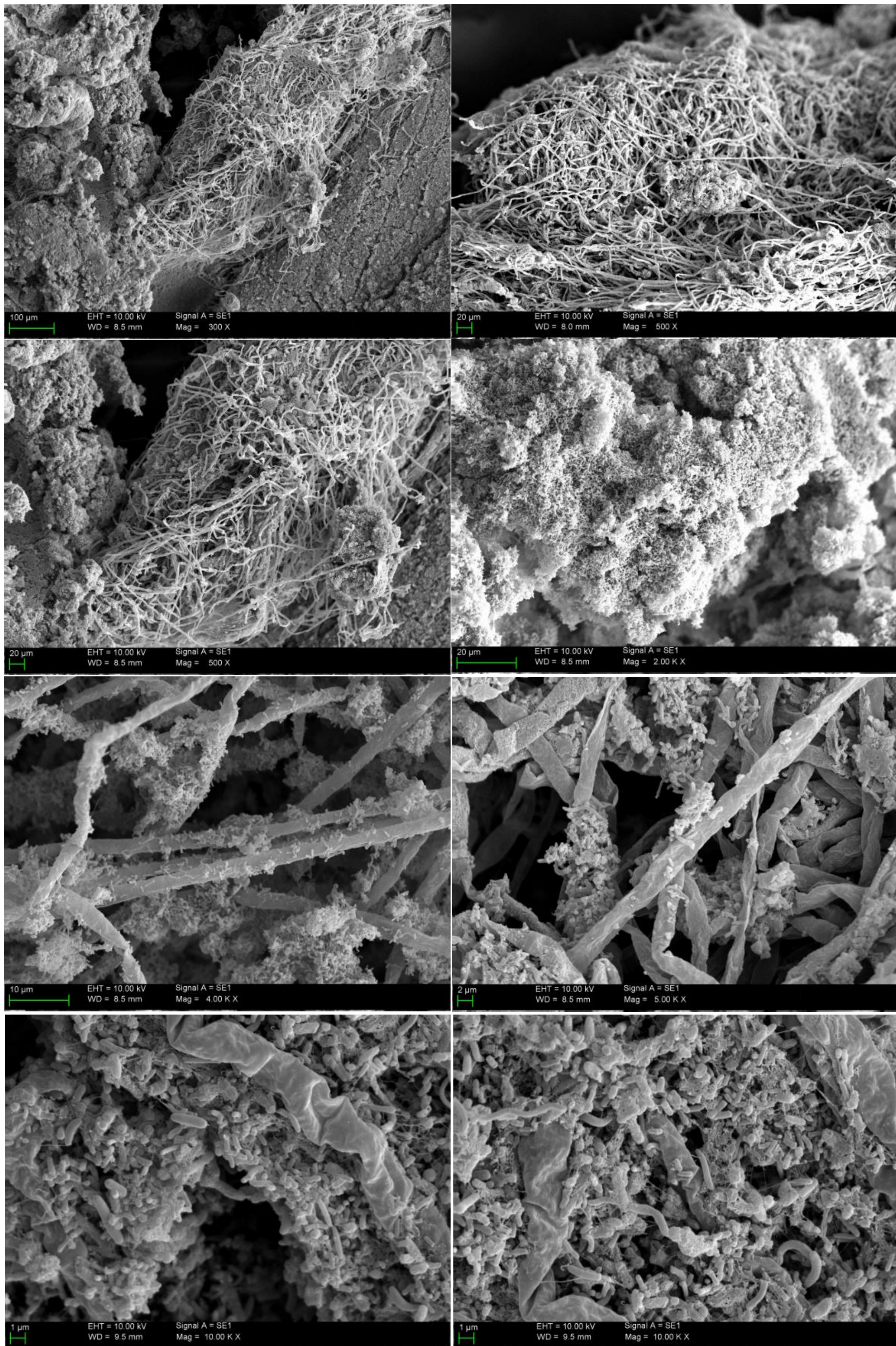


Figure 7.8. SEM pictures of the slime from operation 2.

Elemental analysis

Table 7.4 shows the percentage of carbon, hydrogen, nitrogen and sulfur in the samples analyzed according to Table 7.1. Triplicates of the samples were analyzed, and the results presented are an average of all the measurements. No significant differences can be observed from both samples. Carbon content in both samples is 46 %, hydrogen accounts for a 7 % approximately and nitrogen for a 9.3 %. The sulfur content was lower than 0.6 %.

Table 7.4. Main compounds detected from the lyophilized slime from UASB 1 and UASB 5 by CHNS analysis.

Sample	C (%)	H (%)	N (%)	S (%)
UASB 1	46.8±0.3	7.1±0.1	9.6±0.3	0.5±0.0
UASB 5	46.0±0.3	6.9±0.0	9.2±0.2	0.6±0.1

FTIR spectrum

Infrared spectroscopy is an analytical technique applied to the characterization of molecules, which absorb specific frequencies that are characteristic of their structure. This is the basis of this technique. The strength of the bond causes different absorptions and, changes in the permanent dipole, activate a vibrational mode in the infrared region of a molecule. First, the change in the permanent dipole of a molecule is produced when interaction between this molecule and electromagnetic light takes place. Then, when the frequency of the infrared light is the same as the vibrational frequency of a bond, absorption occurs (Ferrer, n.a.). FTIR has become a valuable tool as it provides the possibility of analyzing and identifying macroscopic or microscopic samples coming from different origins and without destroying them. Moreover, infrared spectrum can be obtained in few minutes and the selectivity of the technique, like a fingerprint of the substance, makes it a first step choice in any conventional analysis.

Due to its sensitivity, an infrared spectrum can be applied to biological systems. The spectra obtained from lyophilized samples is presented in Figure 7.9. There were no easily noticeable differences between the lyophilized slime (SLM1 and SLM2) and granular sludge (GRS) samples having a quick look at the spectra (Figure 7.9), even if the spectra for SLM1 and SLM2 are much more similar than the one corresponding to GRS, what was somehow expected, as differences between slime and granules were being investigated.

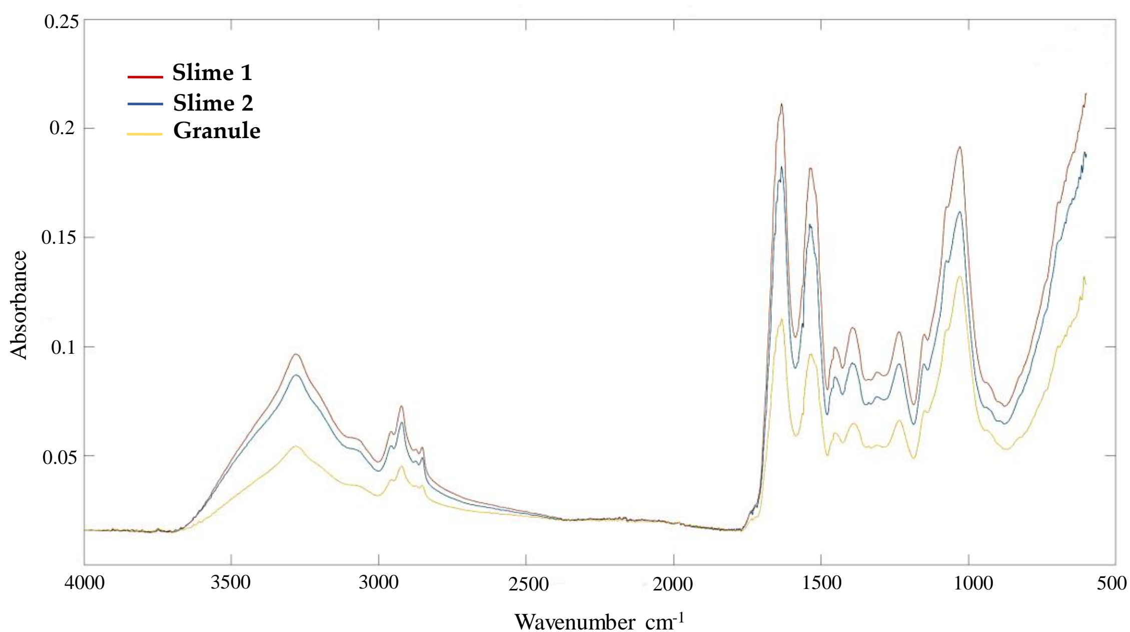


Figure 7.9. FTIR spectrum of the lyophilized samples.

For a more detailed analysis of the spectra, a deeper look at some regions of interest is presented in figure 7.10. In figure 7.10A region 4000-3000 cm^{-1} is shown. In this region O-H stretching and N-H stretching can be seen which can indicate polymeric bonds (strong and broad). It is not very surprising as the carbon source was crude glycerol, and glycerol has three OH groups. The sharper peak observed at 3300 cm^{-1} could indicate N-H groups. Figure 7.10B, region 3000-2800 cm^{-1} is where C-H stretching can be seen, typically absorbance at wavenumbers below 3000 show C-H groups that are sp^3 hybridized, for example saturated fats or CH_3 groups, among others. Between 1700-1600 cm^{-1}

(Figure 7.10C) different bands can be seen that indicate different secondary protein structures (α-helix, β-sheet, β-turn and random coil) reported elsewhere in the literature (Barth, 2007). These are called the amide I bands. One drawback of infrared spectroscopy of aqueous solutions is the strong absorbance of water in this spectral region (near 1645 cm^{-1}) (Venyaminov and Prendergast, 1997), that is why samples were lyophilized. The strong absorbance of water mentioned above, could overlap the important amide I band of proteins and some side chain bands (Barth, 2007). Absorbances of 0.18, 0.21 and 0.11 were measured for SLM1, SLM2 and GRS at 1635 cm^{-1}. SLM contained relatively bigger absorption peaks in this region, which is the amide I band region or protein region. 1740-1700 cm^{-1} shows C=O stretching which means that there could be organic acids. There seems to be a slight difference in ratio of the different bands, but this could also be due to the differences in absorbance between the samples. Amide II band shown in Figure 7.10D (1600-1500 cm^{-1}) is indicative of proteins. Some small differences in the line shape can be seen between SLM1-2 and GRS, which can suggest different protein composition.

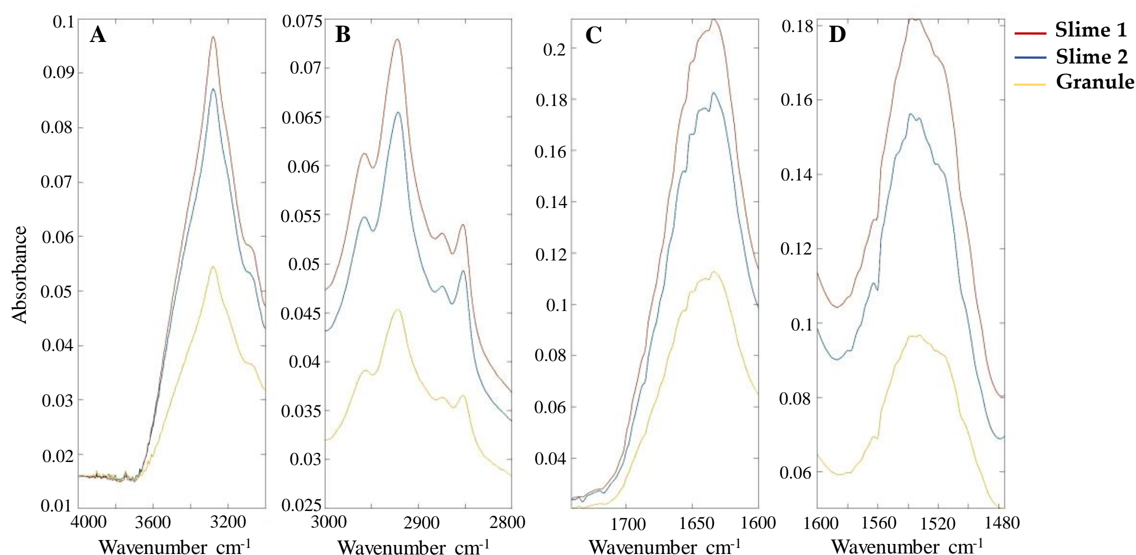


Figure 7.10. Spectra of the lyophilize samples. A) O-H vibration region. B) C-H bond region. C) Amide I band. D) Amide II band.

Peaks around 1235 cm^{-1} can indicate sulfated groups. In our samples the absorbance at 1235 cm^{-1} was 0.90, 0.11 and 0.06 for SLM1, SLM2 and GRS, respectively. Furthermore, the region 1200-1000 cm^{-1}, that can be seen in more detail in Figure 7.11, shows indications of sugar bonds. The 1150 peak is indicative of C-O-C binding like the ones presented typically in sugars. In our samples the absorbance at 1150 cm^{-1} was 0.09, 0.16 and 0.07 for SLM1, SLM2 and GRS, respectively.

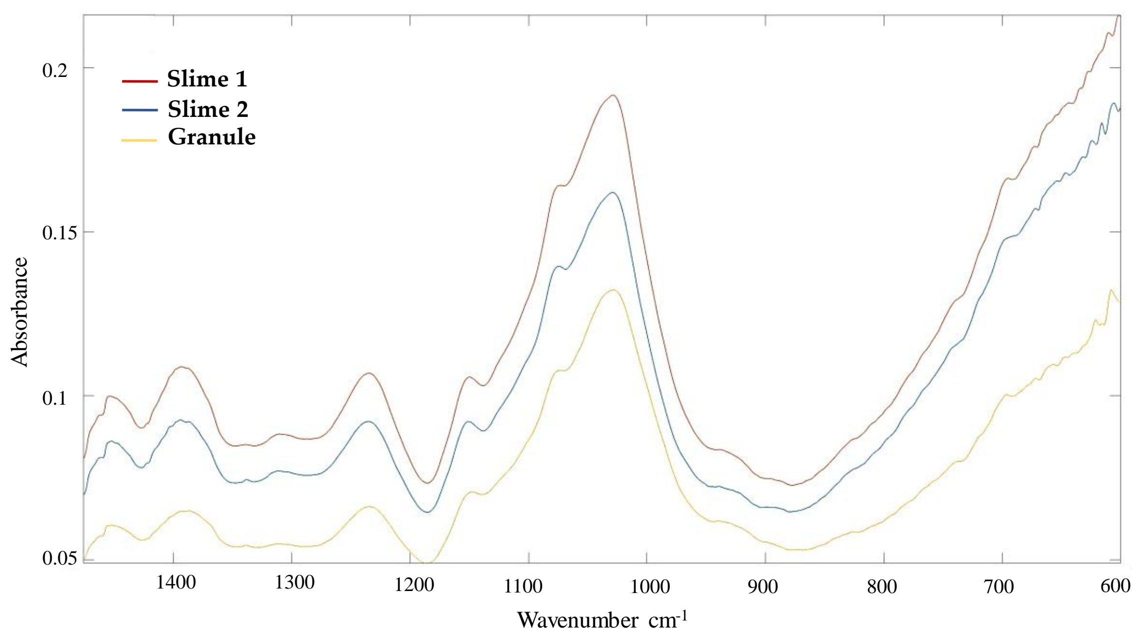


Figure 7.11. Spectra of the lyophilized samples in the 1475-600 cm^{-1} region.

Table 7.5 compares the absorbances of the slime (calculated as an average between SLM1 and SLM2) and the granule for different wavelengths (cm^{-1}). These wavelengths were selected as interesting regions according to the results shown previously. The ratio between them is also shown, which is calculated as the absorbance of the slime divided by the absorbance of the granule. As can be observed, it is almost constant and close to 1.7 along the whole spectrum, meaning that the different in absorbance between slime and granule was maintained.

Table 7.5. Absorbances for the slime and granule samples at different wavelengths along the FTIR spectrum.

ʎ (cm^{-1})	Absorbance (slime)	Absorbance (granule)	Ratio
1150	0.13	0.07	1.82
1235	0.10	0.07	1.52
1540	0.17	0.09	1.77
1634	0.20	0.11	1.76
1650	0.18	0.10	1.74
1740	0.03	0.02	1.68
3300	0.09	0.05	1.72

Proteoglycans (PGs) are biological molecules composed of a specific core protein substituted with covalently linked glycosaminoglycan (GAG) chains. GAGs, that have been measured as described previously, are linear, sulfated, negatively charged polysaccharides, which can be divided into two classes: sulfated GAGs and non-sulfated GAGs (Schaefer and Schaefer, 2010). Sulfated glycoaminoglycans (sGAG) might be produced in our samples. Both, in GRS as in SLM, a similar concentration of sulfated GAG was found, which was around 4 µg sGAG/mg dry sample. The O-sulfated GAG measurement gave a value higher than the total GAG measurement, which is strange, but could be explained by the fact that the total GAG measurement was at the maximum saturation concentration of the dye agent. The O-sulfated GAG determination can give a clue if most of the sGAG is in the form of N-sulfated GAG or O-sulfated. Sulfated GAGs are difficult to synthesize chemically and especially the N-sulfated GAG.

FAMEs

As reported in Chapter 5, crude glycerol derived from the biodiesel production has several impurities that could affect microorganisms. The main impurities in crude glycerol are methanol, heavy metals and soap (Viana et al.,

2012), fatty acids and other organic impurities (Siles López et al., 2009). To gain more knowledge about the slime nature and further determine if the impurities contained in crude glycerol could be present in the slime, FAMEs were analyzed (Table 7.1). Figure 7.12 shows the general chromatograms of the different samples that correspond to the biofilm attached to the reactor walls in the upper part, and to the sludge, collected at different heights (UASB 1-2; UASB 2-3 and UASB 3-4).

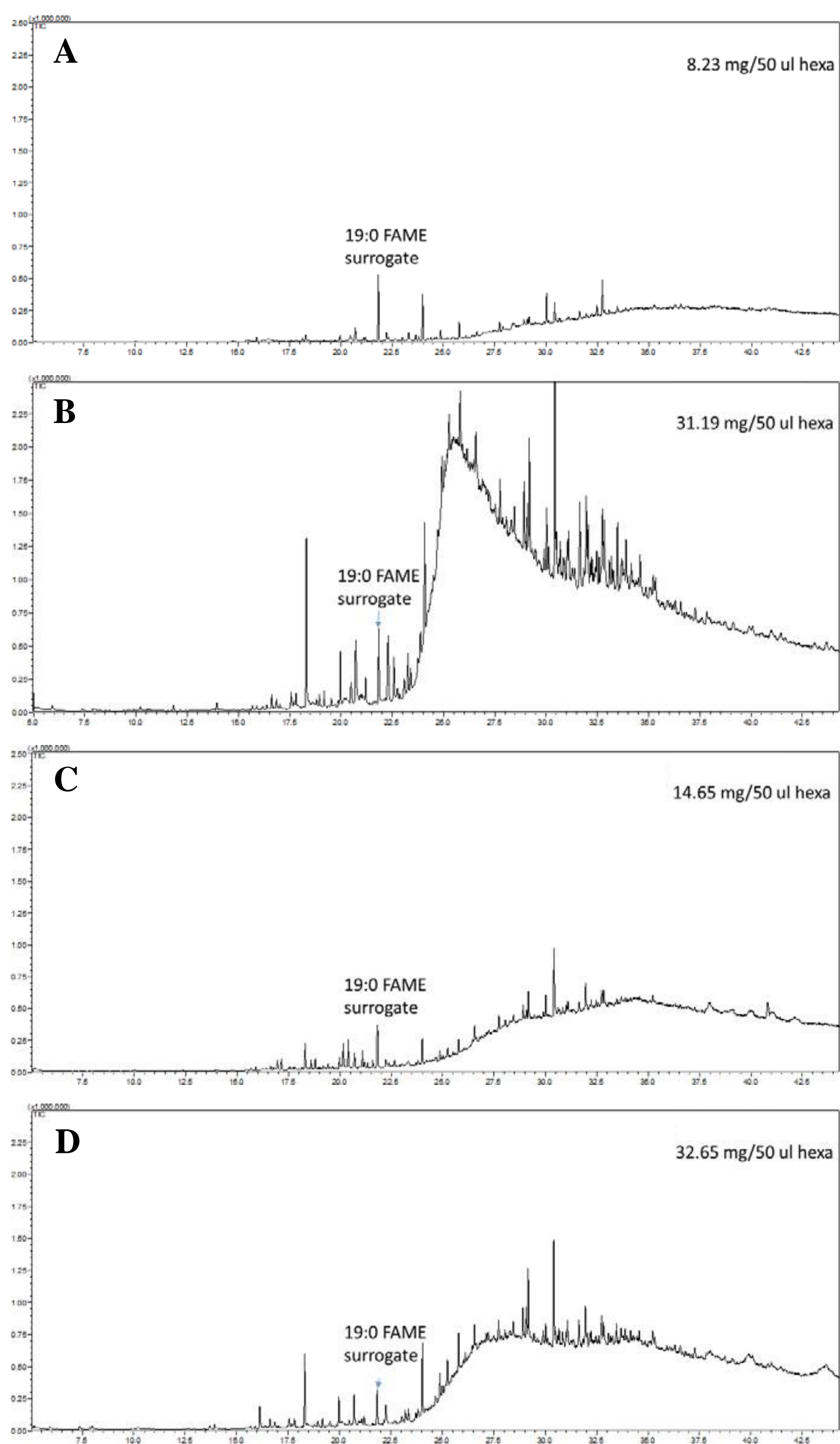


Figure 7.12. General chromatogram of the different samples. A) Biofilm attached to the walls of the reactor. B) UASB 1-2. C) UASB 2-3 D) UASB 3-4.

Different FAMEs were present in the samples, and its concentration is presented in Table 7.6.

Table 7.6. Quantified FAMEs in the slime substance samples obtained from the different reactor heights, expressed mg kg^{-1}.

	Concentration (mg kg^{-1})			
Compounds (FAMEs)	**Attached biofilm**	**UASB 1-2**	**UASB 2-3**	**UASB 3-4**
$C_{14:0}$ (Miristic acid)	1.8	6.1	11.4	6.3
$C_{15:0}$ (Isopentadecanoic acid)	2.5	6.7	6.6	7.4
$C_{15:0}$ (Anteisopentadecanoic acid)	5.0	15.5	18.2	20.2
$C_{15:0}$ (Pentadecanoic acid)	1.7	5.1	7.3	6.5
$C_{16:0}$ (Hexadecanoic acid)	1.0	5.5	4.4	5.8
$C_{16:0}$ (Palmitic acid)	30.5	197.3	125.7	186.3
$C_{17:0}$ (Isoheptadecanoic acid)	2.0	15.1	8.6	28.7
$C_{17:0}$ (Anteisoheptadecanoic acid)	4.1	17.5	13.1	20.1
$C_{17:0}$ (Heptadecanoic acid o margaric acid)	1.3	9.6	8.9	12.3
$C_{17:1}$	4.3	11.1	10.2	12.6
$C_{18:0}$ (Stearic acid)	0.0	26.0	38.5	35.0
$C_{18:1}$ (Oleic acid)	0.0	3.6	17.0	2.9
$C_{18:1}$ (cis-11-octadecanoic acid)	4.3	7.7	3.5	4.7
$C_{20:0}$ (Eicosanoic acid)	0.0	0.8	6.0	2.3
$C_{22:0}$ (Docosanoic acid)	0.0	0.0	2.5	0.0
$C_{24:0}$ (Lignoceric acid/ Tetracosanoic acid)	0.0	0.0	7.7	0.0
Total FAMEs	58.5	327.7	289.4	351.1

Note: $C_{x:y}$: x is the number of carbon in LCFA and y is the number of double bond

Other interesting compounds detected in the samples but not quantified were methyl esters. These compounds could be coming from the crude glycerol used during the operation, which could explain why in UASB 1-2 the highest peaks were detected (data not shown), as this is the feeding point of the reactor. As it was previously seen with SEM images, the slime presented a white cotton-like aspect. Wax esters, that were also detected but no quantification was made, could be responsible of giving this aspect to the slime.

A huge peak could be observed in Figure 7.12B around min 25 that made impossible the quantification of any compound appearing at that retention time. To get a better insight into the different samples, a previous derivatization of each of them was made, and they were injected into a different column to try to quantify the possible alcohols and free fatty acids appearing at that retention time (Section 7.2.4). These results are presented in Table 7.7. According to this table, higher concentrations of free acids, without any metilation, are detected in samples UASB 1-2 and UASB 3-4. Special mention to iso-pentanoic and anteisopentanoic acids, as they are considered bacterial indicators (Kaneda, 1991) and are especially abundant in these samples as well. Propionic, butyric, isovaleric and valeric acids were also detected but no quantification was made.

Table 7.7. Concentrations of free acids in % (weight/weight of sample) found for the different samples.

Compounds	Attached biofilm	UASB 1-2	UASB 2-3	UASB 3-4
$C_{14:0}$	0.4	3.0	0.9	4.0
iso-$C_{15:0}$	1.2	6.3	1.5	8.1
anteiso-$C_{15:0}$	2.8	15.3	5.2	27.0
n-$C_{15:0}$	0.4	1.6	0.5	2.7
$C_{16:0}$ acid without derivatization		3.5		3.6
$C_{16:0}$	4.7	4.5	8.5	11.2
iso-$C_{17:0}$	0.2	1.5	0.8	3.3
anteiso-$C_{17:0}$	0.1	2.2	0.8	4.6
$C_{17:1}$	0.3	2.9	1.2	4.5
n-$C_{17:0}$	0.2	0.6	0.1	1.0
$C_{18:1}$	0.3	0.7	0.2	0.6
$C_{18:0}$	0.2	0.5	0.1	0.7
Total	**10.8**	**42.6**	**19.8**	**71.3**

7.3.3. Biological characterization of the slime

16S Sequencing

The biodiversity found in the slime was examined using complementary microbial ecology methods, 16S rRNA sequencing in this case. As explained in Section 7.2.6, two samples at different operation times were collected for the analysis. Figure 7.13A compares the relative abundances (%) of the most abundant genus detected in the samples of the slime taken from the reactor on day 358 and 538 of operation 2. No significant changes or a shift during slime development can be detected from sample SLM_OP2_358 to sample SLM_OP2_538, even if almost 200 days of operation passed in between. *Dysgonomonas* was the most abundant genus on day 358 with a relative abundance of 14.7 % whereas on day 538 the most abundant one was *Propionispora* with a 21.5 %, that increased from a 11.5 % detected on day 358. On

day 538, *Dysgonomonas* genus decreased to 10 %. The rest of the genera detected maintained their relative abundances from day 358 to day 538 and no other significant changes can be mentioned, taking into consideration that there were no replicates done for any of the samples.

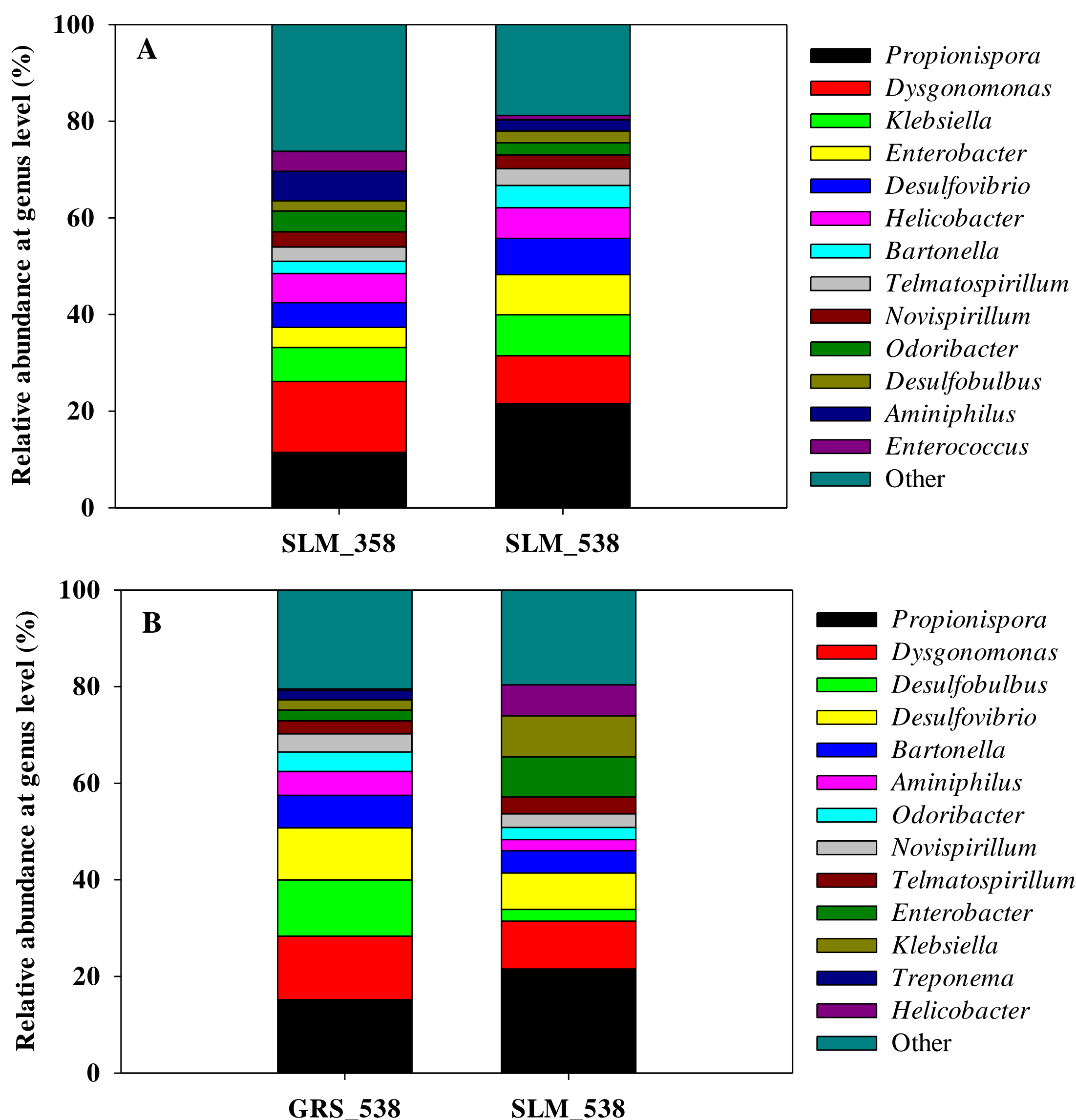


Figure 7.13. Relative abundances (%) of the most abundant genus detected. A) Samples SLM_OP2_358 and SLM_OP2_538. B) Samples GRS_OP2_538 and SLM_OP2_538.

On day 538, granules were also sequenced apart from the slime, and the results are shown in Figure 7.13B. The relative abundance of genus *Propionispora* increased from 15.2 % to 21.5 % if comparing GRS with SLM, respectively. Both, *Desulfobulbus* and *Desulfovibrio* decreased their relative abundances in the SLM compared to GRS from 11.6 % to 2.4 % and from 10.8 % to 7.5 % respectively. By contrast, *Enterobacter*, *Klebsiella*, and *Helicobacter* genera raised their relative abundances in SLM, from 2.2 % to 8.2 %; from 2.1 % to 8.5 % and lastly from 0.3 % to 6.3 % respectively. *Enterobacter* and *Klebsiella* belong to *Gammaproteobacteria* class, whereas *Helicobacter* belongs to *Epsilonproteobacteria* class. Considering that no triplicates were analyzed and therefore, no statistical analysis were performed for the sequencing results, there are no symbolic differences between GRS and SLM in terms of detected genera. *Desulfobulbus* is the genus with a higher difference in terms of relative abundance (%) between both samples, 11.6 % for GRS and 2.4 % for SLM.

7.3.4 Degradability of sulfate and glycerol by the slime

As shown in Figure 7.2, the slime was already covering the walls of the reactor from day 350 onwards. At this point, bottle tests were performed to characterize the predominant mechanisms driven by the slime and to verify its sulfate reduction activity. To get valuable information that could be somehow representative from what was happening in our reactor, conditions set in bottle tests were comparable to those set during the long-term performance of the UASB assessed in Chapter 6 (Section 7.2.7). A set of bottle tests were prepared and monitored to evaluate the biodegradation of sulfate, glycerol and acetate. Concentrations of all the compounds were similar to those set in the influent of the UASB reactor during operation 2. In that way, more accurate conclusions could be inferred on the role of the slime in the reactor and what was its sulfate

reduction and fermenting capacity. Figure 7.14 shows the results from the monitoring of the 4 bottles (replicates) calculated as an average. Results indicate that 8 days after the inoculation of the bottles, the concentration of sulfate was 28.8±3.2 mg L^{-1} (0.9±0.1 mM) and 131.2±16 mg L^{-1} (4.1±0.5 mM) for sulfide, meaning that sulfate reduction was taking place. On the other hand, removal of sulfate or glycerol was not observed in the abiotic control (Figure 7.14B), indicating that the reactions were biotically mediated. Sulfate was almost completely depleted by day 8, so, on day 10 a new dose of sulfate (indicated with an arrow in Figure 7.14A) was added to the bottles, reaching again a concentration of 320 mg L^{-1} (10 mM). During the first 8 days the sulfate reduction rate (SRR) was 12.8 mg L^{-1} (0.4 mM d^{-1}), whereas from day 13 until day 21, it was 9.92 mg L^{-1} (0.31 mM d^{-1}).

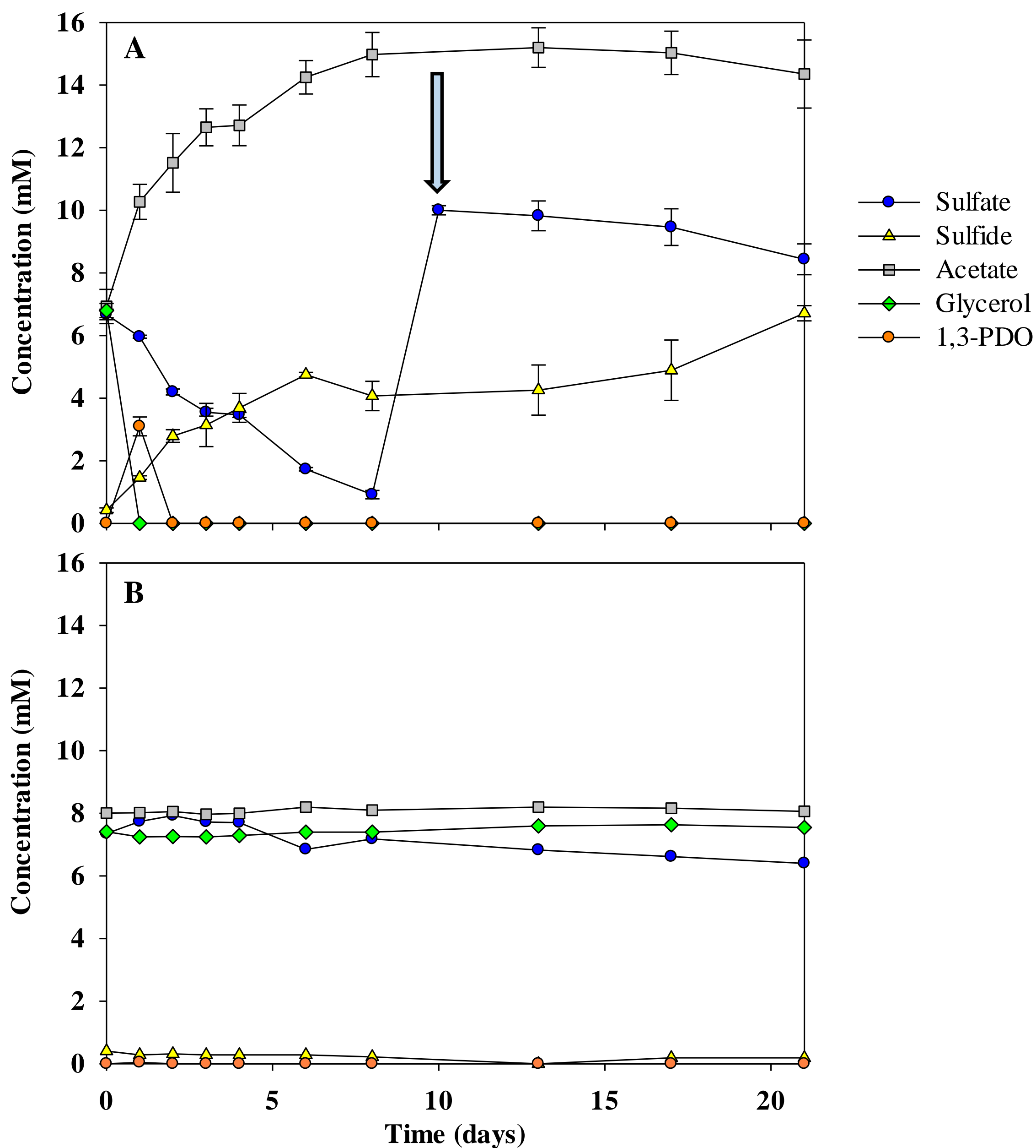


Figure 7.14. A) Test performed with slime. The arrow indicates the addition of sodium sulfate. B) Abiotic control without slime.

7.4 Discussion

Although the UASB reactor owned good performance in operations 1 and 2 for almost 300 days and the feasibility of using crude glycerol as carbon source was demonstrated, in the long-term, both operations ended up failing. Different sulfate loading rates had been tested during operation 1 while for operation 2 the SLR was constant; however, inlet sulfate concentration was not a key factor as all the conditions tested led to a failure. As reported by Omil et al. (1998), methanogenesis is necessary, in addition to sulfate reduction, to achieve a complete removal of the organic matter for wastewaters with COD/sulfate ratios exceeding 0.67 g O_2 g^{-1} SO_4^{2-}. During operation 1 and 2 the COD/sulfate ratio ranged from 1-1.8 g O_2 g^{-1} SO_4^{2-} which would imply that apart from sulfate reduction, methanogenesis would be required for a complete elimination of the organic matter. In accordance to Omil et al. (1998), SRB became predominant over methanogens after 200 days of operation, what was confirmed along Chapter 6. That explained the accumulation of acetate observed in both operations working at the abovementioned ratios. Acetate accumulation implies loss of organic matter in the outlet of the reactor, what is not desirable, but was not the main cause of the failure. Different techniques were used along this chapter to try to explain this breakdown, that will be discussed along this section.

According to our results, granules seem to play an important role in maintaining the stability and removal efficiencies of the reactor. The seed granules used to inoculate the reactor were highly compact and aggregated but in the long-term they became more disintegrated and they changed their color from black to grey. The overgrowth of slime all around the granules and on the walls of the reactor, as shown in Figure 7.1 and 7.2, could have led to the failure of the system. The more disintegrated granules were, and the more slime substance was accumulated, the worst performance was observed. A similar behavior was reported by Lu et al., (2015a), who observed that granules used to

inoculate a UASB reactor fed with methanol, coming from a mesophilic anaerobic digester, were highly compact and aggregated. However, they became very loose and fragile as time elapsed and even showed rupture and dispersion. This not only deteriorated the quality of effluent and gave rise to the reactor imbalance but also increased the risk of failure greatly. His system could operate stably for around 240 days at OLR of 48 g COD $L^{-1}d^{-1}$ and a biogas yield of 18.6 ± 5.7 L L^{-1} d^{-1}.

The hydraulic "selection pressure theory" referred by different authors (Yu et al., 2001; Hulshoff Pol et al., 2004) revealed that heavier granules would remain in the lower part of the sludge bed by self-immobilization while lighter sludge particles would be moved to the upper part or even washed out from the system being operated. Bulky microbial aggregates, as the slime with a high content of FAMEs and other substances detected, may reduce the specific gravity of granules and led to flotation of the sludge even clogging the outlet of the reactor. Lu et al. (2015b) observed intensive flotation of sludge and even washout if biogas-bubbles were not released quickly when reaching the upper part of the UASB and when excess EPS was not consumed effectively. Lu et al. (2015b) also reported that high fraction of polysaccharide content (PS) in EPS of their granules was not advantageous to the long-term stability of reactor because it led to the production of foam and agglomeration of granules.

Another factor that could be affecting our performance could be a mass transfer limitation due to the gelatinous and sticky nature of this slime substance attached to the surface of the granules and to the reactor walls. A similar behavior was found by Lu et al. (2015b) when operating a UASB reactor fed with starch as substrate that mixed with warm waters made sludge particles stick together. A consequent decay in terms of COD removal and biogas production was also reported. The granulation process has been related with up-flow velocities in UASB reactors. Alphenaar et al. (1993) compared the granulation process in two UASB reactors performing anaerobic treatment of sulfate-containing wastewater

and reported higher granule diameters in the reactor with higher up-flow velocities. Reino and Carrera (2017) also related low up-flow velocities (between 0.2-1 m h^{-1}) to external mass transfer problems in their up-flow anammox sludge blanket (UAnSB) reactor. In our case, probably the low up-flow velocity (0.25 m h^{-1}) set along both operations did not promote the granulation process and in the long-term, the massive growth of slime caused mass transfer limitations, even if no calculations were made. However, all the data provided along this chapter made us conclude that FAMEs and other impurities coming from the crude glycerol used, accumulated in the long-term, probably favored by the low up-flow velocity set. This problem was even worsened when methane production ceased and no gas bubbles were produced, so that a much more static sludge was developed.

The overgrowth of this slime and the accumulation of EPS led to a situation presented by SEM images in which many different populations (death or alive) can be seen. It is not clear by these images whether the big strings observed could be fibers coming from the crude glycerol or huge microorganisms. Yamada et al. (2007) also observed a vast number of filamentous cells in the bulking sludge of a full-scale UASB. According to this author, the majority of these kind of fluffy granules (granules surrounded by EPS/slime) were known to be caused by the overgrowth of certain types of filamentous microorganisms (Wu et al., 1993). Factors that generated the filamentous bulking remain unknown, even if some studies reported that filamentous microbes (fluffy sludge granules) overgrew when the carbohydrate concentration in wastewater increases (Wu et al., 1993; Yoda and Nishimura, 1997; Angenent and Sung, 2001; Sekiguchi et al., 2001). Similar pictures as the one presented in this section were captured by Dixon (2018).

In terms of elemental analysis, a similar composition was detected for aerobic granules in Liu et al. (2003). The C: N ratio was 4.9 for UASB 1 and 5 for UASB 5.

Based on the general bacterial cell formula of $C_5H_7NO_2$, the % of the different elements can be calculated, obtaining the following results: 53.1 % C; 6.2 % H; 12.4 % N and 28.3 % O. If compared to our results (Table 7.4), a similar composition can be observed for slime samples at both reactor´s heights (UASB 1 and UASB 5), indicating that there was an important organic fraction in our samples and that probably the slime consisted of biomass with other accumulated compounds. This result could be confirmed by the huge number of bacteria and different colonies that were observed in SEM images.

Having a look at the FTIR spectra described in Section 7.3.2, it seems that there were no meaningful differences between SLM and GRS samples. This may be due to the fact that GRS consisted of cells in a biofilm and, in this biofilm, cells were embedded in an extracellular matrix which was composed of extracellular polymeric substances (EPS). Probably, both, GRS and SLM were biofilms with microbes embedded in an extracellular matrix. Having a look at the different regions previously mentioned, the biggest difference between GRS and SLM was between the ratio of peaks around 3200/1640 and 1030. SLM showed higher peaks around 3200/1640 and GRS showed peaks around 1040. The ratio between the peak at 1635 over peak at 1030 tells relatively how much protein is in the sample and how much sugars. So, for instance a value of Abs1635/Abs1030 = 2 would mean that the peak for proteins is 2 times as high as the peak for sugars. This ratio is 1.1 for SLM and 0.8 for GRS. This indicate that SLM contained relatively more proteins than the GRS, which contained relatively more sugars. A possible explanation for the high value of O-sulfated GAG is related with the presence of polyphosphate, biologically produced, that interferes in the GAG analysis. Polyphosphate (Poly-P) is a linear polymer of orthophosphate, abundant in the environment and it is considered as a key component in wastewater treatment and many bioremediation processes (Khoshmanesh et al., 2012). In the case of wastewater, Poly-P forming microorganisms play an important role in the

removal of excess P, as such bacteria can mediate reaction pathways of P and may control P processes over time (Parkinson and Dust, 2010; Brock and Schulz-Vogt, 2011). Khoshmanesh et al. (2012) found that Poly-P in the settlement showed a broad peak at around 1400-1200 cm^{-1} which can also be observed in our spectra. Previous studies also reported bands above 1200 cm^{-1}, which can be indicative of higher chain Poly-P (He et al., 2007; Ha et al., 2010).

To get a better comprehension of the compounds that could be affecting our performance and that were coming from crude glycerol, FAMEs analyses were performed. Long-chain fatty acids (LCFAs) have been reported as inhibitory substances to a variety of anaerobic microorganisms, frequently ending up in serious operational problems in anaerobic treatment systems (Rinzema et al., 1989; Hwu et al., 1996). Lalman and Komjarova., (2004) reported that oleic and linoleic acids affect glucose fermentation and acetoclastic methanogenic populations, resulting in an accumulation of acetate. Oleic acid, which is detected in samples UASB 1-2, UASB 2-3 and UASB 3-4, has additionally been reported as methanogenic inhibitor (Pereira et al., 2002). This problem was related to granular sludge flotation by Hwu et al., (1998), who reported that these compounds are adsorbed onto the biomass. Pereira et al., (2002) also found that palmitic acid was the main component adsorbed onto the sludge and interestingly, in all our samples, palmitic acid was as well the main LCFA quantified. The relation between LCFA biosorption and sludge flotation in a UASB reactor was investigated by Hwu et al. (1998), who concluded that LCFA biosorption can impede the success of a UASB for wastewater treatment, mainly due to sludge flotation rather than to methanogenesis inhibition.

A multispecies microbial community is the prevailing life form developed in biofilms in most natural and engineered environments (Watnick and Kolter, 2000), what makes it more complex to study. According to Fernández et al. (2008) alpha-*Proteobacteria* remained as the predominant class in a mature biofilm

(where cells are embedded in a matrix of exopolymers) with members of the phyla *Firmicutes*, *Bacteroidete* and *Thermotogae* also detected.

When having a look at the results presented for the 16S rRNA sequencing, trivial differences could be detected between GRS and SLM. A similar result can be observed when comparing SLM_358 with SLM_538 even if 180 have passed by in-between both samples. During the first 180 days of operation 2, significant differences in the microbial population were noticed. As an example, *Desulfovibrio* increased its relative abundance from 0 to 42.8 % and methanogens were almost completely washed out from the system. What can be inferred from these data is that microbial populations evolved quickly during the start-up of the reactor but, once the performance had been already running for one year, no symbolic changes could be mentioned. In our particular case, once the reactor had reached this situation, in which all the factors previously mentioned were affecting the operation, populations became also more "static" (Figure 7.13) what can be also considered a symptom of failured operation, not directly because there were no changes in populations, but because the ones detected were not mainly sulfate reducers. The maintenance of the reactor for such long periods of time gave us the possibility of encountering difficulties in UASB performances that could be undoubtedly interesting before scaling up the sulfate reducing process with crude glycerol.

Hung et al. (2011) studied the microbial relationship and structure of granular sludge in a hydrogen-producing bioreactor and found out that *Clostridium pasteurianum*, *Klebsiella* sp., and *Streptococcus* sp. were the predominant microorganisms and extracellular polymeric substances (EPS) were the main components of biofilms and biological granular sludge. Pugazhendhi et al. (2019) identified the groups *Clostridia*, *Enterobacter* and *Bacilli* sp. as the main ones present in their granular biofilm. According to the investigation of Hung et al. (2011) of the bacterial community structure in granular sludge-forming

bioreactor, *Klebsiella* sp. existed in all phases of granular sludge formation, and Tang et al. (2009) confirmed that *Klebsiella* species can form biofilms. Dixon (2018) revealed seven bacteria as strong biofilm formers: four *Klebsiella* spp. with other strong formers consisting of *Citrobacter*, *Enterobacter* and *Pseudomonas*.

Propionispora is a genus of anaerobic fermentative organisms, in fact, they typically used glycerol, and other carbohydrates, fermenting them to produce propionic and acetic acid, CO_2 and H_2 (Abou-Zeid et al., 2004). They could be competing for the available glycerol with sulfate reducers (*Desulfobulbus* and *Desulfovibrio*) but no concluding remarks can be made according to the results presented. Bacteria belonging to the genus *Desulfobulbus* can oxidize propionate in the presence of sulfate to acetate and ferment pyruvate and lactate to a mixture of acetate and propionate (El Houari et al., 2017). Sulfate reducers, both *Desulfobulbus* and *Desulfovibrio,* decreased their relative abundances in SLM compared to GRS, meaning that these populations were probably mainly living in the core of the granules, what can also explain the decrease in sulfate reducing efficiency when we start losing our "healthy" granules to observed fluffy ones. *Desulfovibrio* spp. only carry out an incomplete oxidation of substrates and they are able to excrete acetate as a product. That could also explain the increase in acetate observed during the experiments performed in serum bottles, as *Desulfovibrio* was the major sulfate reducer detected in the slime as reveled by the sequencing analysis.

Mass transfer limitations previously mentioned could affect these populations, as the granules were completely covered by this slime substance around its surface, what could block or impede the diffusion of the substrate from the medium to the granule itself.

7.5 Conclusions

Overall, results obtained in this chapter demonstrated that long-term performances are important to get valuable overall conclusions. Physical-chemical and biological analyses of the slime were applied to characterize its main properties and biological activities. Different long-chain fatty acids as well as FAMEs were detected in the slime indicating that impurities coming probably from the crude glycerol used, were being accumulated in the system. Many of these substances found in the slime, conferred properties such as viscosity to the slime. This, together with the low up-flow velocity (0.25 m h^{-1}) set during both operations could have favored the accumulation of the slime together with EPS surrounding the granules, what could be observed in SEM images. Bottle test confirmed that the slime had sulfate reducing capacity and sequencing results showed that similar populations were present both in the granules and in the slime. However, FTIR analysis showed differences in the spectra of the granule compared to the slime. The protein content of the slime was higher to that in the granule, which contained relatively more sugars. The slime production was found to be a crucial factor affecting the system. Consequently, problems attributed to mass transfer limitations and granules density could be observed, which negatively affected the sulfate reducing activity and led to failure performances.

Chapter 8: Microbial diversity study towards the improvement of sulfidogenesis in a UASB reactor through the bioaugmentation of acetotrophic-sulfate-reducing bacteria

This work has been performed at the Laboratory of Microbiology, Wageningen University and Research (Wageningen, The Netherlands) under the supervision of Dr. Irene Sanchez Andrea

The motivation of this chapter focused on the optimization of acetate removal during the treatment of sulfate rich wastewaters. Previous chapters have shown that, under non-methanogenic conditions, high concentrations of acetate in the effluent of the UASB reactor are found, leading to a loss of carbon source. Therefore, efforts were directed towards the improvement of sulfidogenesis through the enrichment of acetate-degrading-sulfate-reducing bacteria in serum bottles. In addition, isolation of potential acetate-utilizing sulfate reducers was also persuaded. The experimental part of this chapter was performed at the Laboratory of Microbiology, Wageningen University and Research (Wageningen, The Netherlands).

Abstract

The accumulation of acetate appeared to be as one of the main points to deal with so as to increase the efficiency of the sulfate-reducing process using crude glycerol as carbon source. In this chapter enrichment experiments were performed to study if certain populations of interest could be enriched so as to take advantage of the excess of acetate accumulated in the system. Biomass was collected from the UASB reactor and incubated in serum bottles with sulfate and acetate as a first step to evaluate if sulfate reducers could use acetate to reduce sulfate. Afterwards, a batch of serial dilutions using the dilution-to-extinction technique in liquid medium was used to enrich the degrading cultures with the final purpose of isolating the cultures responsible for the reduction of sulfate with acetate. Lastly, microbial cultures and their evolution were characterized through Illumina platform. All the information collected along the different anaerobic batch test was used to evaluate the effectiveness of the enrichment experiment. After almost one year growing granules with acetate and sulfate in serum bottles, acetotrophic SRB were not identified or were only found with relative abundances below 6 %, presenting low sulfate reduction efficiencies with this carbon source. Therefore, the possibility of extending the experiments to a bigger scale, cultivating sulfate reducers in reactors was discarded. Further analyses are

required for a better understanding towards inoculation techniques that guarantee a successful retention and proliferation of newly added strains or proliferation of existing ones.

8.1 Introduction

Wastewater coming from different industrial activities such as food processing (starch, vegetal oil, etc.), pharmaceutical, mining, chemical and pulp and paper industries, among others, contain high concentrations of sulfate. Biological sulfate reduction performed through anaerobic processes (Visser et al., 1993; Omil et al., 1996; Fang et al., 1997; Percheron and Bernet, 1997), is an option to treat sulfate-laden wastewater. In this case, sulfate-reducing bacteria (SRB) usually outcompete methanogens for common substrates since methanogens only dominate in a low-sulfate environment. In this thesis the carbon source used to perform sulfate reduction was crude glycerol. The metabolic products generated from glycerol fermentation and acetogenesis, mainly acetate and hydrogen, may serve as substrates for methanogens and sulfate reducers; however, under high sulfate concentrations, hydrogen and acetate would be more readily used by hydrogenotrophic and acetate-utilizing sulfate reducers, respectively, because of more favorable substrate affinity (Ks) values of the SRB for these substrates (Stams et al., 2005).

A range of scenarios have been tested before (in Chapters 5 and 6), although limited information about the mechanisms and granular biomass characteristics and activity occurring inside the reactor was obtained. Also, operation of the reactor has been performed always under long-term steady-state conditions to properly assess the impact of tested conditions. However, large amounts of COD are wasted, mainly as acetate. The accumulation of acetate when operating a reactor for biological sulfate removal has already been reported by many authors (Omil et al., 1997; Oude Elferink et al., 1998b; Nagpal et al., 2000; Vallero et al., 2003; Kaksonen et al., 2004; Celis et al., 2009).

Acetate is probably the quantitatively most important physiological electron donor for dissimilatory sulfate reduction. Despite this fact, organisms that can

oxidize acetate with sulfate have been isolated not long ago (Widdel and Pfennig, 1977) because of the requirement of specific conditions for their enrichment and cultivation. Biostimulation of SRB is challenging as they can grow on a variety of carbon sources, probably being less effective than biostimulation of more physiologically constrained microorganisms. Complex communities and the effects of biostimulation on them add an extra level of difficulty as interactions are difficult to predict among the huge amount of microbial groups involved (Pereyra et al., 2012).

Improvement of acetate uptake by SRB is key for sulfidogenesis improvement, thus this chapter pursues the improvement of the use of the carbon source towards sulfate reduction instead of methane production. The latter is important since previous results (Chapters 5 and 6) have shown that CH_4 production is completely blocked at the low C/S ratios tested. In this sense, Chapter 8 proposes to study and promote the growth of acetate-utilizing sulfate reducers, not only to increase the capacity of the UASB, but also to test if different species of SRB can be enriched and maintained on a single energy source (crude glycerol) in a lab-scale sulfidogenic reactor.

8.2 Materials and methods

8.2.1 Inoculum source

Samples were collected from the reactor described in Chapter 6, on day 203 of the long-term operation. Initially, sludge was taken from UASB 1, UASB 2 and UASB 3. A picture of the UASB reactor with the sampling points at different heights is presented in Chapter 6 (Figure 6.1). Biomass was incubated in 250 mL serum bottles containing 150 mL of medium with sulfate and acetate as carbon source, as a first step to evaluate if sulfate reducers could use acetate to reduce sulfate. The composition of the mineral medium was (g L^{-1}): K_2HPO_4 (3),

NH_4Cl (0.2) and Na_2SO_4 (1.15) dissolved in tap water to add macro- and micronutrients and adjusted to pH=8.8-9.0 with NaOH (2 M). Medium was supplemented, per liter, with 300 µg H_3BO_3, 102.4 µg $MnCl_2{\cdot}4H_2O$, 1158.9 µg $FeSO_4{\cdot}7H_2O$, 190 µg CoCl2·6H2O, 23.8 µg $NiCl_2{\cdot}6H_2O$, 41.8 µg $ZnCl_2$, 2 µg $CuCl_2{\cdot}2H_2O$, 18 µg $Na_2MoO_4{\cdot}2H_2O$ and 3 mg EDTA.

To start with, 12 bottles (3 of them with sludge from UASB1, 3 with sludge from UASB 2 and 3 with sludge from UASB 3) with sulfate and 3 control bottles (one per reactor height) without sulfate were inoculated. Acetate concentration when the bottles were fed was between 400-500 mg acetate L^{-1}. Bottles were incubated in a shaker at 37 ºC and 150 rpm. They were sampled once per week for chemical analyses, and once sulfate was consumed, the supernatant was discarded, and bottles were refilled with new mineral medium. Bottles in which no sulfate activity was detected after one month were discarded. Sulfate and thiosulfate concentrations were determined using ion chromatography as described in Section 4.2.2. Concentration of acetate and other compounds measured along this chapter are described also in Section 4.2.2 of Chapter 4.

8.2.2 Enrichment experiments

Enrichments experiments were carried out during a research stay at the Laboratory of Microbiology, Wageningen University and Research (WUR, Wageningen, The Netherlands). The bottles with granular biomass, that had been incubated in UAB with sulfate and acetate as carbon source (Figure 8.1), were sent to WUR were enrichments series were maintained. For these experiments, sludge from UASB 2 was used.

Figure 8.1. Bottles with granular biomass used for the enrichment test. Two bottles were used as experimental ones (fed with sulfate) and the other one was the control bottle (fed without sulfate).

Enrichments with granular biomass were performed in 250 mL serum bottles containing 120 mL of medium and a 1.5 atm N_2 headspace, which were sealed with butyl rubber stoppers and aluminum caps. The composition of the basal medium was (per liter): KH_2PO_4, 0.408g; $Na_2HPO_4·2H_2O$, 0.534g; NH_4Cl, 0.3g; NaCl, 0.3g; $MgCl_2·6H_2O$ 0.1g; yeast extract 0.5 g and resazurin 0.5 mg. Medium was supplemented, per liter, with 61.8 µg H_3BO_3, 61.25 µg $MnCl_2$, 943.5 µg $FeCl_2$, 64.5 µg $CoCl_2$, 12.86 µg $NiCl_2$, 67.7 µg $ZnCl_2$, 13.35 µg $CuCl_2$, 17.3 µg Na_2SeO_3, 29.4 µg Na_2WO_4 and 20.5 µg Na_2MoO_4. For the test bottles (2 replicates), 2.2 g of Na_2SO_4 were added. Medium was prepared, boiled and subsequently cooled on ice under a continuous nitrogen flow. The final pH of the medium was 7.0-7.2. Thereafter, microcosms were amended with sterile anaerobic stock solutions that contained acetate, to achieve a final concentration of 10 mM (600 mg acetate L^{-1}). In the control bottle, granules were cultivated without sulfate to investigate the contribution of other microorganisms to acetate degradation. No more controls were used because of the lack of original granules.

Bottles were monitored periodically (every 7-10 days) to assess sulfide production and acetate consumption and, when the carbon source was depleted, the granules were transferred to new bottles with fresh mineral medium and a new cycle started. Bottles were cultivated in a shaker at 37 ºC and 150 rpm. These microcosms were incubated for almost 10 months (since they were collected from the reactor) during which carbon source and sulfate concentrations were periodically monitored. However, enrichment experiments in a more exhaustive way were additionally carried out during a 5-month research stay at Wageningen University using the dilution-to-extinction technique.

8.2.3 Serial dilution bottles

The dilution-to-extinction technique in liquid medium was used to enrich the degrading cultures. Serial dilutions were performed with the supernatant from the granular bottles and with smashed granules as inoculum, respectively. The basal medium contained the following composition (per liter): KH_2PO_4, 0.408g; $Na_2HPO_4 \cdot 2H_2O$, 0.534g; NH_4Cl, 0.3g; NaCl, 0.3g; $MgCl_2 \cdot 6H_2O$ 0.1g; Na_2SO_4, 2.2 g; yeast extract 0.5 g and resazurin 0.5 mg. Medium was supplemented, per liter, with 61.8 μg H_3BO_3, 61.25 μg $MnCl_2$, 943.5 μg $FeCl_2$, 64.5 μg $CoCl_2$, 12.86 μg $NiCl_2$, 67.7 μg $ZnCl_2$, 13.35 μg $CuCl_2$, 17.3 μg Na_2SeO_3, 29.4 μg Na_2WO_4 and 20.5 μg Na_2MoO_4. The medium was prepared, boiled and subsequently cooled on ice under a continuous nitrogen flow. Bottles of 120 mL were filled with 50 mL of medium and instantly capped with rubber stopper and aluminum cap. The gas phase was exchanged with N_2 resulting in a final pressure of 1.5-1.8 atm. The bottles were then autoclaved and stored at room temperature till further use. Before inoculation, medium was augmented with the following volumes of stock solutions: 1 % v/v of 11 g L^{-1} $CaCl_2 \cdot 2\,H_2O$, 1 % of a vitamin solution containing per liter: biotin 20 mg, nicotinamide 200 mg, p-aminobenzoic acid 100 mg, thiamine (vitamin B1) 200 mg, panthotenic acid 100 mg, pyridoxamine 500 mg,

cyanocobalamine (vitamin B12) 100 mg, riboflavine 100 mg. The medium was reduced by introducing a 5 % v/v of a stock solution containing 4.8 g L^{-1} $Na_2S{\cdot}9H_2O$ and 80 g L^{-1} $NaHCO_3$. The final pH of the medium was 7.0-7.2. For the cultivation, an inoculum volume of 5 % v/v was used. Abiotic controls were performed in the absence of inoculum. Sequential dilutions series were generated through serial 1 in 10 dilutions starting from an active culture and up to 10^{10} dilution (Figure 8.2). Dilutions were incubated at static conditions at 37 ºC and monitored for acetate and sulfate consumption. The bottle with the highest dilution in which sulfate reduction activity was detected, was selected as the inoculum for the next set of serial dilutions.

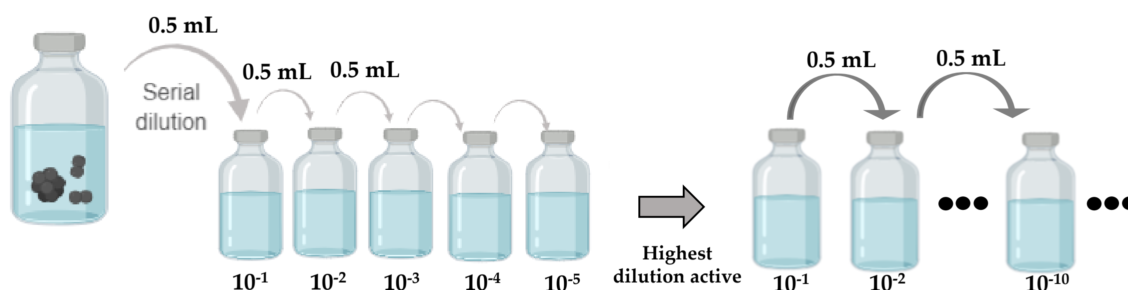


Figure 8.2. Dilution-to-extinction technique followed along the chapter.

8.2.4 Microbial diversity analysis

Identification of the microbial populations were performed using Illumina platform. Table 8.1 presents the different sampling events and names that will be used along the chapter to refer to the different samples. On 21/11/2018 granules were collected from the reactor and transferred to bottles being fed with acetate as the only external carbon source. After 3 months, some granules were collected from the bottles to extract DNA and to perform 16S rRNA sequencing (G1). Almost one year later, on 05/09/2019, some granules were collected (G2) to perform again 16S rRNA sequencing and observe if the populations had evolved over time.

Table 8.1. Biomass sampling days during the bottle's enrichment.

Sample	Sample name	Date of sampling	Analysis performed
Granules from the UASB	G	21/11/2018	Inoculum for enrichment bottles
Granule 1 + Supernatant 1	G1 + S1	3 months post-incubation	16S rRNA sequencing
Granule 2 + Supernatant 2	G2 + S2	10 months post-incubation	16S rRNA sequencing

DNA was extracted from both the granules and the supernatant of the bottles. To obtain DNA for community analysis from the supernatant, samples of 5 mL were centrifuged at 13,400 *g* for 10 min, supernatant was discarded, and the pellet was resuspended in 250 μL of sterile Milli-Q water. Granules from the experimental bottles were mixed and gently crushed. DNA was extracted using the PowerSoil™ DNA isolation kit (MoBio Laboratories, USA), as recommended by the manufacturer. The quantity and quality of the extracted DNA were assessed by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and then DNA samples were preserved at -20 ºC for further analysis. Amplicon sequencing 16S rRNA genes of all samples was performed by "Genomic and Bioinformatics service" on Illumina MiSeq platform at the Universitat Autònoma de Barcelona amplifying the V3-V4 hyper variable region with the universal primers by Illumina (5′-CCTACGGGNGGCWGCAG-3′) and (5′-GACTACHVGGGTATCTAATCC-3′) selected from Klindworth et al. (2013). The coverage of the primers was checked using the test prime tool in Silva's website (www.arb-silva.de), setting the parameters to 1 mismatch for the maximum number of mismatches and 5 bases for the length of 0-mismatch zone at 3′end. As a result, the primer pair presented 66.6 % of coverage for Archaea and 92.4 % for Bacteria. The reference database used for the taxonomic classification of organisms was Greengenes (DeSantis et al., 2006).

8.3 Results

8.3.1 Inoculum source from the long-term UASB operation

The purpose of the experiment in bottles with granular sludge acclimated to acetate was to characterize the SRB populations selectively stimulated when granules were incubated with acetate as the available carbon source. On day 203 of the UASB operation described in Chapter 6, biomass was collected and incubated in serum bottles with sulfate and acetate as a first step to evaluate if sulfate reducers from the UASB could use acetate to reduce sulfate. Sulfate concentration in these bottles was the same one as in the mineral medium used to feed the reactor during Chapter 6 (200-250 mg S-SO_4^{2-} L^{-1}), thus simulating the same conditions. Acetate concentration when the bottles were fed was between 400-500 mg acetate L^{-1}. The performance during the long-term operation of the UASB has been already presented in Figure 6.4 and discussed along Chapter 6. The biomass sampling day (day 203) corresponds to the day when acetate concentration was 584.3 mg L^{-1}, the highest found along the whole operation. As shown in Figure 6.4B, there was no methane production when biomass was collected, and methanogens washout was already confirmed in Chapter 6. S-RE by that day (day 203 of the long-term operation) was 81.3 % and TOC-RE was 30.6 %. Results from Chapters 5 and 6 demonstrated that inlet sulfate concentrations around 250-400 mg S L^{-1} (SLR of 3-5 kg S m^{-3} d^{-1}) at glycerol/S ratios around 5 g O_2 g^{-1} S discontinue methane production and produce a washout of methanogens from the bioreactor. Concomitantly, acetate is accumulated. Even if the non-acetate degrader *Desulfovibrio* was found to be the most abundant SRB genus detected in Chapter 6, a tentative was made to enrich and isolate the sludge in populations able to use acetate and degrade sulfate. Afterwards, if possible, study if these microbial populations would overcompete or coexist with *Desulfovibrio* in the UASB reactor having only acetate as the available carbon source.

8.3.2 Assessment of sulfate reduction rates and microbial diversity of enriched cultures

The strategy proposed to accelerate biological sulfate reduction, and to fully degrade the inlet organic matter, was the stimulation of existing acetate-degrading sulfate reducers (if present) by the addition of acetate as the only available carbon source. A set of bottle tests (2 experimental bottles and one control bottle) were prepared to monitor and assess the biodegradation of sulfate and acetate (Figure 8.1). Granular biomass collected from the UASB reactor described in previous chapters was used for this experiment, as mentioned in Section 8.2.2. Figure 8.3 shows the experimental profile obtained from the monitoring of the 2 bottles (replicates) calculated as an average together with the control bottle. Many cycles were performed but only Figure 8.3 is presented as an example of one of them. A cycle consisted of the period that comprises the time since new mineral medium is added to the bottles until acetate and/or sulfate are consumed and mineral medium is renewed again, discarding the supernatant of the bottles. Table 8.2 presents the starting and ending date of each of the last three cycles performed on the granular bottles. These last three cycles performed along the long-term enrichment of the granules are presented, as they were considered the most representative ones.

Table 8.2. Starting and ending date of the last three cycles performed in the granular bottles along the enrichment experiments.

Cycle	Start date	End date
1	12-June-2019	09-July-2019
2	10-July-2019	01-August-2019
3	02-August-2019	23-August-2019

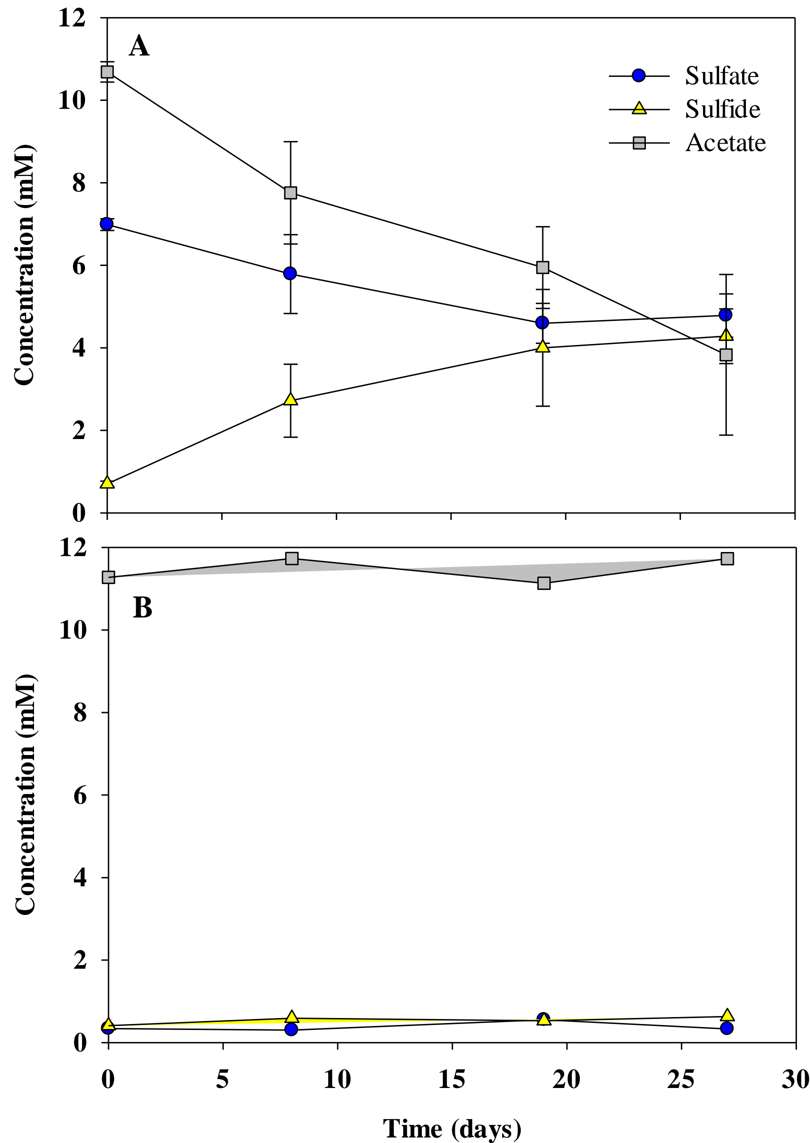


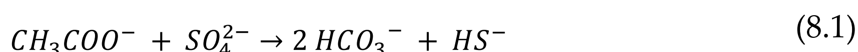
Figure 8.3. A) Granular bottles cultivated with acetate as carbon source. Cycle 1 is presented. B) Control granular bottle fed without sulfate.

Table 8.3 provides the sulfide production rates, acetate consumption rates, ratios and imbalance calculated as an average of the experimental bottles along the complete duration of a cycle. The sulfur imbalance was calculated as the difference between the sulfide produced and the sulfate consumed. It also provides the maximum sulfide production rates and acetate consumption rates, calculated as an average of the experimental bottles considering the first period of the cycle in which rates were the highest.

Table 8.3. Sulfate reduction rates or sulfide production rates, acetate consumption rates, ratios and imbalance calculated as an average of the experimental bottles considering the first period of the cycle in which rates were the highest.

Cycle	Average sulfide production rate (mM S^{2-} d^{-1})	Maximum sulfide production rate (mM S^{2-} d^{-1})	S imbalance (%)	Average acetate consumption rate (mM acetate d^{-1})	Maximum acetate consumption rate (mM acetate d^{-1})	Ratio Acetate/Sulfate
1	0.13±0.03	0.25±0.10	38.4	0.25±0.06	0.37±0.12	1.92
2	0.12±0.02	0.19±0.04	5.1	0.44±0.10	0.62±0.44	3.66
3	0.17±0.01	0.14±0.01	32.2	0.47±0.03	0.53±0.22	2.76

As can be observed from Table 8.3, sulfide production was taking place in the experimental bottles. Moreno-Perlin et al. (2019) reported sulfide production rates of 0.596 mmol d^{-1}, 0.224 mmol d^{-1} and 0.077 mmol d^{-1} at pH 5, 4 and 3 respectively with acetate as electron donor and using sediment historically impacted by acid mine drainage. Removal of acetate was not observed in the control bottle without sulfate (Figure 8.3B), indicating that, there were no other microorganisms using acetate or that they were not able of using it without the presence of sulfate. The theoretical stoichiometric ratio for acetate utilization is presented in Equation 8.1:



$$CH_3COO^- + SO_4^{2-} \rightarrow 2\,HCO_3{}^- + HS^- \tag{8.1}$$

According to this equation, there was more acetate consumed than the one needed. As can be observed from Table 8.3, the ratios acetate/sulfate were always higher than 1, which means that acetate was being used not only for sulfate reduction but for other processes, such as maintenance and growth. Bacterial growth involves two basic reactions, one for energy production and the other for cellular synthesis. Cell maintenance has energy requirements for activities such as cell movement and repair of cellular proteins that decay because of normal resource recycling or through interactions with toxic compounds (Rittmann and Mccarty, 2001). The acetate consumption efficiency was 52.2 % considering the sulfide produced divided by the acetate consumed. In anaerobic ecosystems, non-fermentable carbon compounds such as acetate can be converted into methane and/or carbon dioxide by carbon mineralizers (Montoya et al., 2012), what could also explain the higher consumption of acetate not related with sulfate reduction.

During cycle 1 the average sulfide production rate was 0.13 mM d^{-1}, during cycle 2 it was 0.12 mM d^{-1} and during cycle 3 it was 0.17 mM d^{-1}. At the end of the enrichment process, the sulfide production rate was similar to the one obtained

during previous cycles. This result would indicate that the highest production of sulfide had been reached and that probably our culture was not able to reduce sulfate faster. CO_2 and H_2S were the only products detected in the cultures, indicating that the organic compounds were fully oxidized. Methane was measured when carbon imbalances were found, but it was never detected in the headspace of any bottle.

The results obtained from the 16S rRNA sequencing of the samples in Table 8.1 are presented in Figures 8.4 and 8.5. Figure 8.4 shows the difference between G1 and G2 whereas Figure 8.5 compares S1 and S2.

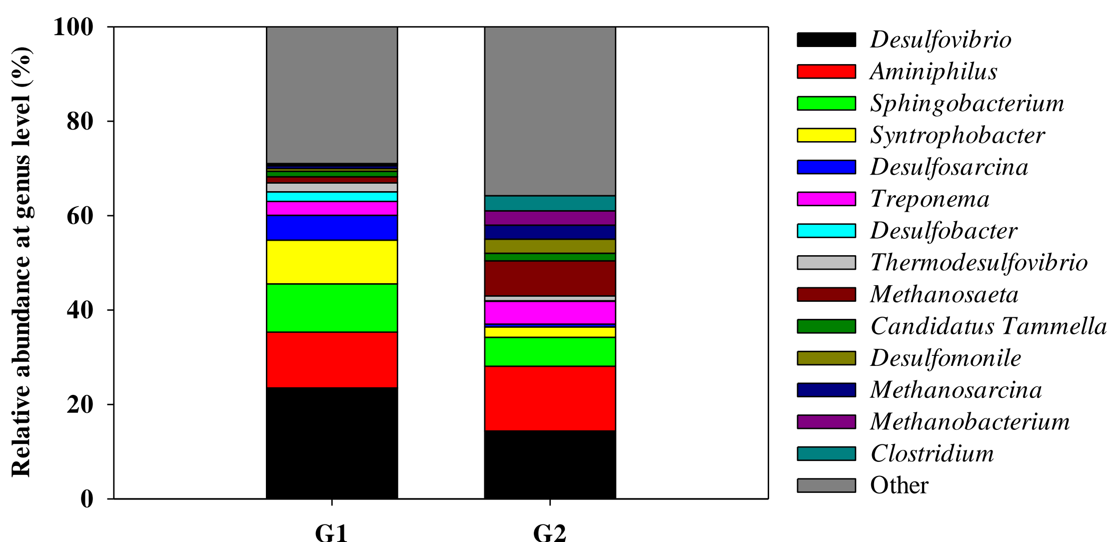


Figure 8.4. Microbial diversity of the most representative genus found for Granule 1 (G1) and Granule 2 (G2).

The three most representative phyla in both samples (G1 and G2) presented in Figure 8.4 were *Proteobacteria*, *Synergistetes* and *Bacteroidetes*. At genus level, differences in the relative abundances can be observed. *Desulfovibrio* decreased its relative abundance from 23.5 % until 12.4 %; *Sphingobacterium* decreased from 10.2 % until 6 %; *Sphingobaterium* decreased from 10.2 % to 6.1 %; *Syntrophobacter* decreased from 9.2 % to 2.2 % and *Desulfosarcina* decreased from 5.3 % to 0.6 %. In general, a decrease in all the major genera presented in G1 is detected in G2,

except from *Methanosaeta*, which significantly increased its relative abundance from 1.3 % to 7.4 %.This finding was surprising as methane could not be detected in the headspace of any bottle, when measured, as previously mentioned.

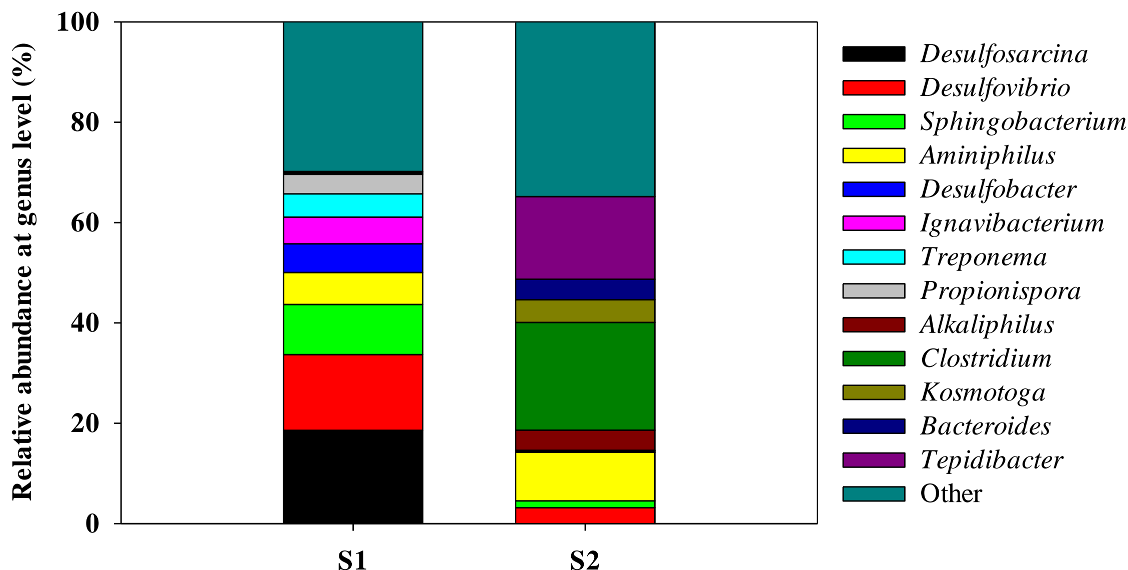


Figure 8.5. Microbial diversity of the most representative genus found for Supernatant 1 (S1) and Supernatant 2 (S2).

In Figure 8.5 the results of the 16S rRNA analysis of the supernatant are presented. In this case a similar tendency to the one in Figure 8.4 is observed. There is a general decrease in all the genera that were more abundant in S1 compared to S2. New genera that were not detected in sample S1 appeared in S2. *Desulfosarcina* decreased its relative abundance from 18.6 % until 0 %; *Desulfovibrio* decreased from 15.1 % until 3.1 %; *Sphingobacterium* decreased from 10 % to 1.4 % and *Desulfobacter* decreased from 5.7 % to 0 %. Genera that increased greatly were *Clostridium* and *Tepidibacter* which relatives' abundances changed from 0 % in both cases in S1 to 21.5 % and 16.5 % respectively in S2.

As a general view, there is a tendency in the main genera detected to decrease from sample 1 to sample 2, both in the granule as in the supernatant. *Desulfovibrio*, *Sphingobacterium* and *Desulfobacter* suffered this huge decrease considering their

relative abundances, meaning that the conditions in which the enrichment experiments were performed did not benefit these populations. Handley et al. (2013) reported that when acetate was added to his system and after an acclimation period, a niche ecosystem for *Desulfobacter* was created and he demonstrated that this genus consumed sulfate coupled to acetate oxidation. This genus has been described as able to reduce sulfate using acetate as their characteristic substrate (Widdel and Pfennig, 1981; Widdel, 1987). In our samples, the relative abundance of this genus was quite low or even undetectable. In G1, *Desulfobacter* represented a 2 %; in G2 it was a 0.1 %, whereas in S1 and S2, it was a 5.7 % and a 0 % respectively. Results suggest that the addition of acetate instead of crude glycerol, which was the carbon source used during the performance of the reactor, led to distinct structures of bacterial community, but not always favoring populations of interest such as the abovementioned *Desulfobacter*. Table 8.4 shows the genera of sulfate-reducing bacteria and archaea that are able to perform a complete or incomplete oxidation of organic electron donors. It also provides with a column to show which genera can use acetate as electron donor. According to this table, *Desulfosarcina* and *Desulfobacter* are the only genera able of using acetate as electron donor that were detected in our samples, both in the granules and in the supernatant. As already mentioned, *Desulfosarcina* decreased its relative abundance from 5.3 % in G1 until 0.6 % in G2 and from 18.6 % in S1 until 0 % in S2; *Desulfobacter* also decreased its relative abundance from 2 % in G1 until 0.1 % in G2 and from 5.7 % in S1 until 0 % in S2. Therefore, acetotrophic SRB populations were not increased according to 16S rRNA results in the experimental bottles.

Table 8.4. Physiological properties of the genera of sulfate-reducing bacteria and archaea (Modified from (Rabus et al., 2013).

Genus	Optimum temperature (°C)	Oxidation of organic electron donors[a]	Acetate as electron donor[b]
Desulfovibrio	30-38	I	-
Desulfomicrobium	28-37	I	-
Desulfobulbus	28-39	I	-
Desulfobacter	28-32	CAC	+
Desulfobacterium	20-35	CO	(+)
Desulfococcus	28-36	CO	(+)
Desulfosarcina	33	CO	(+)
Desulfomonile	37	C	-[c]
Desulfonema	30-32	C	(+)
Desulfobotulus	34	I	-
Desulfoarculus	35-39	CO	(+)
Desulfotomaculum	30-65	I or CO	±
Desulfosporosinus	30-37	I	-
Thermodesulfovibrio	65	I	-
Thermodesulfobacterium	65-70	I	-
Thermodesulforhabdus	60	C	+
Desulfacinum	60	ND	+
Desulforholapus	18-19	I	-
Desulforhabdus	37	C	+
Desulfonatronovibrio	37	ND	-
Desulfonatronum	37-40	I	-
Desulfohalobium	37-40	I	-

Desulfofustis	28	I	+
Desulfocella	34	I	-
Desulfocapsa	20-30	I	-
Desulfobacca	37	C	+
Desulfuromusa	30	C	+
Desulfospira	26-30	C	-
Desulfobacula	28	C	+
Desulfofrigus	10	C	+
Desulfofaba	7	I	-
Desulfotalea	10	I	-

[a]Symbols: C, complete to CO_2 via unknown pathway; CAC, complete oxidation via citric-acid cycle; CO, complete oxidation via carbon monoxide dehydrogenase/C_1 pathway; I, incomplete oxidation to acetate as an end product; ND, not determined or not reported

[b]Symbols: +, utilizes; (+), poorly utilized; ± utilized or no utilized; -, no utilized

[c]May be utilized with thiosulfate as electron acceptor

Another important finding if comparing the evolution in the granules (G1, G2) with the one in the supernatant (S1, S2), was that a more stable structure can be observed in the granules, with a more constant microenvironment, whereas in the supernatant microbial populations suffered more changes. Even if changes were detected, the stability of granules could be demonstrated to a certain degree. Therefore, the activity and stability in the granules could be preserved along time, even if populations of interest were not detected in higher proportions according to the relative abundances found.

Sample G1 was collected from the bottles incubated in acetate. However, if those granules had been growing in the reactor, by the day when the 16S rRNA sequencing of G1 was performed, those granules would be in the day 292 of the long-term operation. Comparing those granules and the ones that were kept in the reactor was possible, as on day 294 of the operation 16S rRNA sequencing was also performed to the granules in the UASB reactor (Section 6.3.2). Figure 8.6 shows this comparison, between granules that are “the same age” but had been growing in a different way and with different carbon sources. *Desulfovibrio* decreased its relative abundance in G1 compared to day 294, from 30.1 % down to 23.5 %; *Dysgonomonas* decreased as well from 13.6 % to 0.5 %. On the contrary, there are many genera that were almost undetectable in the granules inside the reactor but increased their relative abundances when incubated in the bottles with acetate. *Aminiphilus*, *Syntrophobacter* and *Sphingobacterium* increased remarkably reaching a 11.8 %, 9.2 % and 10.2 %, respectively.

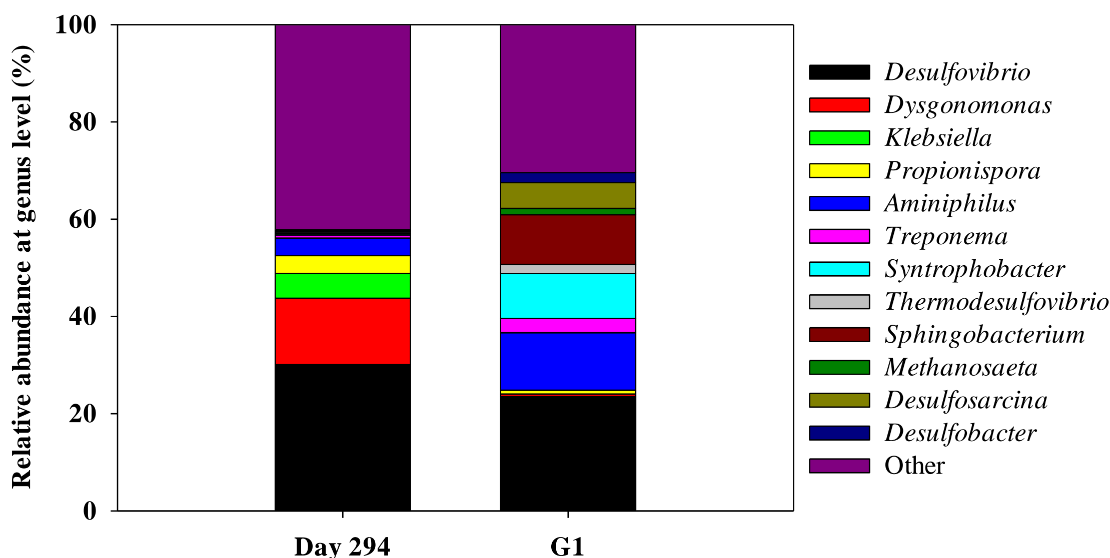


Figure 8.6. Microbial diversity of the most representative genera found for Granule 1 (G1) and for the sample collected on day 294 during the long-term operation described in Chapter 6.

Desulfosarcina and *Desulfobacter* are the SRB that increased their relative abundances when incubated in the bottles to a 5.2 % and a 2 %, which is an important result as it was previously mentioned that these genera are able to use acetate and performed a complete oxidation of the electron donor.

Figure 8.7 is the equivalent to Figure 8.6 but in this case G2 is compared to the granules on day 538 of the operation described in Chapter 6. Genera that suffer a remarkable decrease in their relative abundance while being incubated in the bottles were *Propionispora*, which decreased from 15.2 % on sample from day 538 to 0.7 % in G2; *Dysgonomonas* decreased from 13.2 % to 0.7 % and *Desulfobulbus* decreased from 11.6 % to 0.4 %. On the other hand, there were other genera that were apparently favored by the conditions set in the enrichment bottles: *Aminiphilus* increased from 5 % to 13.7 % in G2 compared to the sample obtained on day 538; *Methanosaeta* increased from 0.7 % to 7.4 % and *Sphingobacterium* increased from 0 % to 6.1 %. *Desulfovibrio* maintained its relative abundance (14.4 % in G2 and 10.8 % on sample from day 538).

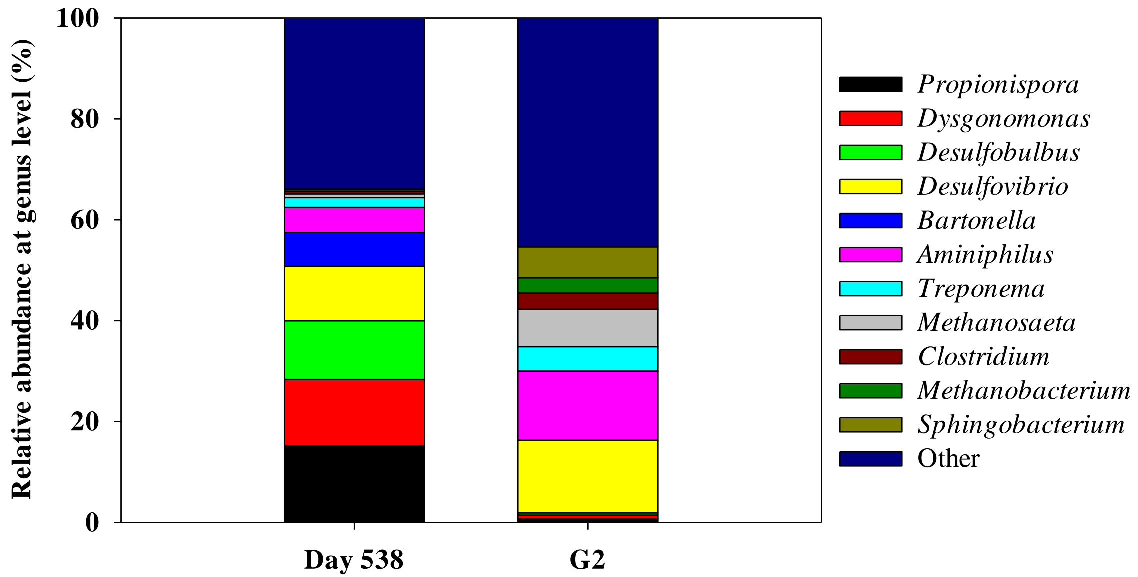


Figure 8.7. Microbial diversity of the most representative genera found for Granule 2 (G2) and for the sample collected on day 538 during the long-term operation described in Chapter 6.

Figure 8.8 was created to have a general view on how the incubation of the granules, in different conditions as the one set in the reactor, affected the diversity of the microbial populations in the long-term.

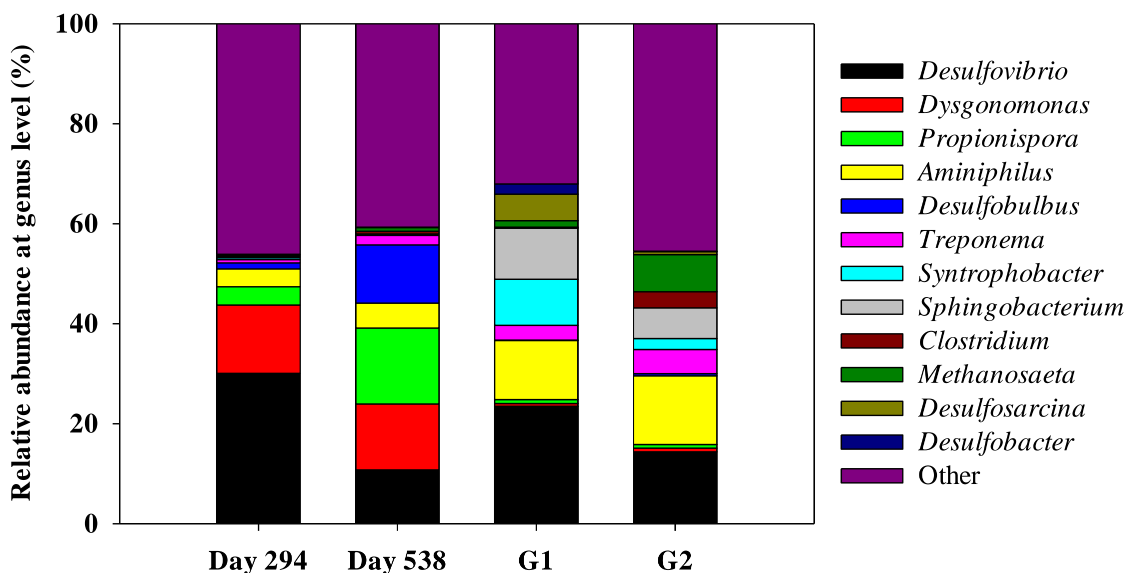


Figure 8.8. Microbial diversity of the most representative genera found for Granule 1 (G1) and 2 (G2) and for the samples collected on day 294 and 538 during the long-term operation described in Chapter 6.

Desulfovibrio, the most abundant SRB found in our reactor, decreased its relative abundance over the time, both in the reactor and in the bottles. The relative abundance progressed from 30 % to a 10.8 % and from 23. 5 % to 14.4 %, respectively, although it seemed that the cultivation in bottles with acetate did not really affect its population, in terms of relative abundance. *Dysgonomonas* maintained its relative abundance along the last period of the operation in 13 %. However, its abundance in the granules of the bottles was around 0.6 % both in G1 and G2, indicating that this population was not able to grow only with acetate as carbon source. *Aminiphilus*, on the contrary, increased its relative abundance in the bottles compared to the granules (4 % to 12 % on average if considering day 294 and 538 and G1 and G2 respectively). Despite our results, Díaz et al. (2007) found out that carbohydrates, formate, acetate, propionate, butyrate, isovalerate and a number of other amino acids were not utilized by *Aminiphilus circumscriptus*. *Propionispora* increased its abundance on day 538 compared to day 294 but its relative abundance in the bottles was constant and almost undetectable. The same tendency was detected for *Desulfobulbus*; its relative abundance increased from 1.2 % on day 294 to 11.6 % on day 538, whereas in the bottles its presence was almost undetectable with 16S rRNA sequencing. This genus can use propionate, lactate, pyruvate, ethanol or propanol as carbon sources, also as electron donors for anaerobic respiration and oxidizes them incompletely to acetate, what can explain why they were not present in the bottles were acetate was the only available carbon source. *Syntrophobacter* and *Sphingobacterium* increased their relative abundances in the bottles compared to the reactor performance but in both cases their abundances decreased when comparing G1 to G2. *Syntrophobacter* decreased from 9.2 % to 2.2 % and *Sphingobacterium* decreased from 10.2 % to 6.1 %.

The case of *Methanosaeta* genus is interesting as its relative abundance was almost undetectable in samples coming from days 294 and 538 as it has been

previously discussed, but it increased on G2 reaching a 7.4 % of relative abundance. Of the many methanogenic genera, only *Methanosaeta* and *Methanosarcina* are known to grow by an acetoclastic reaction, producing methane from acetate (Patel and Sprott, 1990; Barber et al., 2011). The increase in *Methanosaeta* then, could be explained by the fact that they metabolize only acetate and could have been favored by the conditions set in the experimental bottles, where acetate was the only available carbon source.

Desulfosarcina and *Desulfobacter* can both use acetate (Kuever et al., 2015). In the reactor the abundance of these genera was 0 %, but in sample G1 there was a 5.3 % of *Desulfosarcina* detected and a 2 % of sequences that belonged to *Desulfobacter*. It seemed that SRB able of using acetate were growing on the bottles, however, on G2 their relative abundances decreased again to 0 %. SRB are typically a small fraction of all bacteria detected in reactors working with mixed communities, which makes their detection with 16S rRNA gene-targeted fingerprinting methods difficult (Hong et al., 2007). Because of the complexity of these systems, the dominance of uncultured microorganisms and the lack of reliable correlations between phylogeny and function, conclusions about the functional properties of the members of the microbial community is hardly ever possible. However, having a look to the evolution of the populations could be a first approach to obtain information about the main microbial populations that are present and could be playing an important role on the consumption of acetate.

8.3.3 Serial dilutions experiments

Serial dilutions from both granules and supernatant of the experimental bottles were performed to try to isolate the microorganism able of performing sulfate reduction with acetate or at least separate strains from the mixed

population. The first set of serial dilutions performed was using the supernatant of the granular bottles as inoculum. The objective was to assess the relative contribution of planktonic and granular biomass over sulfate reducing activity. This first set lasted 62 days and no sulfate reducing activity was detected in any of the bottles. The serial dilution series was repeated but this time the new set of bottles were maintained for 52 days, again with no sulfate reducing activity detected. After that, a last set of serial dilutions was performed for 14 days. After that time, the concentration of sulfide and acetate was measured, obtaining a sulfate reduction rate of 0.08 mM d^{-1} and an acetate consumption rate of 0.05 mM d^{-1} in the bottle 10^{-4}. This bottle was selected to carry out 16S rRNA sequencing as explained in Section 8.2.5. Figure 8.9 shows the results of this analysis compared to sample S2. There are no significant differences between samples. *Clostridium* is the only genus in which a significant increase was detected accounting for a total of 34.6 % of the total retrieved sequences in the serial dilution bottle compared to S2, where the percentage was a 21.5 %. The species of *Clostridium* comprise a very heterogeneous assemblage of bacteria that do not form a phylogenetically coherent group (Gupta and Gao, 2009). Figure 8.9 demonstrates that sulfate reduction activity was being developed in the granules and not by the biomass suspended in the supernatant. This result also supports the fact that, whenever a cycle was completed in the enrichment experiments (Section 8.2.2), the supernatant was discarded, and new mineral medium was added to the granules. This methodology, therefore, was not affecting the microbial populations of interest.

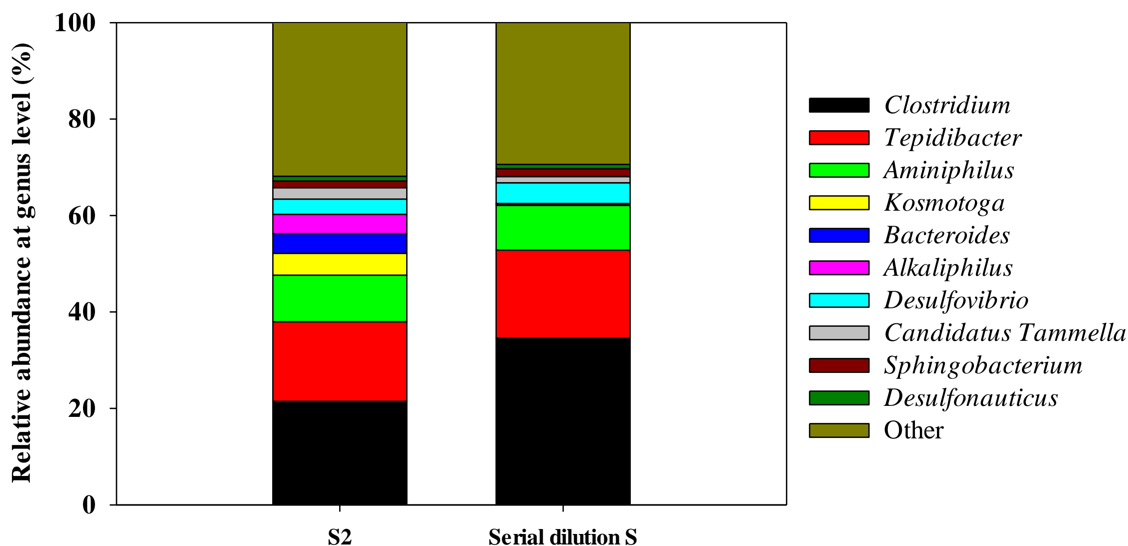


Figure 8.9. Microbial diversity of the most representative genera found for S2 compared to the 10^4 bottle of the serial dilution from the supernatant.

In the case of the smashed granules, the bottle 10^4 of the first set of serial dilutions, in which the highest sulfate reduction activity was detected was transferred to new media (prepared as described in section 8.2.3). After 15 days, the concentration of sulfide and acetate was measured obtaining a sulfate reduction rate of 0.13 mM d^{-1} and an acetate consumption rate of 0.14 mM d^{-1}. This bottle was the one selected for 16S rRNA sequencing, which results are presented in Figure 8.10 compared to G2. *Clostridium* genus increased extraordinarily in the serial dilution sample compared to G2, achieving a 40.4 % whereas in G2 this genus represented only a 2.9 % of the total amount of sequences detected.

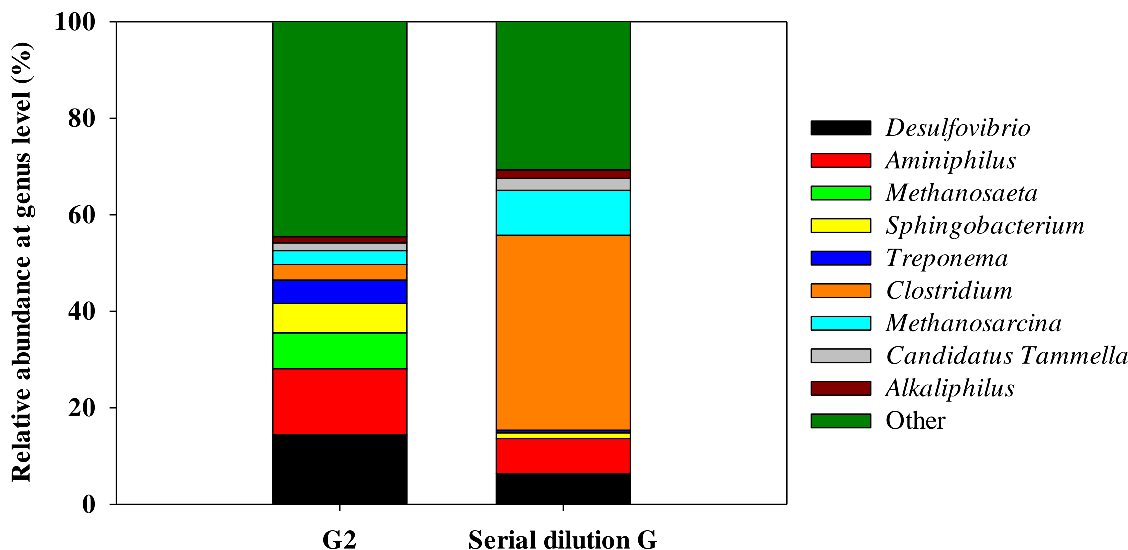


Figure 8.10. Microbial diversity of the most representative genera found for G2 compared to the 10^4 bottle of the first set of serial dilution from smashed granules after the transfer to new media.

Desulfovibrio accounted for a 14.4 % on sample G2, whereas after the serial dilution its relative abundance was 6.4 %. As it has been previously discussed, this genus cannot use acetate as carbon source (Dar et al., 2007a). Therefore, this result was expected as the only available carbon source along the experiments with serial dilution bottles was acetate. *Aminiphilus* suffered a similar decrease from 13.7 % to 7.2 %. *Methanosaeta* accounted for a 7.4 % in G2 but was not detected after the dilution series. On the contrary, *Methanosarcina* increased from 2.9 % in G2 until 9.3 % after the dilution series. This could be due to the fact that *Methanosarcina* has been described as the most versatile of all the mesophilic methanogenic bacteria isolated in pure culture, since it can form methane from H_2 and CO_2 (hydrogenotroph), from methanol and methylamines (methylotroph), and from acetate (acetoclastic) (Rocheleau et al., 1999). *Sphingobacterium* is other genus that almost disappeared after the serial dilution, whereas in G2 it accounted for a 6.1 % of the total retrieved sequences.

8.4 Discussion

The attempt to grow acetotrophic sulfate reducers and incorporate them in the UASB reactor to increase the efficiency of the process, and specially the TOC-RE, was unsuccessful with the tested strategies. Enrichment would be carried out through standard methodologies and characterized for biological activity through anaerobic batch tests. In a second phase, after the bottle experiments, granular sludge from the UASB reactor was expected to be enriched in a batch, magnetically stirred anaerobic reactor of 1L fed with acetate and sulfate. After that, sludge from the enrichment reactor would be added to the UASB reactor to increase the sulfidogenic capacity. However, not even the first step of growing biomass in serum bottles gave promising results. According to our results, enrichment experiments in bottles were not successful. Other researchers have also tried to incorporate different bacteria into reactors and all have highlighted the many obstacles that have to be solved when introducing a new strain in UASB reactors (Omil et al., 1997; O'Flaherty et al., 1999b; Nagpal et al., 2000). As our results showed (Section 8.3.2), sulfate reducing activity was being performed in the granules, even if rates were low. However, entrapment or immobilization of acetotrophic SRB on the surface of mature granules might be a huge challenge. Vallero et al. (2004) studied the bioaugmentation of a bioreactor sludge with the acetate-oxidizing SRB *Desulfobacter halotolerans.* He found out that although some of the *Desulfobacter halotolerans* could possibly be sorbed onto the granules, they did not develop into a population that affected the reactor removal efficiency.

Biostimulation and bioaugmentation are remediation strategies that have been successful in some cases, particularly for dechlorinating microorganisms (Hörber et al., 1998; Tartakovsky et al., 1999; Lanthier et al., 2002). Notwithstanding, many other examples where these strategies failed can be found in the literature. In Wright and Weaver (2004), where biodegradation of crude oil was studied and bioaugmentation was applied, population sizes of heterotrophs and

hydrocarbon-degrading microorganisms were not enhanced or if enhanced, this did not result in an increase of total oil or total petroleum hydrocarbon (TPH) degradation. In Winchell and Novak (2008) two promising polychlorinated biphenyls (PCBs) remediation technologies were investigated, biostimulation and bioaugmentation. In this study, biostimulation experiments resulted in only a slight improvement in the dechlorination. However, they observed a considerable dechlorination after bioaugmenting microcosms with a PCB-dechlorinating enrichment culture. Therefore, this research demonstrate that more work is needed on the physiology of PCB dechlorinators, their synergistic relationships with other organisms, and their long-term activity before successful biostimulation is expected. Bioaugmentation, on the other hand, appears to be an advantageous PCB remediation strategy to further explore.

Results found out along Chapter 8 indicate that SRB are a small fraction of all the other communities, which makes their detection with 16S rRNA gene-targeted fingerprinting methods difficult. Hong et al. (2007) selected a sulfate-reducing mine drainage treatment community for his study because of its known complexity, and the requirement of multiple microbial functional groups to succeed in sulfate reduction with the precipitation of heavy metals at the same time. In that study, capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) was compared versus DGGE for profiling the microbial community. In this chapter, only 16S rRNA sequencing was used as a first attempt to infer if microbial populations were evolving in the desired direction. Taking into consideration the limitation of this techniques, further analyses are required for a better understanding towards inoculation techniques that guarantee a successful retention and proliferation of newly added strains or proliferation of existing ones into anaerobic granules or biofilms. Direct analysis of functional genes as performed in Pereyra et al. (2012) would have been a good

option to understand the microbial community dynamics during start-up and operation of a complex biological system. On that way, much more information and resolution would have been gathered than what was obtained in this study targeting only 16S rRNA genes. Pereyra et al. (2012) observed higher level of similarity when comparing the 16S rRNA gene with the functional genes selected in the study. Therefore, analysis of functional genes captured differences not revealed by 16S rRNA gene patterns.

Vallero et al. (2005) reported that sulfate reduction in a submerged anaerobic membrane bioreactor was carried out using acetate and ethanol as electron donor. The reactor was inoculated with a pure culture of *Desulfobacter*. Interestingly, in that paper, researchers also revealed that when a complete COD removal is required in their reactor, the rate of acetate degradation will define the design of the sulfate reducing reactor as the acetate oxidation is the limiting step. The importance of the acetate degradation rate on the reactor´s performance has been reported previously by other authors for methanol-fed thermophilic (Weijma et al., 2000b; Vallero et al., 2003; Vallero et al., 2005) and VFA-fed mesophilic reactors (Omil et al., 1998). During this thesis, the sulfate reduction step was studied using crude glycerol as electron donor. However, as there were not many references reporting the long-term operation of UASB reactors under the same conditions, the accumulation of acetate could not be predicted. Therefore, this scenario was not considered when designing the reactor. Even so, the accumulation of acetate has been observed in many types of reactors, becoming an inherent problem of sulfidogenic reactors whenever the presence of methanogens or other acetate-consumers is imperceptible (Gallegos-Garcia et al., 2009). Nagpal et al. (2000) proposed the inability of acetate-utilizing sulfate reducers to compete with other sulfate reducers for sulfate. That would favor these populations of SRB, that would lead to an accumulation of substrates such as acetate, that come from an incomplete oxidation. Nevertheless, studies

concerning the competition between acetate-utilizing sulfate reducers and SRB that oxidize the substrate incompletely have to be considered more profoundly.

Desulfobacca acetoxidans, first isolated from a sulfidogenic bioreactor (Oude Elferink et al., 1999), is a Gram-negative SRB that can utilize acetate as the only source of organic carbon and electron donor. Kaksonen et al. (2004) also found *Desulfobacca acetoxidans*-like SRB in their lab-scale fluidized-bed reactors that were fed with a single electron donor, i.e. lactate or ethanol. Two abundant acetate-degrading sulfate reducers, *Desulforhabdus amnigena* and *Desulfobacca acetoxidans*, were isolated from sulfate-reducing bioreactors (Oude Elferink et al., 1995). In this chapter, acetotrophic SRB were not identified or were only found at genus level and with relative abundances below 6 % in the granules. Genus *Desulfovibrio* was almost washout from the bottles where we observed sulfate reduction with no other SRB population identified. One possible explanation would be that, in an inoculum coming from an anaerobic digester, the few initial acetate-degrading sulfate reducers had to compete with huge number of acetoclastic *Methanosaeta* species. These acetotrophic SRB could have been washed out from the system during the start-up period of the long-term operation. SRB have particular growth limitations consequent upon energetic considerations. According to Rittmann and Mccarty, (2001) the maximum specific growth rate of SRB is low compared to aerobic heterotrophs, nitrifying autotrophs, fermenters, sulfide oxidizing autotrophs or even methanogens. The values that reflect the balancing of energy costs for synthesis with the energy that is gained from the donor-to-acceptor energy reaction also shows that in the case of SRB, 95 % of the energy obtained from the carbon source goes to maintenance processes and only 5 % is bound for growth. On the other hand, it is a feature common to all anaerobic organisms that in comparison with their aerobic cousins they have restricted capacity for energy generation from any given substrate (Hamilton, 1998). As an example, Rabus et al. (2013) calculated the energy gain

from dissimilatory sulfate reduction in comparison to aerobic respiration. The results showed that the free energy change (ΔG^o) of the complete oxidation of acetate or lactate with sulfate as electron acceptor is -48 or -128 kJ, respectively, whereas acetate or lactate oxidation with O_2 provides -844 or -1,323 kJ, respectively calculated per mol of the organic substrate.

The identification of functional groups is important because information about the capabilities of the organisms present may be of more interest than phylogenetic identity. The incomplete oxidation of organic substrates by SRB is due to the lack of a mechanism for the terminal oxidation of acetyl-CoA (Rabus et al., 2013). According to this fundamental catabolic difference, two physiological groups have been distinguished and are considered along this chapter, the incomplete and complete oxidizers. However, these are purely physiological or functional groups that overlap only partly with molecular systematic groups. For that reason, the knowledge acquired in this chapter is not enough and further studies are required. In particular, a colleague from GENOCOV research group is already working in that direction to produce kinetic models that describe the degradation mechanisms of both acetotrophic SRB and non-acetotrophic SRB and their interaction with methanogens. A different approach would be necessary to optimize the excess of acetate in a subsequent step for other processes. Further research is needed to enhance the operation in terms of COD consumption in the long-term.

8.5 Conclusions

Crude glycerol has been shown as a proper electron donor for a sulfidogenic UASB during this thesis. A range of scenarios have been tested showing that a start-up using granular sludge from a methane-producing anaerobic digester was feasible. A transition to non-methane producing conditions was reached at

selected sulfate loading rates maintaining the sulfate-reducing efficiency of the UASB. Under such conditions, large amounts of acetate accumulated in the reactor. Along this chapter, SRB populations were incubated under constrained conditions using acetate as the sole external carbon source to selectively stimulate acetotrophic SRB. The dilution-to-extinction technique was performed to try to isolate the microorganism able of performing sulfate reduction with acetate, or at least separate strains from the mixed population. Unfortunately, a culture able of performing sulfate reduction with acetate was not developed in our bottles. However, a stable population along time was observed in the granules after a long incubation period with acetate. 16S rRNA sequencing allowed us to get an idea of the evolution of the populations in our experimental bottles, confirming that acetotrophic SRB were not present. With the results obtained along the chapter and, taking into consideration the limitation of targeting only 16S rRNA genes, further analyses are required for a better understanding towards inoculation techniques that guarantee a successful retention and proliferation of acetotrophic SRB. The different mechanisms for the oxidation of acetate among SRB are a clear demonstration that this group of bacteria are a phylogenetically an extremely diverse collection of organisms. Nonetheless, their dominant characteristic of sulfide production has led to their classification as an homogeneous group with regard to their physiology and ecology. Considering this, much more information and resolution would have been gathered by applying other molecular biology techniques apart from the sequencing such as the study of selected functional genes.

Chapter 9: General conclusions and future work

The main objective of this thesis was to treat synthetic wastewaters with high sulfate content using crude glycerol as carbon source towards the recovery of elemental sulfur. In this section, the main achievements and conclusions withdrawn from this thesis are summarized. Some future research suggestions are also presented, in order to further develop and operate robust systems to improve the sulfate removal and elemental sulfur recovery.

9.1 General conclusions

This thesis shows for the first time the treatment of high-strength sulfate wastewater using crude glycerol as carbon source as a first step towards the recovery of elemental sulfur, a value-added product currently extracted from non-renewable resources. A range of scenarios have been tested showing that a start-up using granular sludge from methane-producing anaerobic digesters, at pH above 8.0 and mesophilic conditions was feasible. A long-term operation was achieved with a UASB reactor using crude glycerol as carbon source at a low up flow velocity (0.25 m h^{-1}) both at constant and variable loading rates. The highest average sulfate elimination capacity (S-EC=4.3 kg S m^{-3} d^{-1}) was obtained at a COD/S-SO_4^{2-} ratio of 5.4 g O_2 g^{-1} S and an OLR of 24.4 kg O_2 m^{-3} d^{-1} with a sulfate removal efficiency of 94 %.

It was not only the COD/S-SO_4^{2-} ratio, but a sum and combination of factors along the operation that determined the competition between SRB and methanogens. FISH and 16S rRNA sequencing allowed observing how microbial communities specialized in more specific functions and SRB populations were selected according to operating conditions. The non-acetate degrader *Desulfovibrio* was found to be the most abundant SRB genus detected. The increase in acetate concentration was related to the wash-out of methanogens together with the inability of the selected SRB to mineralize acetate.

Overall, it was concluded that long-term performances are important to get valuable conclusions in terms of stable sulfidogenic UASB operations. Physical,

chemical and biological characterization of the slime substance formed inside the reactor was applied to investigate its properties, possible interactions with the granules, role in relation to the sulfate-reduction activity and putative mass transfer limitations. After these analyses, it was confirmed that the production of slime could be notably affecting our system performance. Populations colonizing the reactor were present both in the granules and in the slime. But many other substances were found in the slime, such as FAMEs, that conferred viscosity to the slime. Consequently, problems related to mass transfer limitations could be probably occurring, limiting the efficiency of the process in terms of sulfate reducing activity.

Crude glycerol has been shown as a proper electron donor for a sulfidogenic UASB during this thesis. A transition to non-methane producing conditions was reached at selected sulfate loading rates maintaining the sulfate reducing efficiency of the UASB. However, under such conditions, large amounts of acetate were accumulated in the reactor. This accumulation implies a loss of carbon source, which is undesirable, even if crude glycerol was a waste of the biodiesel industry. So as to take advantage of this excess of organic matter in the effluent, efforts were directed towards the incubation of granules from the UASB reactor in serum bottles fed with acetate to selectively promote the growth of acetotrophic SRB. After a long period of incubation under constrained conditions using acetate as the available carbon source, 16S rRNA sequencing was used to observe the microbial evolution of the populations in our experimental bottles, confirming that, unfortunately, acetotrophic SRB were not present.

9.2 Future work

In this thesis an extensive knowledge about biological sulfate reduction and how to operate a UASB reactor for that purpose has been acquired from both technical and biological points of view. However, further investigation is required in order to improve the efficiency of the process in the long-term and the specific use of carbon source for sulfate reduction.

Operational data together with molecular-biology techniques allowed the establishment of a link between the population structure and function of the anaerobic communities in the UASB reactor under certain conditions tested. However, taking into consideration the limitation of targeting only 16S rRNA genes, further analyses are required for a better understanding towards this link.

An optimization of the whole FISH procedure, including the most problematic aspects discussed along Chapter 6, would also be helpful for a better comprehension of the activity of sulfate reducers. On that way, much more information and resolution would have been gathered by applying other molecular biology techniques apart from the ones already mentioned. Further batch activity tests are warranted to properly validate the results obtained herein.

Another common drawback in both long-term operations presented in Chapters 5 and 6 was the accumulation of acetate in our system which implied the loss of carbon source. In this sense, further research is needed to enhance the operation in terms of COD mineralization in the long-term. New cultivation methodologies, both in serum bottles and batch reactors could be applied with new and different cultures. As an alternative, a second reactor could be included after the UASB to take advantage of the effluents with high concentration of acetate to perform other processes. If the use of carbon source could be improved, the costs for the biological sulfate removal would be reduced making it a more competitive technology.

In addition, the main parameters affecting both operations were pointed out with special mention to the slime substance developed inside the sludge bed. Efforts to characterize this substance were made, pointing out the relation between its appearance and the failure of the operation. Modifications in the operation mode of the UASB reactor or even including a recirculation since the start-up of the operation to increase the up-flow velocity should be considered. The influence on the use of crude glycerol and the appearance of the slime substance appeared to be linked, affecting the efficiency of the process. The use of different waste organic sources should be also contemplated, such as cheese whey and vinasses, obtained as well, as byproducts or waste effluents from industrial processes. Mass transfer limitations should be avoided leading to a procedure on how to operate a sulfidogenic UASB reactor in a long-term stable mode, before going to a pilot or greater scale.

Chapter 10: References

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