UNIVERSIDAD DE LA REPÚBLICA
FACULTAD DE INGENIERÍA

Hydrogenotrophic methanogenic activity test: theoretical basis, protocol design discussion and experiences for biosludge at different environmental conditions

Author: Chem. Eng. Evangelina Ripoll González

Maestría en Ingeniería Química - Facultad de Ingeniería

Universidad de la República

Montevideo, Uruguay

2021

UNIVERSIDAD DE LA REPÚBLICA
FACULTAD DE INGENIERÍA

Hydrogenotrophic methanogenic activity test: theoretical basis, protocol design discussion and experiences for biosludge at different environmental conditions

Autora: Ing. Quím. Evangelina Ripoll González

Tesis presentada con el objetivo de obtener el título de Magíster en Ingeniería Química en el marco del Programa de Maestría en Ingeniería Química Plan 2005, Facultad de Ingeniería, Universidad de la República

Directora Académica/Tutora:
Dr. Ing. Quím. Liliana Borzacconi Vidal, Profesor Titular G5

Cotutor:
Dr. Ing. Quím. Iván López Moreda, Profesor Titular G5

Montevideo, Uruguay

2021

PÁGINA DE APROBACIÓN

El tribunal docente integrado por los abajo firmantes aprueba el Trabajo Final:

Título:

Hydrogenotrophic methanogenic activity test: theoretical basis, protocol design discussion and experiences for biosludge at different environmental conditions

Autora:

Ing. Quím. Evangelina Ripoll González

Directora Académica/Tutora:

Dr. Ing. Quím. Liliana Borzacconi Vidal

Cotutor:

Dr. Ing. Quím. Iván López Moreda

Posgrado:

Maestría en Ingeniería Química Plan 2005, Facultad de Ingeniería, Universidad de la República

Puntaje:

……

Tribunal

Profesor……

Profesor……

Profesor……

FECHA ……

AGRADECIMIENTOS

Ha pasado ya tanto tiempo desde el inicio de esta tesis que es difícil no olvidar a ninguno de los que contribuyeron su granito de arena en la investigación y la escritura de esta tesis de maestría… Haré entonces mi mayor esfuerzo. Agradezco profundamente:

A la Agencia Nacional de Investigación e Innovación por financiar mi beca de maestría con la convocatoria POS_NAC_2014_1_102268.

A mis tutores, Liliana Borzacconi e Iván López, por guiarme en esta travesía con largas horas de experiencias de laboratorio y alentarme en esta dura tarea de poner en orden todas las experiencias para dar lugar a este manuscrito. Si he crecido y soy mejor investigadora ahora, se lo debo en gran parte a ustedes.

A mi mentor de alma, Mauricio Passeggi, por inspirarme curiosidad y pasión por lo que hago en cada charla de pasillo y oficina que hemos tenido en estos años de tesis; y por tu ejemplo, que habla más que mil palabras.

A mi familia que me ha apoyado y alentado a través de los años, papá, mamá, mi hermano. Los amo mucho. Papá, desearía que estuvieras aquí para ver el final de esta tesis, así como viste el principio, pero sé que lo estás viendo desde la Eternidad y eso me consuela.

A mi amado esposo Luis. Comencé esta tesis soltera y ahora estoy felizmente apoyada por ti. Gracias por desvelarte largamente conmigo para acompañarme mientras escribía esta tesis. ¡Te amo!

A cada una de mis amigas y a los hermanos de la iglesia, que con sus charlas y mensajes me apoyaron cuando me sentía que nunca iba a acabar.

A todo el grupo BIOPROA, a los ex bioproenses y a mis queridos amigos docentes y no docentes que comparten conmigo los pasillos del Instituto de Ingeniería Química. Si pusiera cada nombre y todo lo que han hecho por mí en este tiempo, escribiría realmente una enciclopedia en vez de una tesis de maestría. Guardo a cada uno en mi corazón.

A Dios por darme gracia y fuerzas para completar esta etapa y crecer.

LIST OF ABBREVIATIONS

ADM1	Anaerobic Digestion Model no. 1
BES	2-Bromoethanesulfonate
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred-Tank Reactor
EGSB	Expanded Granular Sludge Bed
GC	Gas Chromatography
IC	Internal Circulation Reactor
IC50	Half-maximal Inhibitory Concentration
OLR	Organic Loading Rate
SAMA	Specific Acetoclastic Methanogenic Activity
SAO	Syntrophic Acetate Oxidation
SAO-HM	Syntrophic Acetate Oxidation- Hydrogenotrophic Methanogenesis
SAOB	Syntrophic Acetate Oxidizing Bacteria
SHMA	Specific Hydrogenotrophic Methanogenic Activity
SMA	Specific Methanogenic Activity
SMP	Specific Methane Production
SMPR	Specific Methane Production Rate
SRT	Solids Retention Time
TCD	Thermal Conductivity Detector
UASB	Up-flow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VS	Volatile Solids
VSS	Volatile Suspended Solids

LIST OF CONSTANTS AND SYMBOLS (in order of appearance)

n_{CH4}	mol	Moles of methane
n_{gas}	mol	Moles of gas mixture
t	days	Time
P	atm	Pressure inside vial headspace
V_{hs}	L	Volume of vial headspace
R	L atm $K^{-1}mol^{-1}$	Universal constant for gases
T	K	Incubation temperature
X	g_{VSS} L^{-1}	Biomass (microbial) concentration
S	g_{COD} L^{-1}	Substrate concentration (subscript "ac" for acetate and "h2" for H_2)
K_S	g_{COD} L^{-1}	Half-saturation constant
μ_m	d^{-1}	Maximum specific growth rate
r_X	g_{VSS} L^{-1} d^{-1}	Microbial growth rate
r_S	g_{COD} L^{-1} d^{-1}	Substrate consumption rate
$Y_{X/S}$	g_{VSS} g_{COD}^{-1}	Microbial yield
k_S	g_{COD} g_{VSS}^{-1} d^{-1}	Specific substrate consumption rate
k_m	g_{COD} g_{VSS}^{-1} d^{-1}	Monod maximum specific uptake rate
$k_{m,h}$	g_{COD} g_{VSS}^{-1} d^{-1}	Monod maximum specific uptake rate for hydrogenotrophic methanogenesis (based on total biomass)
$k_{m,a}$	g_{COD} g_{VSS}^{-1} d^{-1}	Monod maximum specific uptake rate for acetoclastic methanogenesis (based on total biomass)
V_B	L	Volume of biosludge aliquot
C	g_{COD} mol^{-1}	Conversion factor from moles of methane to gCOD
V_L	L	Volume of liquid in vial
SMP	g_{COD} g_{VSS}^{-1}	Amount of methane produced per gram of biomass
SHMA	g_{COD} g_{VSS}^{-1} d^{-1}	Specific Hydrogenotrophic Methanogenic Activity
SAMA	g_{COD} g_{VSS}^{-1} d^{-1}	Specific Acetoclastic Methanogenic Activity
OLR	g_{COD} g_{VSS}^{-1} d^{-1}	Organic Loading Rate
SMPR	g_{COD} g_{VSS}^{-1} d^{-1}	Specific Methane Production Rate in reactor
r_1	g_{COD} L^{-1} d^{-1}	Substrate consumption rate for acetoclastic methanogens
r_2	g_{COD} L^{-1} d^{-1}	Substrate consumption rate for hydrogenotrophic methanogens
r_3	g_{COD} L^{-1} d^{-1}	Substrate consumption rate for SAOB
r_4	g_{COD} L^{-1} d^{-1}	Substrate consumption rate for homoacetogenesis bacteria
c	g_{COD} g_{VSS}^{-1}	Correction factor of gCOD per gram of VSS for microbial cells
$k_{m,hm}$	g_{COD} g_{VSS}^{-1} d^{-1}	Monod maximum specific uptake rate for hydrogenotrophic methanogens
$k_{m,am}$	g_{COD} g_{VSS}^{-1} d^{-1}	Monod maximum specific uptake rate for acetoclastic methanogens
k_2	g_{COD} g_{VSS}^{-1} d^{-1}	Pseudo-first-order rate constant for hydrogenotrophic methanogenesis
k_4	g_{COD} g_{VSS}^{-1} d^{-1}	Pseudo-first-order rate constant for homoacetogenesis
X_{hm}	g_{VSS} L^{-1}	Concentration of hydrogenotrophic methanogens
S_{CH4}	g_{COD} L^{-1}	Methane concentration
k_{CH4}	g_{COD} g_{VSS}^{-1} d^{-1}	Specific methane production rate (from modelling)
k_{CH4_1}	g_{COD} g_{VSS}^{-1} d^{-1}	Pseudo-first-order rate constant for acetoclastic methanogenesis
k_{CH4_3+2}	g_{COD} g_{VSS}^{-1} d^{-1}	Pseudo-first-order rate constant for SAO-HM pathway
X_{am}	g_{VSS} L^{-1}	Concentration of acetoclastic methanogens
f_1	(dimensionless)	Correction factor to discount homoacetogenesis from SHMA
f_2	(dimensionless)	Correction factor to discount SAO-HM from SAMA
X_{tot}	g_{VS} L^{-1}	Total Concentration of Volatile Solids in a sample from solids digester
x_{CH4}	(dimensionless)	Molar fraction of methane in gas mixture inside vial headspace
f_c	(dimensionless)	Correction factor to consider biomass dilution in the total VS of sample
X_b	g_{VS} L^{-1}	Concentration of biodegradable fraction of substrate
X_{nb}	g_{VS} L^{-1}	Concentration of non-biodegradable fraction of substrate
k_H	d^{-1}	First-order constant for substrate hydrolysis
k_d	d^{-1}	First-order constant for microbial decay
$_T$	d	solids retention time

Note: Subscripts for microbial populations: "am" for acetoclastic methanogens, "hm" for hydrogenotrophic methanogens, "ao" for SAOB, and "ha" for homoacetogenic bacteria (Valid for X, r_X, k_m, K_S, Y). Subscripts: "0" for initial conditions, "in" for inlet of a continuous process (Valid for X and S).

ABSTRACT

Specific methanogenic activity is a cost-effective tool to characterize the biological activity of biosludge, to monitor reactor performance, and study the kinetics of acetate and H_2 conversion to methane in anaerobic-digestion-based systems. More established protocols are applied for acetoclastic activity tests, while hydrogenotrophic activity tests are still less widespread and not standardized. Due to methodological differences, the few results found in the literature for hydrogenotrophic activity are difficult to compare. Drawbacks are observed when analyzing solids digesters, dealing with low activity values associated with biomass dilution by the substrate and inert, long measurement times, and significant microbial growth. Having a standardized technique is of utmost importance, and this thesis intends to contribute to this line. Full calculation and design conditions for hydrogenotrophic activity test are developed over this manuscript, based on kinetics for the H_2/CO_2 conversion to methane. An equation to calculate inoculum size is proposed, suitable for a wide variety of biosludge samples. Zero-order model fitted adequately for hydrogenotrophic activity determined in pilot-scale and full-scale reactor samples, with standard deviations for triplicates between 3 and 12%, based on headspace pressure measures. Kinetics for the four reactions involved in the last step of the anaerobic digestion process was considered to estimate methanogen fractions in microbial population and study pathway contributions during methanogenic activity tests. The inhibitor 2-Bromoethanesulfonate was dosed during methanogenic activity tests, obtaining a IC50 of 3 and 20 mM for acetoclastic and hydrogenotrophic methanogens, respectively, in granular sludge samples. Homoacetogens contributed about 6% of the hydrogenotrophic activity obtained without inhibitor;

whereas, during the acetoclastic activity test, about 23% of methane was produced via syntrophic acetate oxidation, evidencing the importance of studying this route in anaerobic digestion systems. Finally, a case of solid waste digestion was analyzed, and a simple method was proposed to solve drawbacks during hydrogenotrophic test. The evolution of biomass, degradable, and non-degradable substrate was modeled for solids digestion during a batch incubation and then in a CSTR digester. Biomass correction factors were estimated and used as correctors of activity values to reflect microbial activity more accurately and improve the design of methanogenic activity tests.

KEYWORDS: Anaerobic digestion; Biodegradation kinetics; Hydrogenotrophic methanogenesis; Specific methanogenic activity; Waste digestion

RESUMEN

Los ensayos de actividad metanogénica constituyen una herramienta rentable para la caracterización biológica de lodos, el monitoreo de reactores y el estudio cinético de la conversión de acetato e hidrógeno a metano en sistemas de digestión anaerobia. Existen protocolos establecidos para el ensayo de actividad acetoclástica, mientras que el ensayo hidrogenotrófico está poco difundido y carece de protocolo estandarizado, lo que dificulta la comparación de los pocos resultados disponibles en bibliografía. Además, existen inconvenientes en su aplicación para lodos de digestores de sólidos, debido a la dilución de la biomasa por sustrato e inerte, causando actividades bajas y tiempos largos de prueba, con creciemiento microbiano significativo. Con esta tesis, se pretende contribuir a la estandarización de la técnica, abordando la base de cálculo y discutiendo las condiciones de diseño mediante modelos cinéticos simples. Se propone una ecuación para el cálculo del tamaño del inóculo a utilizar en la prueba. Se aplica un modelo de orden cero para obtener la actividad hidrogenotrófica a partir de la presión de cámara del vial, obteniéndose un buen ajuste y desviaciones estándares de 3 a 12% para muestras de reactores industriales y de banco. Se analiza asimismo la cinética de las cuatro reacciones involucradas en la metanogénesis, para estimar las fracciones de metanogénicos en la biomasa y estudiar la contribución de cada vía en los ensayos de actividad. Al aplicar el inhibidor 2-Bromoetanosulfonato de sodio, se obtienen IC50 de 3 y 20 mM para acetoclásticos e hidrogenotróficos en lodo granular, respectivamente. Se observa una contribución de la homoacetogénesis de 6%

en la actividad hidrogenotrófica, mientras que se obtiene 23% de metano por la vía de oxidación sintrófica de acetato acoplada a la metanogénesis hidrogenotrófica durante el ensayo acetoclástico, evidenciando la importancia del estudio de esta ruta. Finalmente, se analiza un caso de digestores de sólidos, modelando la evolución de la biomasa y de las fracciones biodegradables y no biodegradables. Se determinan factores de dilución de biomasa, mostrando un método simple para mejorar el diseño de los ensayos metanogénicos y corregir los valores obtenidos para que reflejen mejor la actividad biológica de lodos de digestores de sólidos.

PALABRAS CLAVE:

Actividad metanogénica específica; Cinética de degradación biológica; Digestión Anaerobia; Digestión de residuos; Metanogénesis hidrogenotrófica

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION 15

1.1. TRACKING PATHWAYS IN ANAEROBIC DIGESTION PROCESS ... 15

1.2. SPECIFIC METHANOGENIC ACTIVITY 19

1.3. AIM OF THE THESIS 20

1.4. A ROADMAP FOR STANDARDIZATION 20

1.5. A DEEPER STUDY BASED ON ACTIVITY TESTS 21

1.6. SOLVING DRAWBACKS OF THE TECHNIQUE 22

1.7. SUMMARIZING OBJECTIVES 23

CHAPTER 2. HYDROGENOTROPHIC ACTIVITY: A TOOL TO EVALUATE THE KINETICS OF METHANOGENS 28

2.1. INTRODUCTION 28

2.2. MATERIALS AND METHODS 32

2.2.1. Detection method selection 32

2.2.2. Kinetic model 33

2.2.3. Inoculum size calculation 35

2.2.4. Incubation temperature selection 36

2.2.5. SHMA assay setup 37

2.2.6. SHMA calculation 39

2.2.7. SAMA assay setup 41

2.3. RESULTS AND DISCUSSION 41

2.3.1. Verification of zero-order conditions for the SHMA assay 41
2.3.2. Verification of zero-order conditions for the SAMA assay 44
2.3.3. Microbial growth 44
2.3.4. Experimental and literature values for SAMA and SHMA 45
2.3.5. Future research 51
2.4. CONCLUSIONS 52
CHAPTER 3. PATHWAYS STUDY BASED ON ACTIVITY TESTS 57
3.1. INTRODUCTION 57
3.2. MATERIALS AND METHODS 65
3.3. RESULTS AND DISCUSSION 69
3.3.1. Hydrogenotrophic methanogens: inhibition in SHMA tests 69
3.3.2. Hydrogenotrophic methanogens: mass fraction estimation 71
3.3.3. Acetoclastic methanogens: inhibition in SAMA tests 73
3.3.4. Acetoclastic methanogens: mass fraction estimation 75
3.3.5. Comments on the order of reaction 76
3.4. CONCLUSIONS 77
CHAPTER 4. HYDROGENOTROPHIC ACTIVITY APPLIED TO SLUDGE FROM SOLIDS DIGESTER 82
4.1. INTRODUCTION 82
4.2. MATERIALS AND METHODS 84
4.2.1. Inoculum 84

4.2.2. Manure bench-scale digester 84
4.2.3. Beverage pilot-scale EGSB reactor 85
4.2.4. SHMA test 85
4.2.5. SAMA test 87
4.2.6. Kinetics model 88
4.3. RESULTS AND DISCUSSION 90
4.3.1. Model simulation results 90
4.3.2. SAMA and SHMA tests 92
4.3.3. Inoculum size estimation for SAMA and SHMA tests 94
4.4. CONCLUSIONS 95
CHAPTER 5. FINAL CONSIDERATIONS 98
GLOSSARY 104
APPENDIX 1 106
APPENDIX 2 114
APPENDIX 3 118
APPENDIX 4 121
APPENDIX 5 126

CHAPTER 1

INTRODUCTION

CHAPTER 1. INTRODUCTION

1.1. TRACKING PATHWAYS IN ANAEROBIC DIGESTION PROCESS

ANAEROBIC DIGESTION (AD) represents widespread technology for waste digestion, water management and renewable energy production (Lier et al., 2015; Vutai et al., 2016). Understanding the phenomena involved in the AD process is crucial to optimize organic matter removal and methane production and to diagnose and solve operational problems. A general scheme for the AD of polymeric substrates is shown in Figure 1-1.

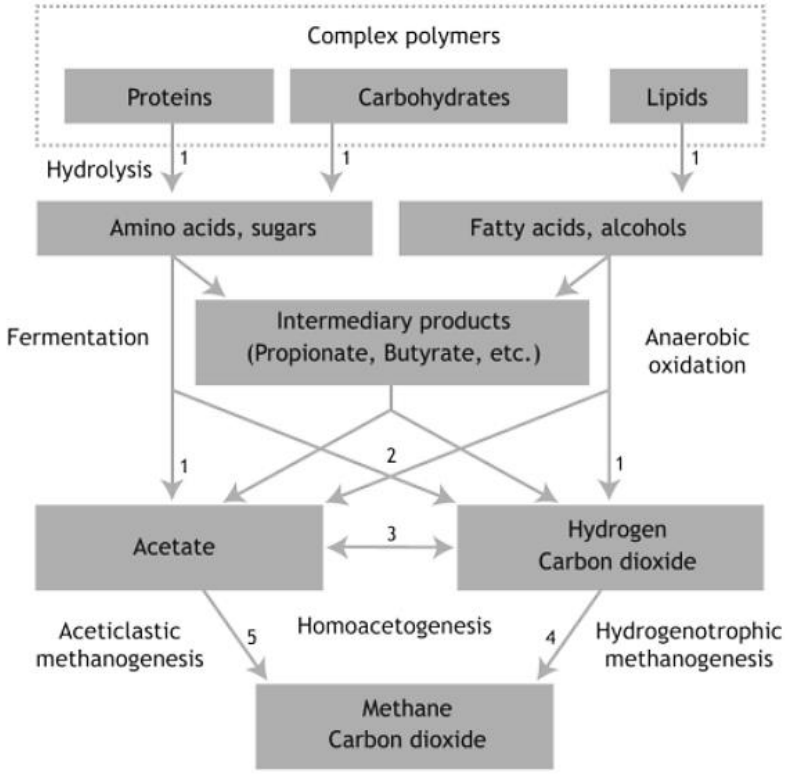

Figure 1-1. Scheme for the AD of polymeric substrates and the groups of microorganisms involved: (1) Hydrolytic and fermentative bacteria; (2) Acetogenic bacteria; (3) Homoacetogenic bacteria and SAOBs; (4) Hydrogenotrophic methanogens; and (5) Acetoclastic methanogens. Extracted from Henze, M.; van Loosdrecht, M. C.; Ekama, G. A.; Brdjanovic (2008), p. 418

Depending on the type of substrate, different steps such as hydrolysis, beta-oxidation, or METHANOGENESIS could be rate-limiting in the AD process. In particular, methanogenesis implies two different pathways: (i) acetoclastic methanogenesis, which represents the conversion of the methyl group of acetate to methane, and (ii) hydrogenotrophic methanogenesis, which involves the reduction of CO_2 coupled to the oxidation of formate or H_2 to produce methane. By the 1960s, researchers studied the mechanisms of these reactions by using radiolabelled compounds: Jeris and McCarty (1965) used labelled bicarbonate, detecting labelled methane associated with hydrogenotrophic methanogenesis and assuming that the totality of non-labelled methane was exclusively produced by acetic acid fermentation (one step). On the other hand, Smith and Mah (1966) used labeled acetic acid in the methyl group instead of labeled bicarbonate, and determined the depletion of that species as a direct measure of the rate of consumption of acetoclastic methanogenesis. In both cases, about 70% of the methane produced was attributed to acetoclastic methanogenesis. However, by then, neither HOMOACETOGENESIS nor SYNTROPHIC ACETATE-OXIDATION (SAO) was known or considered in anaerobic digestion systems (associated with #3 in Figure 1.1). In the 1980s, a syntrophic association was discovered, supporting the idea of methane formation from acetate via hydrogenotrophic methanogenesis (Zinder and Koch, 1984). Years later, the phenomenon of homoacetogenesis, also known as 'reversible acetogenesis' began to be studied more deeply (Zinder, 1994). In the recent years, using acetate labelled in the methyl group (C-2) and measuring the production of $^{14}CH_4$ and $^{14}CO_2$, studies showed that syntrophic acetate-oxidation coupled with hydrogenotrophic methanogenesis (SAO-HM) was a relevant pathway in ANAEROBIC DIGESTERS operating under stress conditions,

such as high levels of total ammonia nitrogen, high levels of VOLATILE FATTY ACIDS (VFA), extreme pH or elevated temperature (Bi et al., 2020; Fotidis et al., 2013; Ho et al., 2013; Oosterkamp et al., 2019; Westerholm et al., 2016). In these studies, the $^{14}CO_2$ produced from [2-^{14}C] acetate remained diluted by the non-labelled CO_2 introduced as sodium bicarbonate, making negligible the $^{14}CH_4$ obtained via hydrogenotrophic methanogenesis, and been able to distinguish SAO-HM from acetoclastic methanogenesis. Kim et al. (2013) found an absolute dominance of hydrogenotrophic methanogens in full-scale digesters for sewage sludge, based on the quantitative Polymerase Chain Reaction (qPCR) of the 16S rRNA gene. Also, Demirel (2014), in a review, identified hydrogenotrophic methanogenesis as the main pathway for methane production in digesters of energy crops, when using Sequence-Specific Oligonucleotide Probe Hybridization. Hence syntrophic acetate-oxidation and hydrogenotrophic methanogenesis drew researchers' attention. On the other hand, Xia et al. (2014) showed by using a metatranscriptomic approach that the acetoclastic step was more active than the hydrogenotrophic step in a thermophilic Sequential Batch Reactor (SBR) for cellulose wastewater treatment, even though acetoclastic members were in a lower abundance. Consequently, the predominance of a particular methanogenic step in anaerobic systems is currently under discussion. More approaches should be incorporated to understand this key step in the AD process. Also, it must be considered that most researchers have studied systems for solid substrate digestion. More in-depth studies in wastewater treatment systems are needed.

In another vein, the hydrogenotrophic pathway became interesting for BIOGAS UPGRADING to biomethane for fuel usage, with the challenge of controlling

the process to favor methane production from the injected hydrogen to enrich the BIOGAS in methane content, instead of producing acetate from hydrogen via homoacetogenesis (Agneessens et al., 2018; Angelidaki et al., 2018). More recently, researchers proposed an alternative method of biogas upgrading by converting CO_2 from biogas into chemicals (e.g., volatile fatty acids) using H_2 and acetogenic mixed culture (Omar et al., 2019).

For a laboratory scale, techniques based on radiolabelled compounds are suitable for rigorous studies, determining the percentages of target species for each trackable pathway; however, they cannot characterize the conversion rate of each substrate, desirable for kinetics study. On the other hand, microbiological tools could provide information on the microbial population in terms of composition and quantity and could distinguish between active and non-active microorganisms according to the method considered (for example, mRNA quantification). The METATRANSCRIPTOMIC APPROACH allows the determination of the transcriptomic activity, represented by the ratio between the reads per kilobase per million mapped reads for RNA and DNA (RPKM-RNA to RPKM-DNA); despite that, it is unable to determine how metabolically active the methanogens are in terms of kinetics (which means in terms of rates). Then, it is relevant to include a tool to measure the consumption rate, study kinetics, and understand system dynamics, and the SPECIFIC METHANOGENIC ACTIVITY (SMA) tests can do so. When considering full-scale ANAEROBIC REACTORS, having a cost-effective tool to monitor their performance and a reasonable kinetic model to predict behaviour is valuable and necessary. SMA could play the role of providing both kinetic parameters and diagnosis information, complementing the characterization of a microbial population, for both pilot and full-scale reactors.

1.2. SPECIFIC METHANOGENIC ACTIVITY

SMA (g_{COD} g_{VSS}^{-1} d^{-1}) is defined as the maximum production rate of methane, expressed in grams of CHEMICAL OXYGEN DEMAND (COD), from a substrate (that could be acetate, VFA, H_2/CO_2, among others), expressed per weight of BIOMASS; thus, it represents a valuable tool to assess the metabolic activity of ANAEROBIC BIOSLUDGE. In this thesis, the term 'biomass' refers to microorganisms present in biosludge, estimated by the VOLATILE SUSPENDED SOLIDS (VSS) content.

Activity tests for both acetoclastic and hydrogenotrophic pathways must be considered to characterize anaerobic biosludge and monitor the performance of reactors. They can provide information about a system imbalance associated with methanogens inhibition, or by the contrary, help to detect a sub-utilization of the reactor capacity. Established protocols are followed by researchers to determine specific acetoclastic methanogenic activity (SAMA), using acetate as the substrate (Dolfing and Bloeman, 1985; Soto et al., 1993; van Loosdrecht et al., 2016). Other authors use a neutralized mixture of acetate, propionate, and butyrate to obtain information related to acetogenic, acetoclastic, and hydrogenotrophic pathways altogether (Gonzalez-Gil et al., 2002; Jeison and van Lier, 2007). There are works reporting values for the specific hydrogenotrophic methanogenic activity (SHMA), but with an evident lack of standardization among the techniques and conditions used (Bassani et al., 2016; Bhattad et al., 2017; Coates et al., 1996; Gonzalez-Estrella et al., 2013; Hao et al., 2017; Keating et al., 2016; Liu et al., 2016; McKeown et al., 2009; Molina et al., 2008; Pereira et al., 2003; Regueiro et al., 2012).

1.3. AIM OF THE THESIS

Having a standardized technique for hydrogenotrophic activity is of utmost importance, and the aim of this thesis is to contribute to this line, with special emphasis on the discussion of test conditions and results. Some years ago while trying to organize an inter-laboratory work between various research groups to standardize this technique, it began to be clear the need of a roadmap that can be used and adapted when test conditions and equipment differed or when analyzing different type of samples.

1.4. A ROADMAP FOR STANDARDIZATION

In Chapter 2 of the present thesis, a full calculation structure is outlined to accomplish the main objective: establish a basis for the design and selection of the test conditions for a wide variety of types of biosludge (from a wastewater treatment plant to solids digesters, from a high-rate reactor to lagoons). The technique introduced by Coates et al. (1996) is the most widespread among scientists; but there is a lack of modelling and the test conditions were adjusted in a purely experimental way using biosludge from a single source. The authors themselves tried without success applying the selected conditions in an essay of biosludge from a different source, a solids digester. The main problem was applying, without any adjustment, the design rules extracted from the essay of biosludge with good activity to biosludge with low activity. In Chapter 2 of the present thesis, a calculation formula to size the INOCULUM used in the SHMA assay is proposed and tested to solve this drawback based on biosludge characteristics and operational data of the source reactor. The kinetics of Monod applied to model the microbial growth rate, and the test designed under zero-

order conditions and negligible growth. The methane production is calculated based on the stoichiometry of the H_2/CO_2 conversion to methane, simplification initially suggested by Coates et al. (1996). A comparison between these hypothesis simplifications and a full calculation based on the organic matter removed to produce methane is also presented in Chapter 2.

1.5. A DEEPER STUDY BASED ON ACTIVITY TESTS

Coates et al. (1996) considered homoacetogenesis to be negligible during the SHMA test, based on an unpublished work of Golden and Colleran. Despite not having found the published version of that work, in an article from the same laboratory, Ryan et al. (2008) published very similar tests that support the idea of Coates et al. (1996), showing a lag phase of about 60 hours that the authors associated with the adaptation of homoacetogens. They claim that the tests were performed under negligible growth conditions; however, there are reports of homoacetogens doubling-times of between 1.75 and 29 hours (Noike and Li, 1989; Saady, 2013). Moreover, there are, for example, reports of homoacetogens been problematic in hydrogen production systems that already have low SOLIDS RETENTION TIME (SRT) (Saady, 2013). Although it is a different system, it illustrates the point that homoacetogens resist low solid retention times. Homoacetogenic microorganisms are diverse and versatile (Schink, 1994), so it is worth studying homoacetogenesis when first-approaching a system or when drastic changes occur affecting the microbial population. Additionally, syntrophic acetate-oxidation bacteria (SAOB) could be important for acetate-consumption when acetoclastic methanogens were inhibited (Oosterkamp et al., 2019; Westerholm et al., 2016). Ryan et al. (2008) already worked with the SHMA test

and complete inhibition of methanogenesis to study homoacetogenesis exclusively. In the present thesis, one of the objectives is to study the four reactions involved in the last step of the AD process: acetoclastic methanogenesis (r_1), hydrogenotrophic methanogenesis (r_2), syntrophic acetate-oxidation (r_3), and homoacetogenesis (r_4) (Figure 1-2). A specific objective is to obtain correction factors to discount the influence of r4 and r3 to feedback AD-model with appropriate parameters for the methanogenic acetoclastic and hydrogenotrophic population. Different levels of a methanogenesis-inhibitor were used in the SAMA and SHMA tests to accomplish objectives; the results and discussion on this topic presented in Chapter 3.

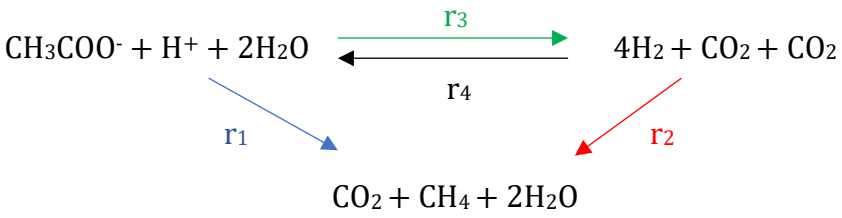

Figure 1-2. Reactions involved in the last step of the AD process

1.6. SOLVING DRAWBACKS OF THE TECHNIQUE

When analyzing a sample from a solids digester, Coates et al. (1996) obtained a time-lapse of around 70 hours, being unable to determine SHMA accurately, due to microbial growth. The problem was attributed to a low quantity of hydrogenotrophic methanogens, which is consistent with the sludge source. Inside solids digesters, microorganisms are diluted by the remaining substrate and the inert material. The challenge is to consider this dilution effect to properly size the inoculum for SHMA and SAMA tests on solids digester samples. Thus, in Chapter 4 of the present thesis, a correction factor for the biomass in the sludge

sample, determined by modeling the solids digester operation, is introduced to the base formula proposed in Chapter 2 to size inoculum. The activities informed for solids digesters are usually significantly less than those reported for wastewater reactors; also, there are reports of activity decrease when increasing solids concentration inside the digester or rising the organic loading rate (Liu et al., 2016). To distinguish whether the efficiency drop is due to an affectation of the microorganisms or is simply due to a dilution effect, the SHMA and SAMA should be expressed per mass of microorganisms instead of grams of VOLATILE SOLIDS (VS), since the VS might englobe microorganisms, substrate, and inert material. The correction factor calculated in Chapter 4 is also applied to adjust SHMA and SAMA and obtain more representative values for the microorganism activities, and see if the performance drop in the digester represents an improvable situation or a collateral effect; this issue is discussed in Chapter 4.

1.7. SUMMARIZING OBJECTIVES

Establish a basis for the design and selection of the test conditions to determine the specific hydrogenotrophic methanogenic activity for a wide variety of types of biosludge. In particular:

- Propose a formula to size inoculum according to sludge characteristics.
- Study the four reactions involved in the last step of the AD process, using activity tests as the main tools.
- Obtain correction factors to discount the influence of SAO-HM and homoacetogenesis from SAMA and SHMA determined in routine tests without inhibitors, to feedback AD-model with appropriate parameters.

- Propose a strategy to consider the dilution effect of substrate and inert material in sludge samples from solids digester to design the activity tests.

REFERENCES OF CHAPTER 1

Agneessens, L.M., Ottosen, L.D.M., Andersen, M., Berg Olesen, C., Feilberg, A., Kofoed, M.V.W., 2018. Parameters affecting acetate concentrations during in-situ biological hydrogen methanation. Bioresour. Technol. 258, 33–40. https://doi.org/10.1016/j.biortech.2018.02.102

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. Biotechnol. Adv. 36, 452–466. https://doi.org/10.1016/j.biotechadv.2018.01.011

Bassani, I., Kougias, P.G., Angelidaki, I., 2016. In-situ biogas upgrading in thermophilic granular UASB reactor: key factors affecting the hydrogen mass transfer rate. Bioresour. Technol. 221, 485–491. https://doi.org/10.1016/j.biortech.2016.09.083

Bhattad, U., Venkiteshwaran, K., Cherukuri, K., Maki, J.S., Zitomer, D.H., 2017. Activity of methanogenic biomass after heat and freeze drying in air. Environ. Sci. Water Res. Technol. 3, 462–471. https://doi.org/10.1039/c7ew00049a

Bi, S., Westerholm, M., Qiao, W., Xiong, L., Mahdy, A., Yin, D., Song, Y., Dong, R., 2020. Metabolic performance of anaerobic digestion of chicken manure under wet, high solid, and dry conditions. Bioresour. Technol. 296, 122342. https://doi.org/10.1016/j.biortech.2019.122342

Coates, J.D., Coughlan, M.F., Colleran, E., 1996. Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. J. Microbiol. Methods 26, 237–246. https://doi.org/10.1016/0167-7012(96)00915-3

Demirel, B., 2014. Major Pathway of Methane Formation From Energy Crops in Agricultural Biogas Digesters. Crit. Rev. Environ. Sci. Technol. 44, 199–222. https://doi.org/10.1080/10643389.2012.710452

Dolfing, J., Bloeman, W.G.B.M., 1985. Acitivity measurements as a tool to characterize the microbial composition of methanogenic environments. J. Microbiol. Methods 4, 1–12. https://doi.org/10.1016/0167-7012(85)90002-8

Fotidis, I.A., Karakashev, D., Kotsopoulos, T.A., Martzopoulos, G.G., Angelidaki, I., 2013. Effect of ammonium and acetate on methanogenic pathway and methanogenic community composition. FEMS Microbiol. Ecol. 83. https://doi.org/10.1111/j.1574-6941.2012.01456.x

Gonzalez-Estrella, J., Sierra-Alvarez, R., Field, J.A., 2013. Toxicity assessment of inorganic nanoparticles to acetoclastic and hydrogenotrophic methanogenic activity in anaerobic granular sludge. J. Hazard. Mater. 260, 278–285. https://doi.org/10.1016/j.jhazmat.2013.05.029

Gonzalez-Gil, G., Kleerebezem, R., Lettinga, G., 2002. Assessment of metabolic properties and kinetic parameters of methanogenic sludge by on-line methane production rate measurements. Appl. Microbiol. Biotechnol. 58, 248–254. https://doi.org/10.1007/s00253-001-0831-5

Hao, X., Liu, R., Loosdrecht, M. Van, Cao, D., 2017. Batch influences of exogenous hydrogen on both acidogenesis and methanogenesis of excess sludge. Chem. Eng. J. https://doi.org/10.1016/j.cej.2017.02.093

Henze, M.; van Loosdrecht, M. C.; Ekama, G. A.; Brdjanovic, D. (Ed.), 2008. Biological wastewater treatment. IWA publishing.

Ho, D.P., Jensen, P.D., Batstone, D.J., 2013. Methanosarcinaceae and Acetate-Oxidizing Pathways Dominate in High-Rate Thermophilic Anaerobic Digestion of Waste-Activated Sludge. Appl. Environ. Microbiol. 79, 6491–6500. https://doi.org/10.1128/AEM.01730-13

Jeison, D., van Lier, J.B., 2007. Thermophilic treatment of acidified and partially acidified wastewater using an anaerobic submerged MBR: Factors affecting long-term operational flux. Water Res. 41, 3868–3879. https://doi.org/10.1016/j.watres.2007.06.013

Jeris, J.S., McCarty, P.L., 1965. The Biochemistry of Methane Fermentation Using C14 Tracers. J. Water Pollut. Control Fed. 37, 178–192.

Keating, C., Chin, J.P., Hughes, D., Manesiotis, P., Cysneiros, D., Mahony, T., Smith, C.J., Mcgrath, J.W., Flaherty, V.O., 2016. Biological Phosphorus Removal During High-Rate, Low-Temperature, Anaerobic Digestion of Wastewater. Front. Microbiol. 7, 1–14. https://doi.org/https://doi.org/10.3389/fmicb.2016.00226

Kim, J., Kim, W., Lee, C., 2013. Absolute dominance of hydrogenotrophic methanogens in full-scale anaerobic sewage sludge digesters. J. Environ. Sci. 25, 2272–2280. https://doi.org/10.1016/S1001-0742(12)60299-X

Lier, J.B., Zee, F.P., Frijters, C.T.M.J., Ersahin, M.E., 2015. Celebrating 40 years anaerobic sludge bed reactors for industrial wastewater treatment. Rev. Environ. Sci. Bio/Technology 14, 681–702. https://doi.org/10.1007/s11157-015-9375-5

Liu, C., Li, H., Zhang, Y., Chen, Q., 2016. Characterization of methanogenic activity during high-solids anaerobic digestion of sewage sludge. Biochem. Eng. J. 109, 96–100. https://doi.org/10.1016/j.bej.2016.01.010

McKeown, R.M., Scully, C., Mahony, T., Collins, G., O'Flaherty, V., 2009. Long-term (1243 days), low-temperature (4–15 8C), anaerobic biotreatment of acidified wastewaters: Bioprocess performance and physiological characteristics 43, 1611–1620. https://doi.org/10.1016/j.watres.2009.01.015

Molina, F., García, C., Roca, E., Lema, J.M., 2008. Characterization of anaerobic granular sludge developed in UASB reactors that treat ethanol, carbohydrates and hydrolyzed protein based wastewaters. Water

Sci. Technol. 57, 837–842. https://doi.org/10.2166/wst.2008.067
Noike, T., Li, Y.Y., 1989. State of the art on anaerobic bacteria for wastewater treatment. 2. Acid-producing bacteria, in: Matsumoto, J. (Ed.), Study on Anaerobic Wastewater Treatment. Japan Society of Civil Engineers, Tokyo, pp. 126–145.
Omar, B., El-Gammal, M., Abou-Shanab, R., Fotidis, I.A., Angelidaki, I., Zhang, Y., 2019. Biogas upgrading and biochemical production from gas fermentation: Impact of microbial community and gas composition. Bioresour. Technol. 286, 121413. https://doi.org/10.1016/j.biortech.2019.121413
Oosterkamp, M.J., Bauer, S., Ibáñez, A.B., Méndez-García, C., Hong, P.Y., Cann, I., Mackie, R.I., 2019. Identification of methanogenesis and syntrophy as important microbial metabolic processes for optimal thermophilic anaerobic digestion of energy cane thin stillage. Bioresour. Technol. Reports 7, 100254. https://doi.org/10.1016/j.biteb.2019.100254
Pereira, M.A., Roest, K., Stams, A.J.M., Akkermans, A.D.L., Amaral, A.L., Pons, M.-N., Ferreira, E.C., Mota, M., Alves, M.M., 2003. Image analysis, methanogenic activity measurements, and molecular biological techniques to monitor granular sludge from an EGSB reactor fed with oleic acid. Water Sci. Technol. 47, 181–188. https://doi.org/10.2166/wst.2003.0314
Regueiro, L., Veiga, P., Figueroa, M., Alonso-Gutierrez, J., Stams, A.J.M., Lema, J.M., Carballa, M., 2012. Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters. Microbiol. Res. 167, 581–589. https://doi.org/10.1016/j.micres.2012.06.002
Ryan, P., Forbes, C., Colleran, E., 2008. Investigation of the diversity of homoacetogenic bacteria in mesophilic and thermophilic anaerobic sludges using the formyltetrahydrofolate synthetase gene. Water Sci. Technol. 57, 675–680. https://doi.org/10.2166/wst.2008.059
Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation : Unresolved challenge. Int. J. Hydrogen Energy 1–20. https://doi.org/10.1016/j.ijhydene.2013.07.122
Schink, B., 1994. Diversity, ecology, and isolation of Acetogenic Bacteria, in: Drake, H.L. (Ed.), Acetogenesis. Chapman and Hall, London, pp. 197–228.
Smith, P.H., Mah, R. a, 1966. Kinetics of acetate metabolism during sludge digestion. Appl. Microbiol. 14, 368–371.
Soto, M., Mendez, R., Lema, J.M., 1993. Methanogenic and non- methanogenic activity tests: theoretical basis and experimental set up. Water Res. 27, 1361–1376.
van Loosdrecht, M.C., Nielsen, P.H., Lopez-Vazquez, C.M., Brdjanovic, D., 2016. Experimental Methods in Wastewater Treatment. IWA Publishing, London.
Vutai, V., Ma, X.C., Lu, M., 2016. The Role of Anaerobic Digestion in Wastewater Management. EM (Pittsburgh. Pa). 0, 12–16.
Westerholm, M., Moestedt, J., Schnürer, A., 2016. Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance. Appl. Energy 179, 124–135. https://doi.org/10.1016/j.apenergy.2016.06.061
Xia, Y., Wang, Y., Fang, H.H.P., Jin, T., Zhong, H., Zhang, T., 2014. Thermophilic microbial cellulose decomposition and methanogenesis pathways recharacterized by metatranscriptomic and metagenomic analysis. Nat. Sci. Reports. https://doi.org/10.1038/srep06708
Zinder, S.H., 1994. Syntrophic Acetate Oxidation and “Reversible Acetogenesis,” in: Acetogenesis. Springer US, Boston, MA, pp. 386–415. https://doi.org/10.1007/978-1-4615-1777-1_14
Zinder, S.H., Koch, M., 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch. Microbiol. 138, 263–272. https://doi.org/10.1007/BF00402133

CHAPTER 2

HYDROGENOTROPHIC ACTIVITY: A TOOL TO EVALUATE THE KINETICS OF METHANOGENS

CHAPTER 2. HYDROGENOTROPHIC ACTIVITY: A TOOL TO EVALUATE THE KINETICS OF METHANOGENS

2.1. INTRODUCTION

Anaerobic digestion (AD) technology has a fundamental role in waste digestion and energy and material recovery (Kougias and Angelidaki, 2018). For decades, researchers have been studying the processes involved in the AD of organic substrates, in particular, the methanogenesis, considered to be the rate-limiting step when hydrolysis is favoured. Understanding the transformation steps that lead to methane production is crucial to optimize the operational conditions of anaerobic reactors for waste digestion and biogas production. Efforts have been made by several researchers to determine the relevance of the hydrogenotrophic and acetoclastic methanogenesis in different anaerobic systems, using tools such as DNA and mRNA quantification, radiolabelled molecules, and methanogenic activities. The use of microbiological tools could provide information on the microbial population composition and quantity and distinguish between active and non-active microorganisms depending on the technique applied (for example, mRNA quantification). However, these tools cannot determine how active microorganisms are. The knowledge of the kinetics involved is essential for understanding the dynamics of methanogenesis, and the tools selected for its study must consider this. The specific methanogenic activity (SMA) is one of these tools, defined as the

maximum rate of methane produced from a substrate and expressed per weight of volatile suspended solids (VSS) as a rough estimation of the biomass (the term ‘biomass’ used to refer to the microorganisms present in biosludge).

In recent years, many studies using radiolabelled sodium acetate have shown that syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis is relevant for methane production in solid digesters operating under stressful conditions, such as high levels of total ammonia nitrogen, high levels of volatile fatty acids, extreme pH or elevated temperature (Bi et al., 2020; Fotidis et al., 2012; Ho et al., 2013; Jiang et al., 2018; Oosterkamp et al., 2019; Westerholm et al., 2016). In another vein, researchers have recently focused their attention on the upgrade of biogas to methane for fuel usage purposes by adding H_2 to convert the remaining CO_2 of the biogas into methane (Angelidaki et al., 2018). In this context, hydrogenotrophic methanogens have a crucial role in proper performance. Hence the utility of studying hydrogenotrophic methanogenesis in biogas production and upgrading systems. In this context, SMA test represents a cost-effective and quickest method to monitor the activity of pilot-scale and full-scale reactors for biogas production and upgrading. It can provide information on-site, where most times, other approaches are not readily available to operators of the AD systems or require significantly more experimental apparatus and expertise. The specific acetoclastic and hydrogenotrophic methanogenic activities

(SAMA and SHMA) indirectly measure the consumption rates of acetate and H_2, respectively. Thus, these two activity values can provide information about a system imbalance associated with methanogens inhibition (low activities) or help to find opportunities to increase the ORGANIC LOADING RATE (OLR) applied to a reactor (high activities). Established protocols are followed by researchers to determine SAMA, using acetate as a substrate (Dolfing and Bloeman, 1985; Soto et al., 1993; van Loosdrecht et al., 2016). Also, some works are reporting SHMA (Bhattad et al., 2017; Gonzalez-Estrella et al., 2013; Hao et al., 2017; Keating et al., 2016; Liu et al., 2016; Regueiro et al., 2012). Nevertheless, there is a lack of standardization among the techniques used to determine SHMA. The novelty of this work mainly consists of outlining the full calculation structure to accomplish the main objective: re-design and select the test conditions for a wide variety of types of biosludge (from a wastewater treatment plant to solid digesters, from a high-rate reactor to lagoons) and lay a basis for a discussion of inter-laboratory work and standardization of the SHMA technique.

Coates et al. (1996) assayed a variety of test conditions for SHMA considering biosludge from a single source and obtained an experimental concentration range for the biosludge inoculum in the batch test. In the same work, when the design was applied to a biosludge sample from a solid digester with low activity, the assay took more than 70 hours, which implied significant microbial growth, distorting the SHMA determination. The same issue is observed in the work of Bhattad et al. (2017), where test bottles

containing a VSS concentration of ≤0.3 g L^{-1} were used, obtaining assays of about 50 to 100 hours, and no adjustment made in the design for those samples for which lower activities were expected. One of the specific objectives of this work is to solve this drawback; thus, a calculation formula is proposed and tested to size the inoculum used in the SHMA assay based on the biosludge characteristics and operational data of the source reactor. In the present Chapter, the basis for the design of the SHMA test is discussed, using the detection method proposed by Coates et al. (1996), but designing the test conditions considering the kinetics of the H_2/CO_2 conversion to methane. The second specific objective of this work is verifying the zero-order model fit to data, and the suitability of the hypothesis simplifications applied when the stoichiometric formula of the H_2/CO_2 conversion to methane is used to calculate the methane production during a SHMA test. The third specific objective is testing the conditions resulting from the SHMA assay design in a wide variety of samples. In this Chapter, eight different biosludge samples collected from pilot-scale and full-scale anaerobic systems are analysed using the design discussed for the SHMA test. Additionally, SAMA is assessed to complete activity characterization in the studied systems. The fourth specific objective is to correlate the SAMA and SHMA of the biosludge to the performance of the source reactor. The results obtained in our laboratory for SAMA and SHMA are compared one to the other and with other values found in the literature. Additionally, a discussion regarding the influence of the operational conditions on the

activity values is introduced in this Chapter. The full paper published in the volume 270 of the Journal of Environmental Management (2020) can be consulted in Appendix 1.

2.2. MATERIALS AND METHODS

2.2.1. Detection method selection

Consider the stoichiometric formula for the H_2/CO_2 conversion into methane:

$$4\,H_{2\,(g)} + CO_{2\,(g)} \rightarrow CH_{4\,(g)} + 2H_2O_{\,(l)} \qquad (2\text{-}1)$$

A decrease in the total number of moles associated with gaseous species is observed during the conversion, producing depletion in the headspace pressure when the reaction is carried out in a closed system. In the literature, two different strategies for hydrogenotrophic activity measurement have been reported. On one hand, Dolfing and Bloeman (1985) recommended a methodology based on the measurement of the pressure headspace combined with the composition analysis of the gas samples by gas chromatography (GC). When those conditions were tested, some drawbacks were identified: (i) Since headspace pressure decreased during the experience not only due to the H_2/CO_2 conversion to methane but also because of the gas sampling, vials needed to be re-pressurized with H_2/CO_2, increasing the safety risks, the gas loss and the length of the assay period. (ii) During the first period of the test, it was found that the detection limit was close to the concentration values obtained for methane when

analysing a gas sample using GC, increasing the relative experimental error associated with the first measures. On the other hand, Coates et al. (1996) proposed a methodology based on the measurement of headspace pressure. Pressure depletion was correlated with methane generation using the stoichiometric relation for the chemical conversion of H_2/CO_2 to methane expressed in Eq. (2-1).

The latter method was chosen for SHMA experiences since it was shown to be more practical, less time-consuming and safer than the method proposed by Dolfing and Bloeman (1985). Then, the moles of methane Δn_{CH4} (mol) produced from H_2/CO_2 during a certain interval of time Δt (days) could be calculated as follows:

$$\Delta n_{CH4} = -\Delta n_{gas}/4 = -\Delta P\, V_{hs}/(4\, R\, T) \quad (2\text{-}2)$$

where ΔP (atm) is the pressure depletion inside headspace; V_{hs} (L) is the headspace volume; R (L atm K^{-1} mol^{-1}) is the universal gas constant; and T (K) is the temperature.

2.2.2. Kinetic model

Considering the Monod kinetics for the microbial growth rate r_X (g_{VSS} L^{-1} d^{-1}):

$$r_X = \mu_m\, X\, S/(K_S + S) \quad (2\text{-}3)$$

where μ_m (d^{-1}) is the maximum specific growth rate; X (g_{VSS} L^{-1}) is the microbial concentration (biomass); S (g_{COD} L^{-1}) is the substrate concentration; and K_S (g_{COD} L^{-1}) is the half-saturation constant.

Then, the substrate consumption rate r_S (g_{COD} L^{-1} d^{-1}) is based on the microbial yield $Y_{X/S}$ (g_{VSS} g_{COD}^{-1}):

$$r_S = r_X/Y_{X/S} = \mu_m X S /[Y_{X/S} (K_S + S)] \quad (2\text{-}4)$$

Thus, the specific substrate consumption rate k_S (g_{COD} g_{VSS}^{-1} d^{-1}) can be expressed as:

$$k_S = r_S/X = r_X /(X Y_{X/S}) = \mu_m S/[Y_{X/S} (K_S + S)] \quad (2\text{-}5)$$

As a result, the expression for the maximum specific activity k_m (g_{COD} g_{VSS}^{-1} d^{-1}) corresponds to:

$$k_m = \mu_m/Y_{X/S} \quad (2\text{-}6)$$

To determine the maximum specific rate for substrate consumption applied to hydrogenotrophic methanogenesis ($k_{m,h}$), the design of the SHMA assay must accomplish the following conditions: (i) substrate concentration must be significantly higher than the half-saturation constant ($S>>K_S$), which means ‘zero-order conditions’; and (ii) microbial growth should be negligible ($X \sim X_0$; where ‘0’ subscript indicates initial conditions). In the hydrogenotrophic activity test, H_2 represents the limiting reagent since CO_2 is in excess due to the use of sodium hydrogen carbonate as the system buffer. For more details, please refer to Section 2 of Appendix 2. The volatile suspended solid (VSS) content is considered a representation of the biomass present in the biosludge.

2.2.3. Inoculum size calculation

The calculation of the amount of biosludge is crucial for a proper design of the duration of the batch assay. The volume aliquot V_B (L) of biosludge added to vial is calculated as:

$$V_B = \Delta n_{CH4}\, C/(\Delta t\, X\, k_{m,h}) \quad (2\text{-}7)$$

where C = 64 g_{COD} mol^{-1} is the conversion factor from moles of methane to grams of COD; X (g_{VSS} L^{-1}) is initially quantified by the VSS determined for the original biosludge sample; and $k_{m,h}$ (g_{COD} g_{VSS}^{-1} d^{-1}) is a preliminary value for SHMA based on historical data and considering the source reactor for the biosludge sample.

Two assumptions are considered for Eq. (2-7): (i) the SPECIFIC METHANE PRODUCTION (SMP) is constant over time and its production rate SHMA is equal to $k_{m,h}$; (ii) the growth during the test is negligible ($X \sim X_0$). The first assumption is accomplished when zero-order conditions are ensured. The second assumption relies on the microbial growth rate for hydrogenotrophic methanogens and the time-length of the batch test, which is associated with the Δt (days) and the number of planned measures. Then, Δn_{CH4} (mol) can be substituted by the expression in Eq. (2-2), obtaining the following expression for V_B (L) calculation:

$$V_B = -\Delta P\, V_{hs} C/(4\, R\, T\, \Delta t\, X\, k_{m,h}) \quad (2\text{-}8)$$

2.2.4. Incubation temperature selection

Activity tests should be carried out under conditions that closely mimic the environment of a given system, for example, temperature, pH and mixing (Angelidaki et al., 2007; Holliger et al., 2016). In the literature, it is possible to find the SMA tests for mesophilic biosludge at different temperatures: (i) 30°C (Gonzalez-Estrella et al., 2013; Karri et al., 2006); (ii) 35°C (Bhattad et al., 2017; Hao et al., 2017; Liu et al., 2016); and (iii) 37°C (Keating et al., 2016; McKeown et al., 2009; Regueiro et al., 2012). Modelling based on the Arrhenius equation, and experiences using VFA or acetate as substrate found in the literature, support the idea of a 50% increase in the SMA at 35°C compared to the SMA at 30°C (Lin et al., 1987; Rittmann and McCarty, 2020; Souto et al., 2010). In mild weather countries, the energy obtained from biogas is not enough to heat full-scale reactors treating diluted wastewater, then its operational temperature rarely exceeds 30°C. On the other hand, it is possible to heat systems treating concentrated wastewater or solid waste due to a positive energy balance and maintain the operational temperature close to the optimal temperature for the mesophilic microbial population (35 to 37°C). Pilot-scale reactors were operated at 30°C. Consequently, the incubation temperature was set at 30°C in the experiments presented in this work to establish correlations between SHMA and SAMA activities and the performance of the reactors analysed.

2.2.5. SHMA assay setup

Biosludge samples from pilot-scale and full-scale anaerobic reactors were assayed. SHMA tests were carried out in triplicate in SCHOTT® 250 mL glass bottles (henceforth referred to as ‘vials’, 308 mL of total volume). For each vial, the screw cap was equipped with a rubber O-ring to improve sealing and a screw nut, a rubber septum, a needle, and a three-way medical stopcock to allow initial gas flushing and pressure measurement. Vial sealing was previously tested by pressurizing each vial to 900 mmHg with N_2. Before the SHMA test, the biosludge sample was flushed with N_2 to displace O_2 inside a SCHOTT® vial (250 mL or 500 mL) and incubated in an air-thermostated chamber at 30°C until the remaining substrate was oxidized. The vial was monitored until methane production was negligible: after 3 days for washed samples from wastewater reactors, at least 7 days for samples from solid digesters; then, the exhausted biosludge was used for the SAMA and SHMA assays. If organic matter present in the inoculum is not eliminated, live blanks using N_2/CO_2 should be run and methane production discounted from the production recorded for each test vial.

The VSS content was determined following a standardized gravimetric method, using a Shimadzu Libror model AEX-200B analytical balance with a resolution of 1E-04 g (Rice et al., 2017). This method was used to determine the VSS content in the biosludge sample before the test, and then in the vials at the end of the test. The biosludge aliquot V_B was calculated using Eq. (2-8), assuming a pressure depletion of 100 mmHg

every 1 or 2 h to minimize the error associated with the pressure measurement. The aliquot of biosludge was dispensed and an aqueous solution of sodium hydrogen carbonate was dosed to reach 100 mL of total volume of liquid (V_L) and a pH of 7.8 to 8.0 in vials, before gas flushing with H_2/CO_2. The pH was expected to decrease after flushing because of the CO_2 dissolution. For initially neutral samples, a concentration of approximately 3 g $NaHCO_3$ L^{-1} was obtained inside the vials. A fourth vial was prepared under the same conditions as for the triplicate test samples to be sacrificed after gas flushing to verify that the pH was close to neutral when the acid-alkaline balance was restored. Next, the vials were pressurized at 900 mmHg (gauge pressure) with H_2/CO_2 (80/20) and depressurized three times to displace the oxygen from the headspace and for a further sealing test. After that, the vials were pressurized at 760 mmHg (gauge pressure) with H_2/CO_2 (80/20), start time was recorded and then vials were incubated in a New Brunswick™ Innova® model 2100 orbital platform shaker at 180 rpm, inside of an air-thermostated chamber at 30°C.

During the SHMA test, the gas pressure in the headspace was measured using a Sper Scientific® model PS100-2BAR pressure transducer (maximum range: 1500 mmHg, resolution: 2 mmHg) every 1 or 2 h according to the design. When necessary, the interval of time elapsed between the two measurements was re-adjusted after observing the biosludge performance during the first period of the test. At the end of the test, the pH was checked to determine whether it was in the optimal range

for the analysed anaerobic system. Additionally, the VSS content (X) was determined at the end of the test for each vial (Rice et al., 2017). Gas samples were taken from vials headspace at the end of the test to analyse its composition using a Shimadzu® gas chromatograph model GC-2014 equipped with a Restek® ShinCarbon model ST 100/120 2 m 1 mm ID 1/16" OD Silco packed column.

2.2.6. SHMA calculation

The pressure depletion ΔP (atm) was calculated with the recorded values of the gauge pressure and applied to determine Δn_{CH4} (mol) using Eq. (2-2) for each interval of time Δt (days) between two consecutive measures. The cumulative value n_{CH4} (mol) could be calculated for the methane produced during a time elapsed t (days), and then, the cumulative specific methane production SMP ($g_{COD}\, g_{VSS}^{-1}$) could be determined as:

$$SMP = n_{CH4}\, C/X\, V_L \quad (2\text{-}9)$$

where C = 64 g_{COD} mol^{-1} is the conversion factor from moles of methane to grams of COD, as in Eq. (2-2) to (2-7); V_L (L) is the volume of liquid inside the test vial. For this calculation, the final VSS determined for each vial at the end of the test is considered a better estimation for X (g_{VSS} L^{-1}) than the initial VSS of the sample, since the aliquot of biosludge might not conserve the original concentration after being dosed in the vial, especially for granular biosludge, and the testing for VSS is destructive.

Eq. (2-2) is valid for a constant ratio of partial pressures for CO_2 and H_2 (p_{CO2}:p_{H2}) of 1:4 and a negligible methane dissolved fraction. Due to acid-

base equilibria, the p_{CO2} is higher than predicted based on the stoichiometric formula (2-1), which implies a lower ΔP. Then, the methane production would be underestimated using Eq. (2-2). On the other hand, methane production is overestimated when assuming that carbon is exclusively converted to methane by not including the microbial yield $Y_{X/S}$ in Eq. (2-2). Since these effects are contraposed, the compensation would depend on the particular assay conditions and the assumed value for $Y_{X/S}$. Performing a comparison between the moles of methane calculated using Eq. (2-2) and the theoretical moles obtained considering the COD removed for methane, based on the microbial yield $Y_{X/S}$, the relative error values were +1.3% ($Y_{X/S}$ of 0.04 g_{COD} g_{COD}^{-1}) to -4.1% ($Y_{X/S}$ of 0.08 g_{COD} g_{COD}^{-1}). Even with the high uncertainties regarding $Y_{X/S}$, the resulting relative error was less than 5% in absolute terms. The experimental error for the SHMA assay was estimated to ponder the order of magnitude of these values (for more details, please consult Section 2 of Appendix 2).

A plot of the SMP (g_{COD} g_{VSS}^{-1}) versus t (days) was constructed for triplicate samples and a least-squares regression line was adjusted to data corresponding to each test vial and confidence interval was calculated for the hydrogenotrophic activity of each sample by using the Data Analysis ToolPak of Microsoft® Office Excel® 2016 Software. The ANOVA F-test was run to check the adequacy of zero-order model to fit data (see Subsection 2.2.2 Kinetic model') and the p-value determined for each test vial. The zero-order model was considered adequate when the p-value was

lower than 1E-3. Then, SHMA ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) was determined as the mean value of slopes calculated for trendlines corresponding to triplicate experiments, whether p-values were lower than 1E-3 for 5 observations. Data from vials with a p-value higher than 1E-3 was dismissed, and the source of discordance was studied.

2.2.7. SAMA assay setup

SAMA tests were carried out in triplicate at 30°C in SCHOTT® 100 mL glass bottles (137 mL of total volume) with an initial concentration of sodium acetate of 3 $g_{COD}\ L^{-1}$. The protocol was based on the methodology of headspace pressure measurement and determination of biogas composition proposed by Soto et al. (1993). These gas samples were analysed using a Shimadzu® gas chromatograph model GC-2014 equipped with a Restek® ShinCarbon model ST 100/120 2 m 1 mm ID 1/16” OD Silco packed column under the following conditions: temperature of injection port, packed column, and TCD were adjusted at 110 °C, current in TCD was set to 60 mA and argon N50 carrier was set at a flow rate of 21 mL min^{-1}.

2.3. RESULTS AND DISCUSSION

2.3.1. Verification of zero-order conditions for the SHMA assay

To verify the zero-order conditions for substrate consumption ($S>>K_S$), the concentration of H_2 dissolved in the aqueous bulk S ($g_{COD}\ L^{-1}$) was calculated at the beginning and the end of the assay. Considering the

solubilization coefficient for H_2 in pure water at 30°C, 1.7E-02 $cm^3\ cm^{-3}\ atm^{-1}$, and the partial pressure for H_2 inside the headspace at the beginning of the test, 1.6 atm absolute (which corresponds to a gauge pressure of 760 mmHg for the gas mixture of H_2/CO_2 80/20), an S_0 of 2.0E-02 $g_{COD}\ L^{-1}$ was obtained. SHMA assays were carried out until gauge pressure in headspace dropped to 200 mmHg. A percent of H_2 close to 60% was found inside headspace, which implied a concentration of dissolved H_2 of about 9.0E-03 $g_{COD}\ L^{-1}$.

Regarding K_S consideration, experimental values of K_S for H_2 consumption collected from the literature showed a wide variation, from 1.2E-07 to 2.1E-03 $g_{COD}\ L^{-1}$ (refer to Table A.1 in Section 1 of Appendix 2). External and internal mass transfer limitations for H_2 could lead to an overestimation of K_S (Giraldo-Gomez et al., 1992). When considering the external mass transfer, Coates et al. (1996) and Dolfing and Bloeman (1985) suggested that the mass transfer of H_2 from the headspace to the liquid could be rate-limiting, and they proposed that vigorous shaking could be applied during vial incubation as an improvement. In addition, the mass transfer from the liquid bulk to the biosludge surface was improved. In our laboratory, vials with a liquid to headspace volume ratio of 1:2 were incubated vertically in a New Brunswick™ Innova® model 2100 orbital shaker at 180 rpm; that was the maximum operative value without causing significant granule disruption in the biosludge samples assayed. When referring to the internal mass transfer, a dependence on the diffusion

phenomenon was observed, related to the physical properties of the biosludge. On this issue, Dolfing (1985) found a positive correlation between the size of the granules and apparent K_S determined using formate or acetate. Coates et al. (1996) reported a K_S of 3.6E-04 g_{COD} L^{-1} for granular biosludge at 37 °C. This value is one order of magnitude higher than the values for pure cultures and non-granular biosludge reported in the literature, even when considering different incubation temperatures. The value of K_S assumed in the ANAEROBIC DIGESTION MODEL NO. 1 (ADM1) development, 2.5E-05 g_{COD} L^{-1} at 35 °C in mesophilic high-rate systems (Batstone et al., 2002), is one order of magnitude lower than the value reported by Coates et al. (1996). Based on these considerations, the K_S value reported by Coates et al. (1996) was considered as a conservative value for granular sludge, when there was no external mass transfer limitation. The S values were two orders of magnitude higher than the K_S, considering a pressure depletion inside headspace from 760 to 200 mmHg (gauge pressure) during the assay. The test conditions ensured S values that were one order of magnitude higher, even compared with values of K_S that were affected by external mass transfer. When the influence of temperature over Ks was introduced, the results still indicating the accomplishment of the zero-order condition during the whole testing period; by performing a conservative calculation, a value of 1E-03 g_{COD} L^{-1} at 30°C was obtained (Donoso-Bravo et al., 2009; Lawrence and McCarty, 1969). Robinson and Tiedje (1982) obtained an K_S of 9.1E-05 g_{COD} L^{-1} for biosludge

from a solid digestor at 30°C, one order lower than the value obtained by Coates et al. (1996) for granular sludge, probably due to the difference in the structure of biosludge. Thus, it can be deduced the zero-order conditions accomplishment during the SHMA test from 760 to 200 mmHg (gauge pressure) for solid digesters biosludge (valid for non-granular sludge).

2.3.2. Verification of zero-order conditions for the SAMA assay

At the beginning of the SAMA batch test, the concentration of sodium acetate was 3 g_{COD} L^{-1} inside each vial. By the end of the test, the concentration of acetate in each vial was higher than 1 g_{COD} L^{-1}. Thus, the kinetics of Monod for microbial growth at zero-order conditions were accomplished when a half-saturation constant for acetate of 0.05 to 0.30 g_{COD} L^{-1} was considered for mesophilic acetoclastic *archaea* (Batstone et al., 2002), obtaining the maximum specific activity for the acetoclastic methanogenesis (see Eqs. (2-3) to (2-6) applied to this step).

2.3.3. Microbial growth

Based on the design proposed for the SHMA assay, a range of headspace pressures from 760 to 200 mmHg and an interval of time between measurements of 1.5 or 2 h were suitable for full data recording; the time elapsed to fulfil the test was 8 to 10 h. In most cases, the aliquot V_B contained from 500 to 800 mg of VSS depending on the biosludge characteristics. Microbial growth during the test was estimated in 0.8 to 1.2% of the initial VSS dosed in vials when considering an $Y_{X/S}$ of 0.06 g_{COD}

g_{COD}^{-1} for the hydrogenotrophic population (Batstone et al., 2002); therefore, the effect of microbial growth over VSS was negligible when comparing to experimental error for SHMA assay (for more details about experimental error see Section 2 of Appendix 1). Also, cell decay is negligible since it is considered to be 10% of the cell growth (from ADM1) (Batstone et al., 2002).

2.3.4. Experimental and literature values for SAMA and SHMA

The temperature inside the reactor, the type of substrate and the organic loading rate OLR (g_{COD} g_{VSS}^{-1} d^{-1}) applied, and the presence of inhibitors are among the factors that shape the microbial population, affecting the relative abundance and activity of the acetoclastic and hydrogenotrophic microorganisms. In this work, biosludge samples from eight different anaerobic reactors were analysed to determine their SAMA and SHMA. For SHMA assays, plots showing the specific methane production accumulated SMP (g_{COD} g_{VSS}^{-1}) over time t (d) for the different biosludge samples are presented in Figure 2-1 and Figure 2-2. Standard deviations associated with triplicate measurements were between 3 and 10% for SHMA and between 3 and 12% for SAMA. Whether one slope calculated from these graphs is significantly higher and has no concordance with the other two, the corresponding vial is under suspicion of gas loss. Therefore, it is advisable to test the sealing of the suspect vial to rule out this problem. When adjusting data graphed in Figure 2-1 and Figure 2-2 by least-squares regression, the R^2 values obtained were between 0.9924 and 0.9999, except for the vial 3 of sample (h) (R^2 of 0.9594). The ANOVA F-

test was performed for data from each vial. The p-values of the F-test were lower than 1E-3 (from 4E-6 to 9E-4), except for the vial 3 of sample (h) (p-value of 0.02). These provide evidence of an adequate fit to the zero-order kinetic model, accordingly with verification performed previously based on the K_S and S magnitudes (see Subsection 2.3.1 Verification of zero-order conditions for the SHMA assay). The VSS concentration inside the vial 3 of the sample (h) doubles the VSS concentration inside the other two vials (vial 1 and 2). The last two measures were under 200 mmHg, and the zero-order model was unaccomplished. Then, only the slopes from vials 1 and 2 were considered in the calculation of SHMA for sample (h). The confidence intervals were between ±2.0% and ±8.2% for samples (a) to (g) for a confidence level α of 95%; on the other hand, sample (h) had a confidence interval of ±16.6%. The error associated with the application of Eq. (2-2) for methane production calculation was on the same order of magnitude as the experimental error involved in the SHMA assay (refer to Subsection 2.2.6 of the present text and Section 2 of Appendix 2).

The results for these methanogenic activity tests, as well as the organic loading rate OLR and the SPECIFIC METHANE PRODUCTION RATE in each reactor SMPR ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$), when available, are presented in Table 2-1. The OLR per gram of VSS (henceforth 'OLR') is used instead of per volume since it allows for distinguishing between reactors with the same working volume but with different amounts of biomass. The SMPR was calculated considering the methane production and VSS in the reactor.

Additionally, the SMPR could be determined using the specific OLR, the efficiency of COD removal observed in the reactor and assuming the value of microbial yield of 0.10 $g_{COD}\ g_{COD}^{-1}$ for complex substrates and 1.42 $g_{COD}\ g_{VSS}^{-1}$ for microbial cells (from ADM1) (Batstone et al., 2002).

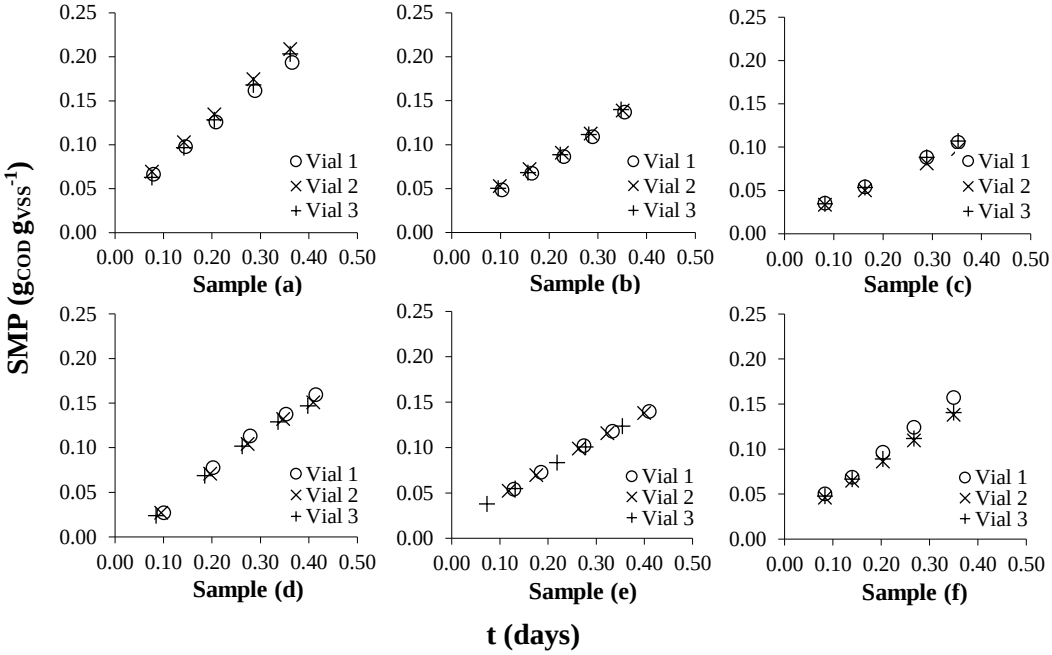

Figure 2-1. Specific methane production (SMP) accumulated during the SHMA assay: (a) Dairy full-scale UASB; (b) Dairy pilot-scale EGSB; (c) Beverage pilot-scale EGSB; (d) Brewery full-scale IC; (e) Beverage bench-scale EGSB; and (f) Protein bench-scale EGSB

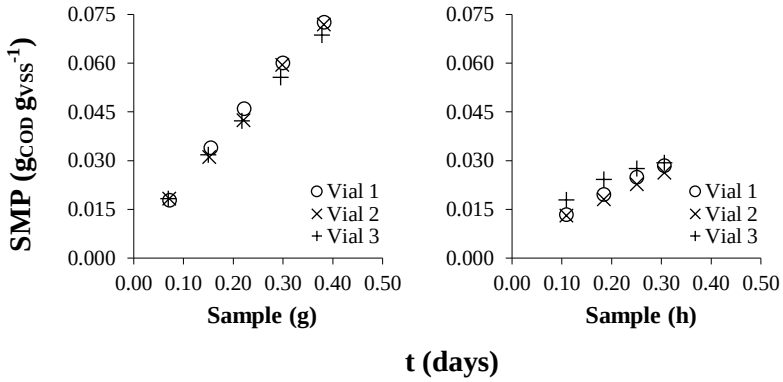

Figure 2-2. Specific Methane Production accumulated during the SHMA assay for biosludge with low activity: (g) Slaughterhouse lagoon; and (h) Manure bench-scale CSTR

In Table 2-1, samples indicated with (a) to (f) correspond to granular biosludge; sample (g) corresponds to a flocculent sludge from an anaerobic lagoon and sample (h) to a solids digester. For samples (b), (e) and (h) shown in Table 2-1, the SMPR ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) observed in the reactor was higher than the corresponding value for SAMA ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$), even though activities were measured at optimal conditions. The former evidenced that the SAMA gave insufficient information about the biological characteristics of the biosludge to explain the performance observed in the reactor; hence, the relevance of including both methanogenic activities SAMA and SHMA in the study to consider the acetoclastic and hydrogenotrophic methanogenesis. When comparing the SAMA and SHMA ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) from Table 2-1, for samples (c) and (f), the SHMA values were lower than those for SAMA, representing 85 to 93% of SAMA. Even in these samples it is still relevant to study both activities. In the other cases, the SHMA was higher than the SAMA; for example, SHMA was double the SAMA for samples (a) and (h). Due to the source of sample (g), an anaerobic lagoon, biosludge had a higher age than samples from high-rate reactors, and the OLR applied is lower. The higher concentration of inert organic material dilutes microbial fraction in the total VSS, producing lower activities (both SAMA and SHMA) than the activities observed for high-rate wastewater reactors, as in samples (a) to (f) of Table 2-1. Additionally, this effect can also be seen by comparing the slopes of the graphs presented in Figure 2-1 and Figure 2-2.

Table 2-1. SAMA and SHMA at 30°C obtained for anaerobic biosludge from different sources and operational data. The standard deviation is reported between brackets for the triplicate test samples, except for sample (h) marked with (*) that corresponds to duplicates

Biosludge sample ID	SAMA ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	SHMA ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	OLR ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	SMPR ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)
(a) Dairy full-scale UASB	0.22 (0.02)	0.48 (0.03)	0.18 (0.03)	0.14 (0.03)
(b) Dairy pilot-scale EGSB	0.28 (0.01)	0.35 (0.01)	0.48 (0.05)	0.41 (0.06)
(c) Beverage pilot-scale EGSB	0.27 (0.01)	0.26 (0.01)	0.19 (0.03)	0.15 (0.03)
(d) Brewery full-scale IC	0.34 (0.04)	0.41 (0.01)	0.26 (0.04)	0.19 (0.04)
(e) Beverage bench-scale EGSB	0.27 (0.01)	0.31 (0.01)	0.35 (0.03)	0.30 (0.03)
(f) Protein bench-scale EGSB	0.54 (0.02)	0.48 (0.03)	0.47 (0.05)	0.39 (0.05)
(g) Slaughterhouse lagoon	0.091 (0.003)	0.159 (0.007)	< 0.20	< 0.15
(h) Manure bench-scale CSTR	0.031 (0.003)	0.072 (0.007)(*)	0.08 (0.01)	0.05 (0.01)

In Table 2-2, activity values from the literature are summed. Standard deviations obtained by Bhattad et al. (2017), Gonzalez-Estrella et al. (2013), Hao et al. (2017), Keating et al. (2016), and McKeown et al. (2009) are similar to those obtained in our laboratory for SAMA and SHMA. However, in the other cases shown in Table 2-2, values for the standard deviation of approximately 20% are observed. The assay temperature must be considered when comparing the activity values from Table 2-1 and Table 2-2. When the incubation temperature Is raised in a suitable range for the microbial population, the specific substrate consumption rate is expected to increase. In most cases of Table 2-2, SHMA and SAMA were carried out at 37°C, leading to an increase in the biosludge activity when compared to the values obtained at 30°C. The kinetics variation for SAMA and SHMA with temperature could differ between each other, producing changes in the SHMA:SAMA ratio when the temperature increased from 30 to 37°C.

Table 2-2. SAMA and SHMA values for mesophilic anaerobic systems found in the literature. Standard deviation is reported between brackets for the triplicate test samples. References: [1] Molina et al. (2008), [2] McKeown et al. (2009), [3] Regueiro et al. (2012), [4] Gonzalez-Estrella et al. (2013), [5] Liu et al. (2016), [6] Keating et al. (2016), [7] Bhattad et al. (2017), [8] Hao et al. (2017). Reference of the SHMA protocol applied: (I) Coates et al. (1996); (II) Soto et al. (1993); (III) Internal

Biosludge Sample	Type of waste	T (°C)	SAMA ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	SHMA ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	Ref.
Lab-scale UASB	Ethanol-based	37	0.44 (0.10)	1.10 (0.20)	[1](III)
Lab-scale UASB	Carbohydrate	37	0.72 (0.08)	1.87 (0.04)	
Lab-scale UASB	Protein-based	37	0.57 (0.06)	1.87 (0.36)	
Full-scale IC	Alcohol	37	0.223 (0.003)	0.146 (0.006)	[2](I)
Lab-scale EGSB.AF	VFA	37	1.06 (0.08)	0.85 (0.03)	
Full-scale CSTR	Sewage sludge	37	0.15 (0.01)	0.55 (0.15)	[3](II)
Full-scale UASB	Brewery	37	0.33 (0.05)	0.79 (0.04)	
Full-scale CSTR	Dairy/fish	37	0.29 (0.02)	0.84 (0.08)	
Lab-scale CSTR	Glycerine/pig manure	37	0.01 (0.00)	0.37 (0.07)	
Full-scale CSTR	Sugar process	37	0.23 (0.02)	0.45 (0.06)	
Full-scale CSTR	Yeast process	37	0.05 (0.02)	0.83 (0.08)	
Full-scale UASB	Brewery	30	0.32 (0.03)	0.57 (0.03)	[4](III)
Lab-scale CSTR high TS	WWTP Sludge	35	0.027 (0.001)	0.018 (0.001)	[5](III)
Lab-scale CSTR low TS	WWTP Sludge	35	0.054 (0.001)	0.021 (0.004)	
Lab-scale hybrid FF	Sewage-based	37	0.50 (0.07)	0.91 (0.10)	[6](I)
Lab-scale CSTR	Not-fat-milk	35	0.21 (0.01)	0.72 (0.07)	[7](I)
Full-scale CSTR	WWTP Sludge	35	0.014 (0.005)	0.045 (0.002)	[8](I)

Analyzing the activity values presented in Table 2-2, it can be noted that there are cases where the SHMA was lower than the SAMA, from 39 to 80% of SAMA (Liu et al., 2016; McKeown et al., 2009). However, there are examples of the SHMA being higher than the SAMA in Table 2-2, in some cases by one order of magnitude (Gonzalez-Estrella et al., 2013; Molina et al., 2008; Regueiro et al., 2012).

When considering biosludge from solids digesters, the microbial fraction in the total VSS is diluted by the presence of inert materials and

substrate; thus, in Table 2-1, sample (h) shows lower methanogenic activities than samples (a) to (f), in some cases by one order of magnitude. SAMA and SHMA for sample (h) are of the same order of magnitude as the values reported by Liu et al., as seen in Table 2-2 (Liu et al., 2016). It is also important to note that the retention time for the biosludge and the OLR in solid digesters are markedly different from those in wastewater reactors. Therefore, data from solid digesters and wastewater reactors should be analysed separately. During the design of the SHMA test for samples (g) and (h), the aliquot of biosludge V_B determined using Eq. (2-8) implied concentrations of VSS in vials of 16 g_{VSS} L^{-1} and 35 g_{VSS} L^{-1} respectively, significantly higher than the ones suggested by Coates et al. (1996) (1.7 to 8 g_{VSS} L^{-1}). Based on this design, the time elapsed in the SHMA assay of these samples was 7 to 10 h.

2.3.5. Future research

For the SHMA calculation, the conversion of H_2/CO_2 to methane was supposed to be carried out by hydrogenotrophic archaea. The contribution of homoacetogenic population was considered negligible (Coates et al., 1996). This assumption must be verified. Selective inhibitors could be incorporated into batch activity tests to puzzle down the different steps involved in methanogenesis, including acetoclastic and hydrogenotrophic methanogenesis, homoacetogenesis, and syntrophic oxidation of acetate. Similarly, syntrophic acetate oxidation must be considered when assessing SAMA. The standardization of this type of technique (SHMA and SAMA) is

crucial to compare results between different reactors and laboratories. Inter-laboratory experiences would enrich the discussion and provide a basis to analyse the influence of different variables and consider variations between available equipment to achieve comparable conditions. As an example, external mass transfer limitations should be avoided or made comparable. Additionally, further studies must be performed to determine the influence of temperature on the SHMA and SAMA. This information would provide a basis to weight this influence and decide whether a standardized temperature must be defined and become widely used in the scientific community. Also, it is necessary to adjust the SHMA technique for biosludge from psychrophilic and thermophilic reactors since kinetic parameters could vary significantly with changes in the temperature, especially associated with changes in the microbial population.

2.4. CONCLUSIONS

This work presented full calculation and laid a basis for a discussion of inter-laboratory work and a standardization of the SHMA technique. The equation proposed to size inoculum made the SHMA determination possible for low activity biosludge within 7 to 10 hours, as well as for biosludge from high-rate reactors, with negligible microbial growth. The zero-order model showed an adequate fit to data, and hypothesis simplifications used for the calculation of methane production during the SHMA test introduced an error comparable to the experimental error. SHMA, together with SAMA, gives a

better explanation to the performance of the biosludge in the reactors (represented by the parameter of specific methane production rate in the reactor, SMPR).

REFERENCES OF CHAPTER 2

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, L., Guwy, A., Jenicek, P., Kalyuzhnui, S., van Lier, J., 2007. Anaerobic biodegradation, activity and inhibition (ABAI) Task Group Meeting 9th to 10th October 2006, in Prague. Kgs. Lyngby: Institute of Environment & Resources, Technical University of Denmark.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. Biotechnol. Adv. 36, 452–466. https://doi.org/10.1016/j.biotechadv.2018.01.011

Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S. V., Pavlostathis, S.G., Rozzi, a., Sanders, W.T., Siegrist, H., Vavilin, V. a., 2002. The IWA Anaerobic Digestion Model No 1 (ADM1). Water Sci. Technol. 45, 65–73.

Bhattad, U., Venkiteshwaran, K., Cherukuri, K., Maki, J.S., Zitomer, D.H., 2017. Activity of methanogenic biomass after heat and freeze drying in air. Environ. Sci. Water Res. Technol. 3, 462–471. https://doi.org/10.1039/c7ew00049a

Bi, S., Westerholm, M., Qiao, W., Xiong, L., Mahdy, A., Yin, D., Song, Y., Dong, R., 2020. Metabolic performance of anaerobic digestion of chicken manure under wet, high solid, and dry conditions. Bioresour. Technol. 296, 122342. https://doi.org/10.1016/j.biortech.2019.122342

Coates, J.D., Coughlan, M.F., Colleran, E., 1996. Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. J. Microbiol. Methods 26, 237–246. https://doi.org/10.1016/0167-7012(96)00915-3

Dolfing, J., 1985. Kinetics of methane formation by granular sludge at low substrate concentrations - The influence of mass transfer limitation. Appl. Microbiol. Biotechnol. 22, 77–81. https://doi.org/10.1007/BF00252160

Dolfing, J., Bloeman, W.G.B.M., 1985. Acitivity measurements as a tool to characterize the microbial composition of methanogenic environments. J. Microbiol. Methods 4, 1–12. https://doi.org/10.1016/0167-7012(85)90002-8

Donoso-Bravo, a., Retamal, C., Carballa, M., Ruiz-Filippi, G., Chamy, R., 2009. Influence of temperature on the hydrolysis, acidogenesis and methanogenesis in mesophilic anaerobic digestion: Parameter identification and modeling application. Water Sci. Technol. 60, 9–17. https://doi.org/10.2166/wst.2009.316

Fotidis, I.A., Karakashev, D., Kotsopoulos, T.A., Martzopoulos, G.G., Angelidaki, I., 2012. Effect of ammonium and acetate on methanogenic pathway and methanogenic community composition. FEMS Microbiol. Ecol. 83, 38–48. https://doi.org/10.1111/j.1574-6941.2012.01456.x

Giraldo-Gomez, E., Goodwin, S., Switzenbaum, M.S., 1992. Influence of mass transfer limitations on determination of the half saturation constant for hydrogen uptake in a mixed-culture CH4-producing enrichment. Biotechnol. Bioeng. 40, 768–776. https://doi.org/10.1002/bit.260400704

Gonzalez-Estrella, J., Sierra-Alvarez, R., Field, J.A., 2013. Toxicity assessment of inorganic nanoparticles to acetoclastic and hydrogenotrophic methanogenic activity in anaerobic granular sludge. J. Hazard. Mater. 260, 278–285. https://doi.org/10.1016/j.jhazmat.2013.05.029

Hao, X., Liu, R., Loosdrecht, M. Van, Cao, D., 2017. Batch influences of exogenous hydrogen on both acidogenesis and methanogenesis of excess sludge. Chem. Eng. J. https://doi.org/10.1016/j.cej.2017.02.093

Ho, D.P., Jensen, P.D., Batstone, D.J., 2013. Methanosarcinaceae and Acetate-Oxidizing Pathways Dominate in High-Rate Thermophilic Anaerobic Digestion of Waste-Activated Sludge. Appl. Environ. Microbiol. 79, 6491–6500. https://doi.org/10.1128/AEM.01730-13

Holliger, C., Alves, M., Andrade, D., Angelidaki, I., Astals, S., Baier, U., Bougrier, C., Buffière, P., Carballa, M., De Wilde, V., Ebertseder, F., Fernández, B., Ficara, E., Fotidis, I., Frigon, J.C., De Laclos, H.F., Ghasimi, D.S.M., Hack, G., Hartel, M., Heerenklage, J., Horvath, I.S., Jenicek, P., Koch, K., Krautwald, J., Lizasoain, J., Liu, J., Mosberger, L., Nistor, M., Oechsner, H., Oliveira, J.V., Paterson, M., Pauss, A., Pommier, S., Porqueddu, I., Raposo, F., Ribeiro, T., Pfund, F.R., Strömberg, S., Torrijos, M., Van Eekert, M., Van Lier, J., Wedwitschka, H., Wierinck, I., 2016. Towards a standardization of biomethane potential tests. Water Sci. Technol. 74, 2515–2522. https://doi.org/10.2166/wst.2016.336

Jiang, Y., Banks, C., Zhang, Y., Heaven, S., Longhurst, P., 2018. Quantifying the percentage of methane formation via acetoclastic and syntrophic acetate oxidation pathways in anaerobic digesters. Waste Manag. 71, 749–756. https://doi.org/10.1016/j.wasman.2017.04.005

Karri, S., Sierra-Alvarez, R., Field, J.A., 2006. Toxicity of copper to acetoclastic and hydrogenotrophic activities of methanogens and sulfate reducers in anaerobic sludge. Chemosphere 62, 121–127. https://doi.org/10.1016/j.chemosphere.2005.04.016

Keating, C., Chin, J.P., Hughes, D., Manesiotis, P., Cysneiros, D., Mahony, T., Smith, C.J., Mcgrath, J.W., Flaherty, V.O., 2016. Biological Phosphorus Removal During High-Rate, Low-Temperature, Anaerobic Digestion of Wastewater. Front. Microbiol. 7, 1–14. https://doi.org/https://doi.org/10.3389/fmicb.2016.00226

Kougias, P.G., Angelidaki, I., 2018. Biogas and its opportunities—A review. Front. Environ. Sci. Eng. 12, 14. https://doi.org/10.1007/s11783-018-1037-8

Lawrence, A.W., McCarty, P.L., 1969. Kinetics of Methane Fermentation in Anaerobic Treatment. J. Water Pollut. Control Fed. 41, R1–R17.

Lin, C.Y., Noike, T., Sato, K., Matsumoto, J., 1987. Temperature Characteristics of the Methanogenesis Process in Anaerobic Digestion. Water Sci. Technol. 19, 299–300. https://doi.org/10.2166/wst.1987.0210

Liu, C., Li, H., Zhang, Y., Chen, Q., 2016. Characterization of methanogenic activity during high-solids anaerobic digestion of sewage sludge. Biochem. Eng. J. 109, 96–100. https://doi.org/10.1016/j.bej.2016.01.010

McKeown, R.M., Scully, C., Mahony, T., Collins, G., O'Flaherty, V., 2009. Long-term (1243 days), low-temperature (4–15 8C), anaerobic biotreatment of acidified wastewaters: Bioprocess performance and physiological characteristics 43, 1611–1620. https://doi.org/10.1016/j.watres.2009.01.015

Molina, F., García, C., Roca, E., Lema, J.M., 2008. Characterization of anaerobic granular sludge developed in UASB reactors that treat ethanol, carbohydrates and hydrolyzed protein based wastewaters. Water Sci. Technol. 57, 837–842. https://doi.org/10.2166/wst.2008.067

Oosterkamp, M.J., Bauer, S., Ibáñez, A.B., Méndez-García, C., Hong, P.Y., Cann, I., Mackie, R.I., 2019. Identification of methanogenesis and syntrophy as important microbial metabolic processes for optimal thermophilic anaerobic digestion of energy cane thin stillage. Bioresour. Technol. Reports 7, 100254. https://doi.org/10.1016/j.biteb.2019.100254

Regueiro, L., Veiga, P., Figueroa, M., Alonso-Gutierrez, J., Stams, A.J.M., Lema, J.M., Carballa, M., 2012. Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters. Microbiol. Res. 167, 581–589. https://doi.org/10.1016/j.micres.2012.06.002

Rice, E.W., Baird, R.B., Eaton, A.D. (Eds.), 2017. Standard Methods for the Examination of Water and Wastewater, 23rd ed. APHA, AWWA, WEF, Washington DC, USA.

Rittmann, B.E., McCarty, P.L., 2020. Environmental biotechnology: principles and applications, 2nd ed. McGraw-Hill, New York.

Robinson, J.A., Tiedje, J.M., 1982. Kinetics of hydrogen consumption by rumen fluid, anaerobic digestor sludge, and sediment. Appl. Environ. Microbiol. 44, 1374–1384. https://doi.org/10.1128/aem.71.11.7483-7492.2005

Soto, M., Mendez, R., Lema, J.M., 1993. Methanogenic and non- methanogenic activity tests: theoretical basis and experimental set up. Water Res. 27, 1361–1376.

Souto, T.F., Aquino, S.F., Silva, S.Q., Chernicharo, C. a L., 2010. Influence of incubation conditions on the specific methanogenic activity test. Biodegradation 21, 411–424. https://doi.org/10.1007/s10532-009-9311-x

van Loosdrecht, M.C., Nielsen, P.H., Lopez-Vazquez, C.M., Brdjanovic, D., 2016. Experimental Methods in Wastewater Treatment. IWA Publishing, London.

Westerholm, M., Moestedt, J., Schnürer, A., 2016. Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance. Appl. Energy 179, 124–135. https://doi.org/10.1016/j.apenergy.2016.06.061

CHAPTER 3

PATHWAYS STUDY BASED ON METHANOGENIC ACTIVITIES TESTS

CHAPTER 3. PATHWAYS STUDY BASED ON ACTIVITY TESTS

3.1. INTRODUCTION

The four bioreactions involved in the last step of the AD process and the corresponding free energy are detailed in Figure 3-1. Blue circles include chemical species associated with methane formation from acetate, identified as r_1 (acetoclastic methanogenesis); whereas, red circles mark chemical species involved in methane formation from H_2/CO_2, referred to as r_2 (hydrogenotrophic methanogenesis). On the other hand, green boxes include chemical species associated with acetate oxidation to H_2/CO_2, noted as r_3 (SAO), and its reverse reaction, acetate formation from H_2/CO_2, identified as r_4 (homoacetogenesis). Note that r_3 is an endergonic reaction at standard conditions (positive $\Delta G°'$). In anaerobic environments, r_3 and r_2 occur together to obtain an exergonic global reaction from acetate to methane (Zinder, 1993; Zinder and Koch, 1984). Therefore, the term 'syntrophy' applies to the relationship between bacteria performing SAO (SAOB) and hydrogenotrophic methanogens, hence the difficulty of isolating SAOB in the laboratory (Müller et al., 2016; Wei et al., 2020).

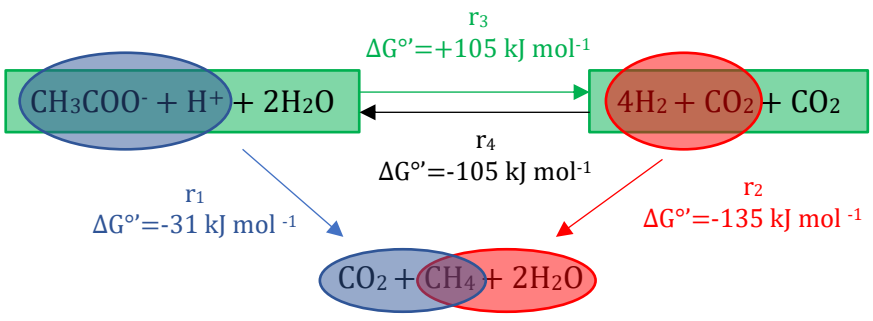

Figure 3-1. Free energy of reactions in the last step of the AD process (Zinder, 1993)

As explained in Chapter 2, a first step forward understanding the relevance of acetoclastic and hydrogenotrophic microorganisms involved in methane formation is to measure their specific activities, SAMA and SHMA, associated with r_1 and r_2 respectively (Colleran et al., 1992; Soto et al., 1993). Nevertheless, as r_3 and r_4 could be also present, a fraction of acetate could be converted into H_2/CO_2 and viceversa, during the activity tests. Consequently, the methane produced in SAMA and SHMA tests could be the addition of the methane produced by both reactions, r_1 and r_2. In this context, decoupling the four steps is necessary to determine the importance of these reactions in the methane formation under different conditions. When acetoclastic methanogens are inhibited or under extreme conditions, SAO-hydrogenotrophic (SAO-HM) pathway, associated to r_3+r_2, becomes relevant and the presence of SAOB crucial for recovering systems performance (Ho et al., 2013; Oosterkamp et al., 2019; Shimada et al., 2011; Westerholm et al., 2016). During this thesis, a case of one bench-scale EGSB reactor treating beverage-based wastewater was studied where SAMA decreased by 20% due to AGV inhibition, and SHMA increased by 35%. Despite the good hydrogenotrophic activity, the reactor lowered its performance by almost 35%. For more details, please consult Appendix 3 containing full paper exposed orally in XIII Latin American Workshop and Symposium on Anaerobic Digestion, Medellín (2018). Given the literature reports about the SAO-HM pathway being relevant in case of inhibition, questions were raised regarding the presence and role of SAOB

in this reactor, and SAMA and SHMA were not enough to answer them. Then, complementary tools are needed to describe the behavior of the reactor and answer these questions. In this context, an inhibitor of the acetoclastic methanogenesis can be used in the SAMA test to study the SAO-HM pathway.

On the other hand, homoacetogenesis (r_4) is considered relevant for the degradation of some substrates (e.g. butyrate) under suppressed methanogenesis (Siriwongrungson et al., 2007). Despite this, Xu et al. (2010) showed that when methanogenic pathways r_1 and r_2 were working properly, r_4 was disadvantaged during the anaerobic degradation of the remaining substrate in a mixture of mesophilic biosludge, due to the low absolute value of the ΔG of the homoacetogenic reaction compared to the energy required for the synthesis of 1/3 ATP. However, in the presence of an inhibitor of the methanogenesis, Xu et al. (2010) found that gauge pressure increased until 1000 Pa during batch tests, and homoacetogenesis occurred. According to the design proposed in Chapter 2 for the SHMA test, gauge pressures handled are higher than 1000 Pa; then, based on the report of Xu et al. (2010), r_4 might be feasible, and r_4+r_1 could distort results obtained from SHMA tests that will not exclusively correspond to the hydrogenotrophic methanogen population (r_2). By the contrary, reports from Coates et al. (1996) and Ryan et al. (2008) suggest that the performance of homoacetogens became noticeable hours after the end of the SHMA test, about 60 h for a 12-hour test; therefore, it would not have an impact on the

determination of activity. Ryan et al. (2008) associated this phenomenon with a delay in the adaptation of homoacetogens. Nevertheless, given the diversity of homoacetogenic bacteria and its low doubling-times between 1.75 and 29 h according to Noike and Li (1989) and Schink (1994), these reports might not universalizable. Although homoacetogenesis seems not to be relevant in the operating conditions of reactors, it could be so in the SHMA test. Then, its contribution should be discounted to obtain kinetic parameters corresponding exclusively to hydrogenotrophic methanogens, to make adequate modeling of reactors. The same applies to obtain kinetics for acetoclastic methanogens from the SAMA test, where it is necessary to discount the influence of SAO-HM to feedback the AD-model with appropriate parameters for each microbial population. The objective of the present work was obtaining correction factors to discount the influence of SAO-HM and homoacetogenesis from SAMA and SHMA values determined in routine tests without inhibitors. Another objective was to determine the fraction of biomass associated with each methanogenic population. These objectives respond to the aim to generate valuable kinetic parameters for methanogens and feed the modeling of reactors.

In this context, the 2-Bromoethanesulfonate (BES) can be applied as a specific inhibitor of the methanogenesis since it is an analog of the coenzyme M (2-mercaptoethanesulfonate) involved in the reduction of methyl-coenzyme M to methane (Gunsalus et al., 1978). Some acetoclastic methanogens lack a protective envelope and are easily penetrated by

toxicants; whereas, some hydrogenotrophic methanogens can synthesize coenzyme M and exhibit lower rates of transport of external coenzyme M into the cell (Gerardi, 2003; Xu et al., 2010). Then, BES is expected to inhibit acetoclastic methanogens at lower concentrations than in the case of hydrogenotrophic methanogens. On the other hand, the reactions r_3 and r_4 would occur in the presence of BES since there are performed by *bacteria* and not by *archaea*, and *bacteria* are not inhibited by using BES.

In SAMA tests, acetate is added at the beginning of the test (indicated with green-colored boxes in Schemes #1 to #3 from Figure 3-2); then, acetate could be consumed through both pathways r_1 and r_3+r_2, as illustrated Scheme #1 from Figure 3-2. It is necessary to inhibit acetoclastic archaea to weigh the influence of r_3+r_2 on acetate consumption. BES concentrations inhibitory for acetoclastic *archaea* (r_1) without affecting hydrogenotrophs (r_2) are represented with the 'level 1' in Scheme #2 from Figure 3-2. In both Schemes #1 and 2, H_2 and CO_2 are intermediate compounds (enclosed in dotted line boxes), which ultimately lead to methane formation, keeping H_2 pressure low, since r_3 and r_2 occur together in symphony. By applying BES at inhibitory concentrations for hydrogenotrophic *archaea*, r_2 is canceled, preventing the occurrence of r_3 due to its positive ΔG. In this situation of complete inhibition of methanogens, represented as 'level 2' in Scheme #3, no methane production nor acetate consumption is expected.

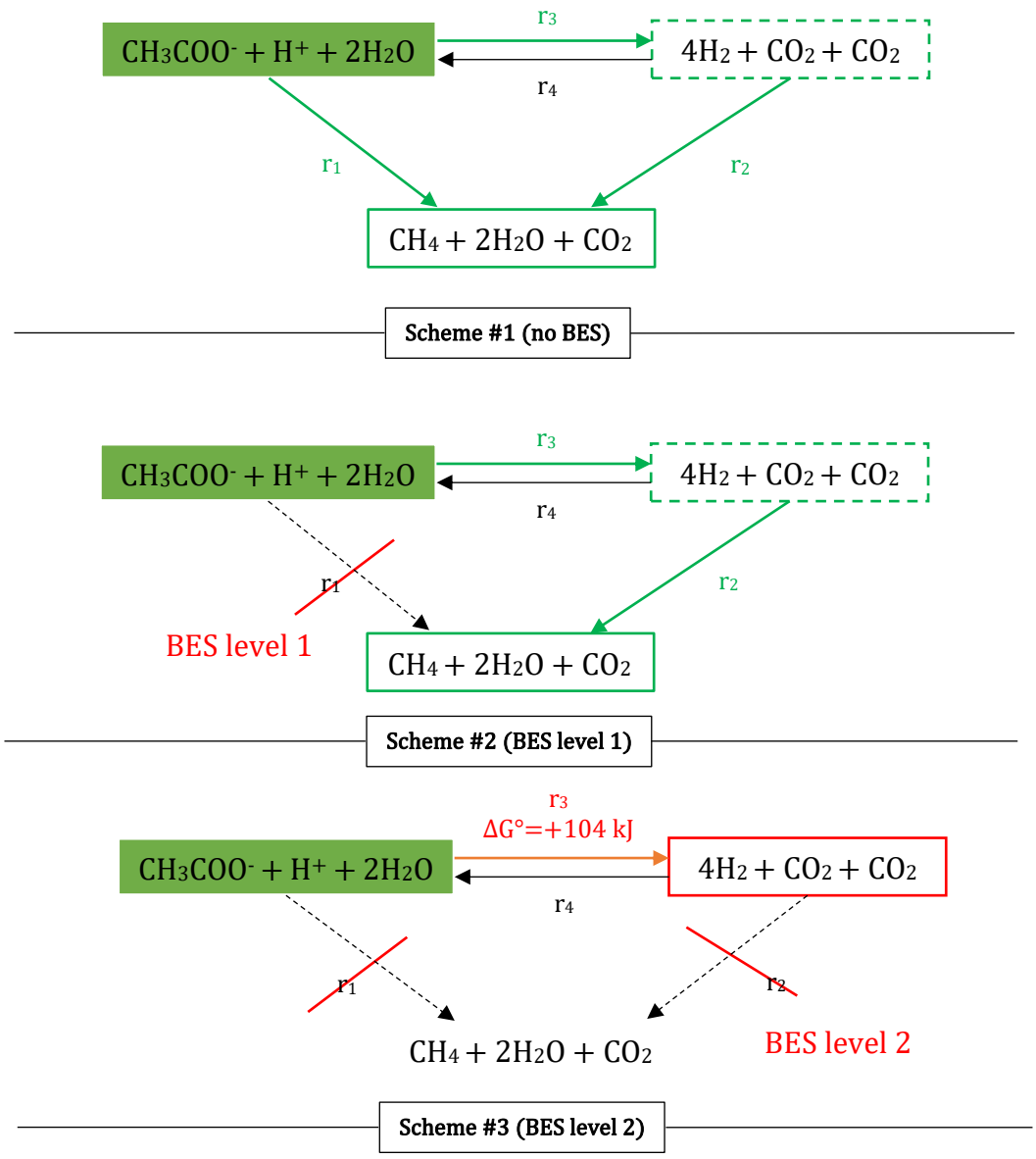

Figure 3-2. Schemes of reactions for SAMA test: # 1 in the absence of BES; # 2 for a BES concentration at level 1 with inhibited acetoclastic archaea; and # 3 for a BES concentration at level 2 with inhibited hydrogenotrophic archaea

Similarly, schemes #4 to #6 are presented for SHMA tests in Figure 3-3. During the SHMA assay, H_2/CO_2 could be potentially consumed by both pathways r_2 and r_4+r_1, as shown in Scheme #4. When applying BES at inhibitory concentrations for acetoclastic *archaea* (level 1), r_1 is prevented

from occurring, acetate accumulated due to r_4 and methane formed through r_2 (Scheme #5). Then, for BES concentrations corresponding to complete inhibition of methanogens (level 2), only r_4 occurs, consuming H_2/CO_2 and accumulating acetate with no methane production (Scheme #6).

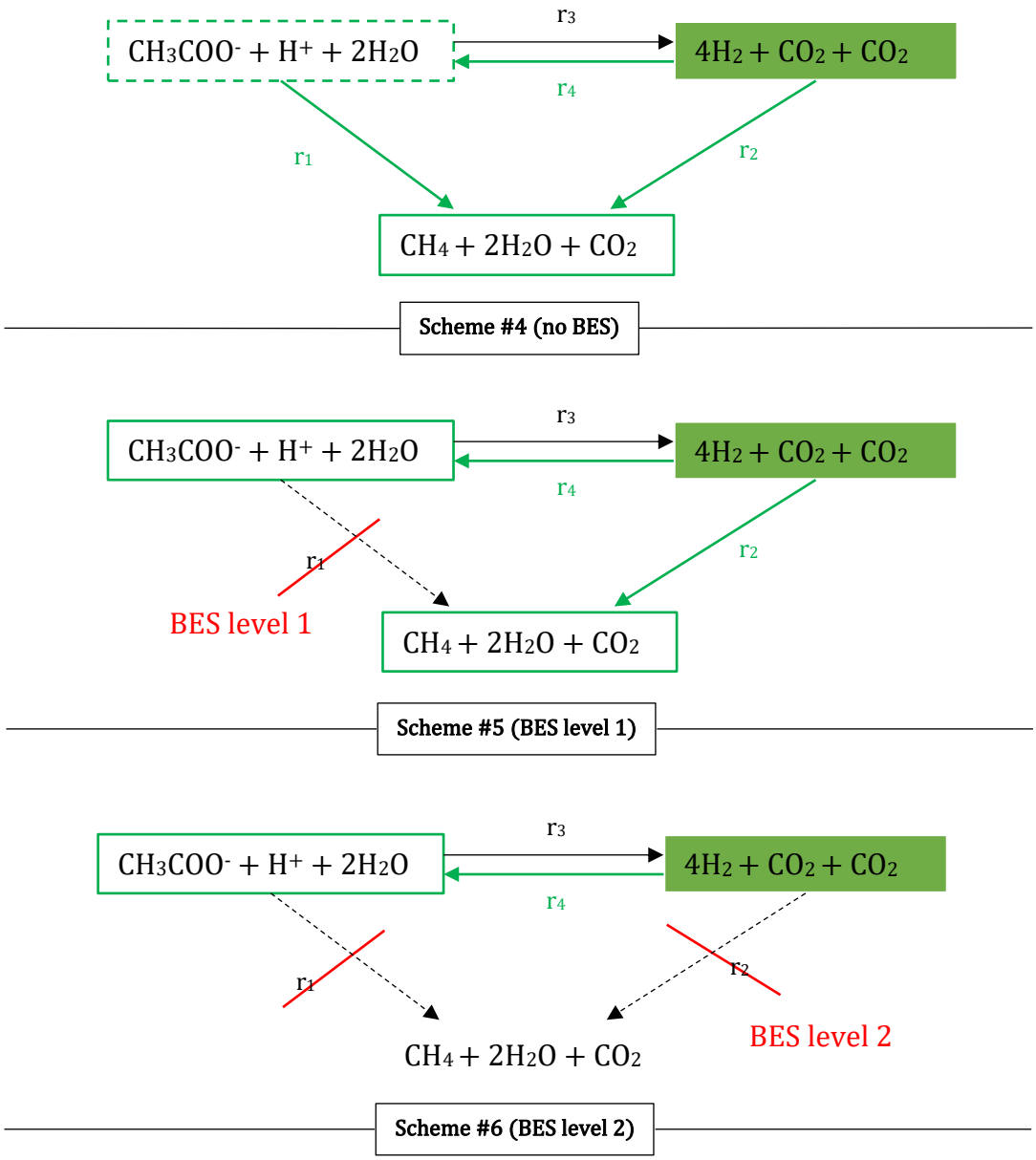

Figure 3-3. Schemes of reactions for SHMA test: #4 in the absence of BES; # 5 for a BES concentration at level 1; and # 6 for a BES concentration at level 2

Data were consulted in the literature to determine the concentrations associated with levels 1 and 2 for experimental design. Zinder et al. (1984) found that a BES concentration of 1mM caused complete inhibition of the acetoclastic methanogenesis, confirmed using sodium [2-^{14}C] acetate as the substrate and collecting the $^{14}CH_4$ in short-term (1 to 2 h) and long-term (24 h) batch tests for a thermophilic biosludge sample extracted from a digester treating lignocellulosic waste at 58°C; on the contrary, little or non-inhibition of the CO_2 reduction (hydrogenotrophic methanogenesis) was observed at a BES concentration of 10 mM. A BES concentration of 50 mM was required for the complete inhibition of methanogenesis from CO_2 with acetate, H_2, and ethanol accumulation, verified using H_2/CO_2 and $NaH^{14}CO_3$. The work of Zinder et al. (1984) is considered in the frame of reference with the serious exception that thermophilic conditions were used; methanogenic population is expected to be radically different in mesophilic reactors. However, working at mesophilic conditions, Xu et al. (2010) obtained similar results than those from Zinder et al. (1984), using 50 mM of BES with a 9:1 mixture of biosludge from a mesophilic sludge digester from a WWTP and an IC reactor treating citric acid wastewater tested at 35°C. In another vein, BES has been used to select microbial population for bioelectrochemical wastewater treatment in lab-scale reactors, suppressing methanogens in the inoculum (Chae et al., 2010; Haavisto et al., 2019; Mona et al., 2020; Varanasi et al., 2019). However, the addition of BES is not practical for the continuous operation of the microbial electrolysis cells

(MEC), and other techniques are considered to control methanogens, limiting the use of BES to batch-test studies (Parameswaran et al., 2009). Chae et al. (2010) reported complete inhibition of acetoclastic *Methanosarcinaceae* and *Methanosaetaceae* at BES concentrations between 0.1 to 0.27 mM, present in the biofilm from the anode of a MEC operating in a temperature-controlled room at 28 ± 1°C (hydrogenotrophic *Methanobacteriales* not found in the sample); whereas, Parameswaran et al. (2009) informed that a BES concentration of 50 mM was needed for suppressing methanogenesis since hydrogenotrophic methanogens *Methanobacteriales* were found in the anode compartment from MEC operating at 30°C. Note that the biosludge samples considered by Chae et al. (2010), Parameswaran et al. (2009), Xu et al. (2010), and Zinder et al. (1984) are not granular; granular biosludge might have a different response to BES due to its structure. In this chapter, the aim is to study the influence of SAO-HM on the SAMA assay as well as homoacetogenesis on the SHMA determination focused on granular biosludge. This work was presented as a poster in 16th World Congress on Anaerobic Digestion, Delft (2019). A brief version of this paper can be found in Appendix 4.

3.2. MATERIALS AND METHODS

Granular biosludge was sampled from a dairy full-scale UASB reactor, washed with distilled water, flushed with N_2, and incubated at 30°C for 72 h to consume the remaining substrate. Then, sodium 2-

bromoethanesulfonate 98% (BES, molecular weight: 211,01 g/mol) was added at concentrations of 1, 2, 5, 20, 50, and 100 mM and left during 48 h to favor its diffusion inside the granules. Both SAMA and SHMA tests were carried out in triplicate at 30°C in an air-thermostated chamber, for each concentration of BES and also in the absence of BES using the same methodology as described in Chapter 2 of the present thesis: 3 g_{COD} L^{-1} of sodium acetate initially added for SAMA, and gauge pressure of 760 mmHg of a mixture H_2/CO_2 (80/20) for SHMA (Ripoll et al., 2020; Soto et al., 1993). In SAMA tests, headspace pressure and gas composition were determined; whereas, only headspace pressure was measured, and the stoichiometric formula for the H_2/CO_2 conversion to methane was considered to calculate methane production during SHMA tests, as shown in Chapter 2. Then, for each run, the maximum slope for the specific methane production during the test time was calculated to report SAMA and SHMA and plot them as a function of BES concentration. The contributions of the SAO-HM pathway on SAMA and homoacetogenesis on SHMA were estimated, assuming zero-order conditions and maintaining calculation rules proposed in the test protocols, except for the SHMA values obtained after inhibiting hydrogenotrophic methanogenesis, that were corrected by considering stoichiometric formula for r_4 instead of r_2. Also, HALF-MAXIMAL INHIBITORY VALUES (IC50) were calculated for both acetoclastic and hydrogenotrophic methanogens, and the fraction of the solids content for each population was calculated based on typical kinetical values from the literature and kinetics

equations for high hydrogen concentrations in SHMA tests (Eqs. 3-1 to 3-3) and high acetate concentrations in SAMA tests (Eqs. 3-4 to 3-6). Considering the Monod kinetics for microbial growth rates r_X ($g_{VSS}\ L^{-1}\ d^{-1}$) of the populations involved in reactions from Figure 3-1, substrate consumption rates r_1, r_2, r_3, and r_4 ($g_{COD}\ L^{-1}\ d^{-1}$) could be expressed as:

$$r_2 = \frac{r_{X,hm}}{Y_{hm}} = \frac{\mu_{m,hm}\, X_{hm}\, S_{h2}}{Y_{hm}\,(K_{s,hm}+S_{h2})} = k_{m,hm} X_{hm} \frac{S_{h2}}{K_{s,hm}+S_{h2}} \cong k_{m,hm} X_{hm} \quad (3\text{-}1)$$

$$r_4 = \frac{r_{X,ha}}{Y_{ha}} = \frac{\mu_{m,ha}\, X_{ha}\, S_{h2}}{Y_{ha}\,(K_{s,ha}+S_{h2})} = k_{m,ha} X_{ha} \frac{S_{h2}}{K_{s,ha}+S_{h2}} \cong k_{m,ha} X_{ha} \quad (3\text{-}2)$$

Then, mass balance for hydrogen in SHMA batch-test corresponded to:

$$-V_L \frac{dS_{h2}}{dt} = \left(k_{m,hm} X_{hm} + k_{m,ha} X_{ha}\right) V_L \quad (3\text{-}3)$$

Analogously,

$$r_1 = \frac{r_{X,am}}{Y_{am}} = \frac{\mu_{m,am}\, X_{am}\, S_{ac}}{K_{s,am}+S_{ac}} = k_{m,am} X_{am} \frac{S_{ac}}{K_{s,am}+S_{ac}} \cong k_{m,am} X_{am} \quad (3\text{-}4)$$

$$r_3 = \frac{r_{X,ao}}{Y_{ao}} = \frac{\mu_{m,ao}\, X_{ao}\, S_{ac}}{K_{s,ao}+S_{ac}} = k_{m,ao} X_{ao} \frac{S_{ac}}{K_{s,ao}+S_{ac}} \cong k_{m,ao} X_{ao} \quad (3\text{-}5)$$

Thus, mass balance for methane in SAMA batch-test is expressed as:

$$V_L \frac{dS_{CH4}}{dt} = \left[(1 - c\, Y_{am}) k_{m,am} X_{am} + (1 - c\, Y_{hm})(1 - c\, Y_{ao}) k_{m,ao} X_{ao}\right] V_L \quad (3\text{-}6)$$

Where: μ_m (d^{-1}) is the Monod maximum specific growth rate; Y ($g_{VSS}\ g_{COD}^{-1}$) is the microbial yield; c corresponds to 1.42 $g_{COD}\ g_{VSS}^{-1}$ for microbial cells; k_m ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) is the Monod maximum specific uptake rate (μ_m/Y); X ($g_{VSS}\ L^{-1}$) is the biomass concentration; K_S ($g_{COD}\ L^{-1}$) is the half-saturation constant; subscripts for X, r_X, k_m, K_s, and Y represent different microbial

populations: hydrogenotrophic methanogens (hm), homoacetogens (ha), acetoclastic methanogens (am), and SAOB (ao). S (g_{COD} L^{-1}) is the substrate or product concentration, and different subscripts are used to represent acetate (ac), hydrogen (h2), or methane (CH4). V_L (L) is the working volume for the liquid phase in the batch-tests. Zero-order conditions ($S>>K_S$) and negligible microbial growth ($X \sim X_0$) were considered in all cases. Additionally, r_3 was considered rate-limiting in the SAO-HM pathway, and the methane produced is estimated considering the microbial yield of both populations Y_{hm} and Y_{ao}.

Finally, the specific substrate consumption rate k_S (g_{COD} g_{VSS}^{-1} d^{-1}) could be defined as follows:

$$k_S = \frac{r_S}{X} \tag{3-7}$$

Where: r_S (g_{COD} L^{-1} d^{-1}) is the substrate consumption rate, to know r_1, r_2, r_3, and r_4 (g_{COD} L^{-1} d^{-1}) for each reaction in Figure 3-1, resulting on the specific substrate consumption rates k_1, k_2, k_3, and k_4 (g_{COD} g_{VSS}^{-1} d^{-1}), respectively; X (g_{VSS} L^{-1}) is the concentration of VSS in the biosludge sample that represents total biomass.

Analogously, the specific methane production rate k_{CH4} (g_{COD} g_{VSS}^{-1} d^{-1}) could be expressed as:

$$k_{CH4} = \frac{1}{X}\frac{dS_{CH4}}{dt} \tag{3-8}$$

These k values (g_{COD} g_{VSS}^{-1} d^{-1}) could be associated with the means of the maximum slopes for plots of substrate consumption or cumulative specific

methane production over time (SMP-t plot) from SAMA and SHMA tests with and without BES (for details on SMP-t plots, refer to Chapter 2). In this context, the pseudo-first-order rate constant for hydrogenotrophic methanogenesis k_2 could be obtained considering the SHMA raw results from tests with 0 mM and level 2 of BES; whereas, the pseudo-first-order rate constant for homoacetogenesis k_4 could be calculated from SMP-t plot for SHMA test at level 2 of BES. Analougsly, the pseudo-first-order rate constant for acetoclastic methanogenesis $k_{CH4,1}$ could be estimated from SAMA at 0 mM and level 1 of BES; whereas, the pseudo-first-order rate constant for SAO-HM pathway $k_{CH4,3+2}$ could be extracted from SMP-t plot for SAMA at level 1 of BES.

3.3. RESULTS AND DISCUSSION

3.3.1. Hydrogenotrophic methanogens: inhibition in SHMA tests

In SHMA tests, involving high hydrogen concentrations and no acetate addition, r_3 could be neglected. If there is some contribution to methane formation via r_1, it is due to acetate formed by r_4. As observed in Figure 3-4, results for SHMA tests showed a lineal drop when the BES concentration was increased from 0 to 20 mM (p-value of 1E-08 for n=12 in ANOVA F-test), obtaining an IC50 value of about 20 mM. This behaviour agrees with reports observed in the literature, with complete inhibition of methanogenesis at 50 mM (Parameswaran et al., 2009; Xu et al., 2010;

Zinder et al., 1984). No lag phase was observed for SHMA tests at 50 or 100 mM of BES, counterposing the 60 hours informed by Ryan et al. (2008).

In calculations, the stoichiometric formula for r_4 used for 50 and 100 mM of BES, implying the decrease of the total moles of gas by 6 for every 4 moles of hydrogen consumed, instead of 4 as happens in r_2. Whereas, for the values for 5, 10, and 20 mM of BES represented in Figure 3-4, it was assumed a stoichiometry of 4 moles of total gas depletion for every 4 moles of hydrogen consumed. However, the stoichiometric factor is expected to change from 4 to 6 while hydrogenotrophic methanogenesis is progressively inhibited.

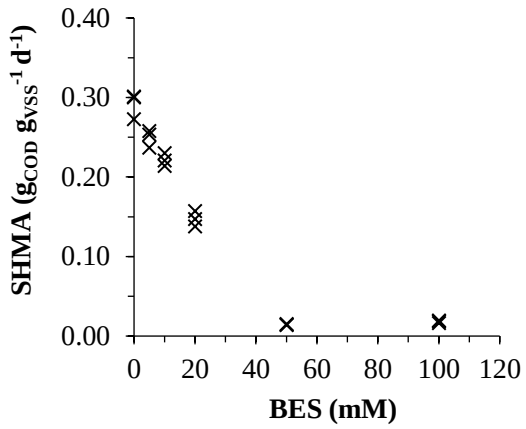

Figure 3-4. SHMA values for triplicate at different concentrations of BES

For the BES concentrations from 5 to 20 mM, acetate, hydrogen, and methane should be monitored rather than total pressure to analyze the contribution of r_2 and r_4. This analysis is beyond the objective of the present work; for this work, the relevant points are those for 0 and 50 mM of BES, where methanogenesis is not inhibited or is fully inhibited, to obtain a factor

f_1 and discount the effect of homoacetogenesis from the SHMA obtained in a routine test run without BES ($SHMA_{0mM}$), and estimate the activity purely associated with hydrogenotrophic methanogens ($SHMA_{corrected}$), as follows:

$$\mathrm{SHMA}_{\mathrm{corrected}} = \mathrm{f}_1 \ \mathrm{SHMA}_{\mathrm{0mM}} \quad (3\text{-}9)$$

In this case, the confidence interval for a confidence level α of 95%, considering the entire data set for 50 and 100 mM of BES, was 0.016 ± 0.002 $g_{COD}\ g_{VSS}^{-1}\ d^{-1}$ and represented 6% of the value obtained for the SHMA test without BES, being associated with the H_2 consumption exclusively by r_4. Then, a factor f_1 of 0.94 ± 0.01 was obtained considering error propagation, and a corrected SHMA of 0.275 ± 0.021 $g_{COD}\ g_{VSS}^{-1}\ d^{-1}$ was determined as an estimation of the hydrogenotrophic methanogens activity. This correction factor might change for samples from the same reactor at different operational conditions, as well as for biosludge from other sources.

3.3.2. Hydrogenotrophic methanogens: mass fraction estimation

A confidence interval for a confidence level α of 95% of 0.291 ± 0.019 $g_{COD}\ g_{VSS}^{-1}\ d^{-1}$ was obtained based on the SMP-t data for triplicates in the SHMA test without BES. Then, k_2 and k_4 ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) were estimated, based on SHMA tests at 0 and 50 mM of BES:

From Eqs. 3-1 and 3-7, at 50 mM $\mathrm{k}_4 = \frac{\mathrm{r}_4}{\mathrm{X}} \cong \frac{\mathrm{k}_{\mathrm{m,ha}}\mathrm{X}_{\mathrm{ha}}}{\mathrm{X}} = 0.016\ \mathrm{g}_{\mathrm{COD}}\ \mathrm{g}_{\mathrm{VSS}}{}^{-1}\mathrm{d}^{-1}$

From Eqs. 3-2 and 3-7, considering SHMA at 0 mM and 50 mM:

$$k_2 = \frac{r_2}{X} \cong \frac{k_{m,hm} X_{hm}}{X} = (0.291 - 0.016)\ g_{COD}\ g_{VSS}^{-1} d^{-1}$$

Then, $\frac{k_{m,ha} X_{ha}}{k_{m,hm} X_{hm}} = \frac{0.016}{0.291-0.016}$ thus, $k_{m,ha} X_{ha} = 0.059\ k_{m,hm} X_{hm}$ (3-10)

Substituting Eq. 3-10 into Eq. 3-3:

$$-V_L \frac{dS_{h2}}{dt} = \left(k_{m,hm} X_{hm} + k_{m,ha} X_{ha}\right) V = 0.941\ k_{m,hm} X_{hm} V_L$$

The following expression for X_{hm} ($g_{VSS}\ L^{-1}$) is obtained:

$$X_{hm} = \frac{0.941\ (-\frac{dS_{h2}}{dt})}{k_{m,hm}} \quad (3\text{-}11)$$

Zehnder and Wuhrmann (1977) reported μ_m of 0.058 h^{-1} (1.4 d^{-1}) and Y_{hm} of 2.53 $g_{VSS}\ mol^{-1}$ (0.04 $g_{VSS}\ g_{COD}^{-1}$) for a *Methanobacterium* Strain AZ from supernatant of digested sewage sludge, using H_2/CO_2 at a pH of 7.0 and 33 °C, obtaining $k_{m,hm}$ of 35 $g_{COD}\ g_{VSS}^{-1}\ d^{-1}$, cited by Gujer and Zehnder (1983), and later referenced in well-known modeling works (Batstone et al., 2002; Pavlostathis and Giraldo-Gomez, 1991). For the SHMA test at 0 mM of BES, an experimental concentration of biomass X of 8.2 $g_{VSS}\ L^{-1}$ inside vials was quantified, and a value of 2.39 $g_{COD}\ L^{-1}\ d^{-1}$ was determined for hydrogen consumption rate ($-dS_{h2}/dt$); then, an X_{hm} of 0.064 $g_{VSS}\ L^{-1}$ was obtained from Eq. 3-11. Therefore, the population of hydrogenotrophic methanogens represents 0.8% of the solids content associated with biomass in the biosludge sample (there are not enough inputs available to propagate the error associated with value).

3.3.3. Acetoclastic methanogens: inhibition in SAMA tests

In SAMA tests, since high acetate and low hydrogen concentrations are involved, r_4 is negligible. Nevertheless, methane production could occur by either r_1 or r_3+r_2. Experiences showed different behaviour at low and high BES concentrations, as shown in Figure 3-5 where SAMA, calculated as the mean of the maximum slopes from the SMP-t plot for triplicates, is plotted versus BES concentrations. A confidence interval for a confidence level α of 95% of 0.201 ± 0.008 $g_{COD}\ g_{VSS}^{-1}\ d^{-1}$ was obtained based on the SMP-t data for triplicates in the SAMA test without BES. A linear drop was observed in SAMA when BES concentration was increased from 0 to 5 mM, with a p-value of 3.7E-07 for ANOVA F-test (n=9, triplicates for 0 mM and duplicates for 1, 2, and 5 mM). This drop could be mainly associated with inhibition of r_1; r_2 inhibition could be reasonably dismissed based on results from SHMA tests, where the activity decreased by 14% when BES was increased from 0 to 5 mM. For higher concentrations of BES, 20 and 50 mM, r_1 is completely inhibited, and the drop in SAMA could be associated with r_2 inhibition, affecting r_3+r_2. SAMA dropped until zero when BES increased to 50 mM, coherent with complete inhibition of hydrogenotrophic methanogens and positive ΔG of r_3. The IC50 for acetoclastic methanogens was close to 3 mM of BES. When comparing to the literature, especially to the values found by Chae et al. (2010) working at a similar temperature, the IC50 value obtained was higher than expected; this could be related to the granular structure of the biosludge assayed. It is also relevant to mention that it was

necessary to leave the BES in contact with biosludge for 48 hours before SAMA and SHMA tests to obtain reproducible results, probably due to the diffusion of the inhibitor in the granule of the BES molecule, with a molecular weight of 211.01 g/mol.

Analogous to Eq. 3-9, an f_2 could be defined as the correction factor to estimate the activity purely due to acetoclastic methanogens (SAMA $_{corrected}$) from the SAMA value obtained at 0 mM (SAMA $_{0mM}$):

$$\mathrm{SAMA}_{\mathrm{corrected}} = \mathrm{f}_2 \ \mathrm{SAMA}_{\mathrm{0mM}} \quad (3\text{-}12)$$

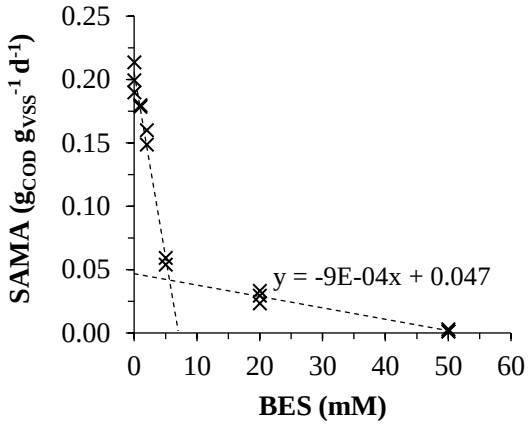

Figure 3-5. SAMA values at different concentrations of BES

In this case, the contribution of the SAO-HM pathway represented about 23% of the methane produced from acetate. A factor f_2 of 0.77±0.12 was obtained considering the error propagation, and a corrected SAMA of 0.156±0.030 $g_{COD}\ g_{VSS}^{-1}\ d^{-1}$ was estimated for acetoclastic methanogenic population.

3.3.4. Acetoclastic methanogens: mass fraction estimation

Considering Eqs. 3-6 and 3-8, and the intercept of the lineal regression involving SAMA values for 20 and 50 mM, 0.047 ± 0.010 $g_{COD}\ g_{VSS}^{-1}\ d^{-1}$, as an estimation of the contribution of the SAO-HM pathway, the specific methane production rate associated with r_3+r_2 pathway, $k_{CH4,3+2}$ ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$), can be expressed as follows:

$$k_{CH4,3+2} = \frac{(1-c\ Y_{hm})(1-c\ Y_{ao})k_{m,ao}X_{ao}}{X} = 0.047\ g_{COD}\ g_{VSS}{}^{-1}d^{-1}$$

Since hydrogenotrophs were partially affected at the different concentration of BES, level 1 was not verified experimentally. Raw results of the SAMA test without BES and the intercept of a trendline constructed with SAMA at 20 and 50 mM were used to calculate the pseudo-first-order constant associated with r_1, $k_{CH4,1}$ ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$), as follows:

$$k_{CH4,1} = \frac{(1-c\ Y_{am})k_{m,am}X_{am}}{X} = (0.201-0.047)\ g_{COD}\ g_{VSS}{}^{-1}d^{-1}$$

Therefore, $\frac{(1-c\ Y_{hm})(1-c\ Y_{ao})k_{m,ao}X_{ao}}{(1-c\ Y_{am})k_{m,am}X_{am}} = \frac{0.047}{0.201-0.047} = 0.305$

$$(1-c\ Y_{hm})(1-c\ Y_{ao})k_{m,ao}X_{ao} = 0.305\ [(1-c\ Y_{am})k_{m,am}X_{am}] \quad (3\text{-}13)$$

Substituting Eq. 3-13 into 3-6: $V_L\frac{dS_{CH4}}{dt} = 1.305\ (1-c\ Y_{am})\ k_{m,am}X_{am}V_L$

The following expression for X_{am} ($g_{VSS}\ L^{-1}$) is obtained:

$$X_{am} = \frac{1}{1.305\ (1-c\ Y_{am})\ k_{m,am}}\ (\frac{dS_{CH4}}{dt}) \quad (3\text{-}14)$$

For SAMA test without BES, an X of 2.30 g_{VSS} L^{-1} was determined, and the methane production rate (dS_{CH4}/dt) was estimated in 0.462 g_{COD} L^{-1} d^{-1}. Thus, considering the values obtained by Lawrence and McCarty (1969), Y_{am} of 0.054 g_{VSS} g_{COD}^{-1} (c of 1.42 g_{COD} g_{VSS}^{-1} for microbial cells) and $k_{m,am}$ of 4.8 $g_{CH3COOH}$ g_{VSS}^{-1} d^{-1} (equivalent to 5.1 g_{COD} g_{VSS}^{-1} d^{-1}) for a mixed culture at 30°C, an X_{am} of 0.075 g_{VSS} L^{-1} is obtained from Eq. 3-14. Therefore, acetoclastic methanogens represent 3.3% of the solids content associated with biomass in the biosludge sample (there are not enough inputs available to propagate the error associated with value). Then, the methanogenic population (hm+am) represent about 4% of the total biomass in coherence with the literature (Soto et al., 1993).

3.3.5. Comments on the order of reaction

The literature was reviewed in search of values for the half-saturation constant K_S of Monod kinetics for homoacetogens to study the order of reaction for homoacetogenesis under SHMA test conditions. Ni et al. (2011) informed a K_S of 3.7E-05 ± 3.1E-06 g_{COD} L^{-1}, whereas Liu et al. (2016) reported a K_S of 0.23 ± 0.1 g_{COD} L^{-1}, both at 35 °C, along with a K_S for hydrogenotrophic methanogens of 0.035 ± 0.007 g_{COD} L^{-1}; none of these samples corresponded to granular biosludge. In addition, the methodology used by Ni et al. (2011) for the selection of homoacetogens in the inoculum is more than rough, making the results questionable. On the other hand, despite the methodology being more reasonable, the values presented by Liu et al. (2016) seem to be affected by hydrogen mass transfer limitation,

evidenced through the comparison of the K_S obtained for hydrogenotrophic methanogens with others found in the literature (for more understanding, see table A1 from Appendix 2 and the discussion about K_S from Chapter 2). There is even less reliable information about SAOBs. Therefore, the need for a precise determination of K_S for the homoacetogens and SAOBs present in the granular sludge is not yet satisfied, and the study of the kinetics associated with homoacetogenesis and SAO remains unfulfilled and pending for future approaches on this subject, escaping from the scope of the current work. However, all the SHMA runs were carried out in similar ranges of total pressures, especially at the beginning of the test. The same is true for SAMA concerning acetate concentration. As SHMA and SAMA determinations were based on initial rates, substrate concentrations in each test were comparable, being in the same order of reaction, despite not having a detailed description of the r_3 or r_4 kinetics. Then, activity values derived from complete inhibition tests were comparable and applicable for the calculation of factors for the correction of SAMA and SHMA obtained from routine tests without BES.

3.4. CONCLUSIONS

During SMA tests, IC50 values of 3 and 20 mM were observed for acetoclastic and hydrogenotrophic methanogens, respectively, in granular sludge samples exposed to BES. Homoacetogens contributed about 6% of the value of SHMA obtained without inhibitor, whereas the SAO-HM

pathway contributed about 23% of the methane produced from acetate during the SAMA test. Therefore, correction factors were introduced to adjust values from both SAMA and SHMA routine tests to represent methanogens activity. The specific biomass concentration was calculated based on kinetics data obtained. The contributions of methanogenic acetoclastic and hydrogenotrophic archaea were estimated in 0.8% and 3.3% of the VSS content of the biosludge sample, respectively.

REFERENCES OF CHAPTER 3

Batstone, D.J., Keller, J., Angelidaku, I., Kalyuzhnyio, S.V., Pavlostathis, S.G., Rozzi, A., Saners, W.T.M., Siegrist, H., Vavilin, V.A., 2002. Anaerobic Digestion Model No. 1, Scientific and Technical Report No. 13. IWA Task Group for Mathematical Modelling of Anaerobic Digestion Processes. London.

Chae, K., Choi, M., Kim, K., Ajayi, F.F., Park, W., Kim, C., Kim, I.S., 2010. Bioresource Technology Methanogenesis control by employing various environmental stress conditions in two-chambered microbial fuel cells. Bioresour. Technol. 101, 5350–5357. https://doi.org/10.1016/j.biortech.2010.02.035

Coates, J.D., Coughlan, M.F., Colleran, E., 1996. Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. J. Microbiol. Methods 26, 237–246. https://doi.org/10.1016/0167-7012(96)00915-3

Colleran, E., Concannon, F., Golden, T., Geoghegan, F., Crumlish, B., Killilea, E., Henry, M., Coates, J., 1992. Use of Methanogenic Activity Tests to Characterize Anaerobic Sludges, Screen for Anaerobic Biodegradability and Determine Toxicity Thresholds against Individual Anaerobic Trophic Groups and Species. Water Sci. Technol. 25, 31–40. https://doi.org/10.2166/wst.1992.0136

Gerardi, M.H. (Ed.), 2003. Methane-forming bacteria, in: The Microbiology of Anaerobic Digesters. John Wiley and Sons Inc., pp. 17–29.

Gujer, W., Zehnder, A.J.B., 1983. Conversion Processes in Anaerobic Digestion. Water Sci. Technol. 15, 127–167. https://doi.org/10.2166/wst.1983.0164

Gunsalus, R.P., Romesser, J.A., Wolfe, R.S., 1978. Preparation of coenzyme M analogs and their activity in the methyl coenzyme M reductase system of Methanobacterium thermoautotrophicum. Biochemistry 17, 2374–2377. https://doi.org/10.1021/bi00605a019

Haavisto, J.M., Kokko, M.E., Lakaniemi, A., Mira, L.K., Puhakka, J.A., 2019. The effect of start-up on energy recovery and compositional changes in brewery wastewater in bioelectrochemical systems. Bioelectrochemistry 107402. https://doi.org/10.1016/j.bioelechem.2019.107402

Ho, D.P., Jensen, P.D., Batstone, D.J., 2013. Methanosarcinaceae and Acetate-Oxidizing Pathways Dominate in High-Rate Thermophilic Anaerobic Digestion of Waste-Activated Sludge. Appl. Environ. Microbiol. 79, 6491–6500. https://doi.org/10.1128/AEM.01730-13

Lawrence, A.W., McCarty, P.L., 1969. Kinetics of Methane Fermentation in Anaerobic Treatment. J. Water Pollut. Control Fed. 41, R1–R17.

Liu, R., Hao, X., Wei, J., 2016. Function of homoacetogenesis on the heterotrophic methane production with exogenous H2/CO2involved. Chem. Eng. J. 284, 1196–1203. https://doi.org/10.1016/j.cej.2015.09.081

Mona, S., Kumar, S.S., Kumar, V., Parveen, K., Saini, N., Deepak, B., Pugazhendhi, A., 2020. Green technology for sustainable biohydrogen production (waste to to energy): A review. Sci. Total Environ. 138481. https://doi.org/10.1016/j.scitotenv.2020.138481

Müller, B., Sun, L., Westerholm, M., Schnürer, A., 2016. Bacterial community composition and fhs profiles of low- and high-ammonia biogas digesters reveal novel syntrophic acetate-oxidising bacteria. Biotechnol. Biofuels 9, 48. https://doi.org/10.1186/s13068-016-0454-9

Ni, B.J., Liu, H., Nie, Y.Q., Zeng, R.J., Du, G.C., Chen, J., Yu, H.Q., 2011. Coupling glucose fermentation and homoacetogenesis for elevated acetate production: Experimental and mathematical approaches. Biotechnol. Bioeng. 108, 345–353. https://doi.org/10.1002/bit.22908

Noike, T., Li, Y.Y., 1989. State of the art on anaerobic bacteria for wastewater treatment. 2. Acid-producing bacteria, in: Matsumoto, J. (Ed.), Study on Anaerobic Wastewater Treatment. Japan Society of Civil Engineers, Tokyo, pp. 126–145.

Oosterkamp, M.J., Bauer, S., Ibáñez, A.B., Méndez-García, C., Hong, P.Y., Cann, I., Mackie, R.I., 2019. Identification of methanogenesis and syntrophy as important microbial metabolic processes for optimal thermophilic anaerobic digestion of energy cane thin stillage. Bioresour. Technol. Reports 7, 100254. https://doi.org/10.1016/j.biteb.2019.100254

Parameswaran, P., Torres, C.I., Lee, H., Krajmalnik-brown, R., Rittmann, B.E., 2009. Syntrophic Interactions Among Anode Respiring Bacteria (ARB) and Non-ARB in a Biofilm Anode: Electron Balances. Biotechnol. Bioeng. 103, 513–523. https://doi.org/https://doi.org/10.1002/bit.22267

Pavlostathis, S.G., Giraldo-Gomez, E., 1991. Kinetics of anaerobic treatment. Water Sci. Technol. 24, 35–59.

Ripoll, E., López, I., Borzacconi, L., 2020. Hydrogenotrophic activity : A tool to evaluate the kinetics of methanogens. J. Environ. Manage. 270. https://doi.org/10.1016/j.jenvman.2020.110937

Ryan, P., Forbes, C., Colleran, E., 2008. Investigation of the diversity of homoacetogenic bacteria in

mesophilic and thermophilic anaerobic sludges using the formyltetrahydrofolate synthetase gene. Water Sci. Technol. 57, 675–680. https://doi.org/10.2166/wst.2008.059
Schink, B., 1994. Diversity, ecology, and isolation of Acetogenic Bacteria, in: Drake, H.L. (Ed.), Acetogenesis. Chapman and Hall, London, pp. 197–228.
Shimada, T., Morgenroth, E., Tandukar, M., Pavlostathis, S.G., Smith, a., Raskin, L., Kilian, R.E., 2011. Syntrophic acetate oxidation in two-phase (acid-methane) anaerobic digesters. Water Sci. Technol. 64, 1812–1820. https://doi.org/10.2166/wst.2011.748
Siriwongrungson, V., Zeng, R.J., Angelidaki, I., 2007. Homoacetogenesis as the alternative pathway for H2 sink during thermophilic anaerobic degradation of butyrate under suppressed methanogenesis. Water Res. 41, 4204–4210. https://doi.org/10.1016/j.watres.2007.05.037
Soto, M., Mendez, R., Lema, J.M., 1993. Methanogenic and non- methanogenic activity tests: theoretical basis and experimental set up. Water Res. 27, 1361–1376.
Varanasi, J.L., Veerubhotla, R., Pandit, S., Das, D., 2019. Biohydrogen Production Using Microbial Electrolysis Cell : Recent Advances and Future Prospects, Biomass, Biofuels, Biochemicals. Elsevier B.V. https://doi.org/10.1016/B978-0-444-64052-9.00035-2
Wei, Y., Wu, Y., Zhang, L., Zhou, Z., Zhou, H., Yan, X., 2020. Genome recovery and metatranscriptomic confirmation of functional acetate-oxidizing bacteria from enriched anaerobic biogas digesters. Environ. Pollut. 265, 114843. https://doi.org/10.1016/j.envpol.2020.114843
Westerholm, M., Moestedt, J., Schnürer, A., 2016. Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance. Appl. Energy 179, 124–135. https://doi.org/10.1016/j.apenergy.2016.06.061
Xu, K., Liu, H., Chen, J., 2010. Effect of classic methanogenic inhibitors on the quantity and diversity of archaeal community and the reductive homoacetogenic activity during the process of anaerobic sludge digestion. Bioresour. Technol. 101, 2600–2607. https://doi.org/10.1016/j.biortech.2009.10.059
Zehnder, A.J.B., Wuhrmann, K., 1977. Physiology of a Methanobacterium strain AZ. Arch. Microbiol. 111, 199–205. https://doi.org/10.1007/BF00549357
Zinder, S.H., 1993. Physiological Ecology of Methanogens, in: Methanogenesis. Springer US, Boston, MA, pp. 128–206. https://doi.org/10.1007/978-1-4615-2391-8_4
Zinder, S.H., Anguish, T., Cardwell, S.C., 1984. Selective inhibition by 2-bromoethanesulfonate of methanogenesis from acetate in a thermophilic anaerobic digestor. Appl. Environ. Microbiol. 47, 1343–1345.
Zinder, S.H., Koch, M., 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch. Microbiol. 138, 263–272. https://doi.org/10.1007/BF00402133

CHAPTER 4

HYDROGENOTROPHIC ACTIVITY APPLIED TO SLUDGE FROM SOLIDS DIGESTER

CHAPTER 4. HYDROGENOTROPHIC ACTIVITY APPLIED TO SLUDGE FROM SOLIDS DIGESTER

4.1. INTRODUCTION

The influence of factors over solids digesters performance, such as temperature, pH, type of digestion, and substrate characteristics, has been reported in the literature (Bi et al., 2020; Fotidis et al., 2013; Liu et al., 2016; Westerholm et al., 2016). Solids digesters, dealing with complex type of substrates such as manure or the organic fraction of municipal solid waste, usually have problems with ammonia inhibition at high OLR; since hydrogenotrophic methanogens are less sensitive to the presence of ammonia than acetoclastic methanogens, SAO-HM is crucial for the AD process in solids digester under ammonia inhibition (Fotidis et al., 2014; Yenigün and Demirel, 2013). Methanogenic activity tests could lead to a better comprehension of the pathways involved in AD process inside digesters and provide a more holistic diagnosis of its performance, as discussed in Chapter 1 and 2.

In Chapter 2, a formula to calculate the inoculum size was proposed for SHMA batch-assays considering selected conditions, such as incubation temperature and headspace volume in vials, and characteristics of the sludge sample: the VSS content and an estimated value for SHMA; then, samples with low VSS or low SHMA required larger inoculum sizes using this formula, which worked properly for washed and exhausted sludge from

wastewater reactors, where no significant amount of inert or substrate was found and the VSS content could be used as a direct quantification of the biomass. However, when considering samples from solids digesters, substrate cannot be completely separated from microorganisms (refer to as ‘biomass’) by washing or exhausting; then, substrate and inert dilutes biomass concentration, and a greater inoculum size would be needed to assess SHMA in a reasonable period with negligible microbial growth. Additionally, since total VS roughly represents both biomass and substrate, and SHMA is usually expressed per gVSS of the sample, the hydrogenotrophic activity would be undervalued. In the present Chapter, the work developed on this issue is exposed.

The main objective of the present work was to study the dilution effect of substrate and inert presence over biomass concentration in biosludge samples from solids digester and improve the methodology for SHMA and SAMA determination. In this context, the specific objective was to determine a correction factor to recalculate the inoculum size formula, and activity results for a better estimation of the biomass activity. This work was presented orally in XII Latin American Workshop and Symposium on Anaerobic Digestion, Cuzco (2016) and the full paper can be consulted in Appendix 5.

4.2. MATERIALS AND METHODS

4.2.1. Inoculum

Sludge from a slaughterhouse anaerobic lagoon was provided to inoculate two different systems: (i) a bench-scale CSTR for manure digestion; (ii) a pilot-scale EGSB reactor for wastewater treatment in a beverage industry. Inoculum was mostly flocculent but had some incipient granules. SHMA, SAMA, and VSS were determined for the inoculum ('sample 0'). Also, methanogenic activities for both inoculated systems were analysed to compare solids digester case, where biomass dilution effect might be relevant, to the EGSB reactor where such dilution does not exist.

4.2.2. Manure bench-scale digester

The inoculum (X) was incubated in batch conditions for adaptation to the substrate with manure (S) from a milking-yard farm at an initial ratio of 2.0 $g_{VS\_X}\ g_{VS\_S}^{-1}$ for 30 days. Manure characterization can be seen in Table 4-1. At the end of this 30-day-batch incubation, the incubated sludge was sampled for SHMA, SAMA, and VS analysis ('sample 1', t=30 d). A CSTR digester (3.0 L of working volume) was inoculated with the incubated sludge and operated at an OLR of 2.9 $g_{COD}\ L^{-1}d^{-1}$ and an SRT of 30 days, obtaining a COD removal efficiency of 54% (period 1). The digester was sampled at the end of period 1 after 2 SRT (Solid Retention Time) for SHMA, SAMA, and VS analysis ('sample 2', t= 103 d). Then, OLR was increased to 4.4 $g_{COD}\ L^{-1}d^{-1}$ (period 2), achieving a COD removal efficiency of 51%.

Table 4-1. Manure characterization (mean and standard deviation for triplicates). Analytical methods: BMP and biodegradability - Angelidaki et al. (2009); VS - Rice et al. (2017); total COD - Yadvika et al. (2006)

VS-s (mg/g)	COD (g_{COD}/g_{VS})	BMP (mL_{CH4}/g_{VS})	Biodegradability (%)
139 ± 3	1.36 ± 0.06	307± 9	65 ± 2

Another sample was taken from CSTR digester after 3 SRT in period 2 ('sample 3', t=196 d). Details about the operation of the digester named as ‘reactor 2’ can be consulted in Benzo (2016).

4.2.3. Beverage pilot-scale EGSB reactor

After inoculation and during the start-up, the pilot-scale EGSB reactor (15 m^3) treating beverage wastewater at an OLR of 5 $kg_{COD}\ m^{-3}\ d^{-1}$ (0.3 $kg_{COD}\ kg_{VSS}^{-1}\ d^{-1}$) was sampled for SHMA, SAMA, VSS analysis ('sample 4').

4.2.4. SHMA test

SHMA tests were carried out by triplicate using the adapted protocol discussed in Chapter 2 (Ripoll et al., 2020). This protocol is based on monitoring the depletion of gas pressure in vial headspace (ΔP), using a 0-2 barg pressure transducer (Sper Scientific® model PS100-2BAR; maximum range: 1500 mmHg; resolution: 2 mmHg), and calculations considering the stoichiometric relation for the H_2/CO_2 conversion to methane (Coates et al., 1996). The volume aliquot of biosludge V_B(L) added to each vial was calculated as:

$$V_B = \frac{-\Delta P \, V_{hs} \, C}{4 \, R \, T \, \Delta t \, X \, k_{m,h}} \quad \text{with X estimated as: } X = X_{tot} \, f_c \qquad (4\text{-}1)$$

Where C = 64 g_{COD} mol^{-1} is the conversion factor from moles of methane to grams of COD; ΔP (atm) is the pressure depletion inside headspace desirable between two consecutive measures; V_{hs} (L) is the headspace volume; R (L atm K^{-1} mol^{-1}) is the universal gas constant; and T (K) is the temperature; Δt (days) is the time planned to be elapsed between two consecutive measures; X (g_{VSS} L^{-1}) is the concentration of biomass (microorganisms) in the sample; X_{tot} (g_{VSS} L^{-1}) is the total content of VS in the sample taken from a solids digester; and $k_{m,h}$ (g_{COD} g_{VSS}^{-1} d^{-1}) is a preliminary value for SHMA based on historical data and considering the source reactor for the particular biosludge sample. The original SHMA protocol was adapted to consider the dilution of the biomass with the solid substrate and inert by using a correction factor f_c that corrects the VS concentration determined for the sample by the standardized protocol presented in Rice et al. (2017) to obtain an estimation of the biomass concentration. This correction factor was calculated by using kinetics model, as explained in the following subsection.

Before SHMA tests, samples 0 and 4 were exhausted at 30ºC for 3 days, whereas samples 2 and 3 were left 6 days at 37ºC; sample 1 was not exhausted. SCHOTT® 250mL flasks (308 mL of total volume) were used as test vials in SHMA assay, with a headspace volume V_{hs} of 208 mL. Vials containing sludge were gassed to 760 mmHg (gauge pressure) with H_2/CO_2 (80/20) and incubated at 30ºC in a New Brunswick™ Innova® model 2100

orbital platform shaker at 150 rpm. Measures for pressure inside headspace (P) were taken every 2 hours.

4.2.5. SAMA test

SAMA tests were carried out in triplicate at 30°C inside SCHOTT® 100 mL glass bottles (137 mL of total volume) with an initial concentration of sodium acetate of 3 g_{COD} L^{-1} so as to work at zero-order conditions (see Chapter 2). The protocol was based on the methodology of headspace pressure measurement and determination of biogas composition proposed by Soto et al. (1993). These gas samples were analysed using a Shimadzu® gas chromatograph model GC-2014 equipped with a Restek® ShinCarbon model ST 100/120 2 m 1 mm ID 1/16" OD Silco packed column under the following conditions: temperature of injection port, packed column, and TCD were adjusted at 110°C, current in TCD was set to 60 mA and argon N50 carrier was set at a flow rate of 21 mL min^{-1}.

In this case, the volume aliquot of sludge V_B (L) could be determined analogously than in SHMA tests, as follows:

$$V_B = \frac{-\Delta P \, x_{CH4} \, V_{hs} \, C}{R \, T \, \Delta t \, X \, k_{m,a}} \quad \text{with X estimated as: } X = VS_{sample} \, f_c \qquad (4\text{-}2)$$

Where C = 64 g_{COD} mol^{-1}, x_{CH4} is the molar fraction of methane in the gas mixture inside the headspace; $k_{m,a}$ (g_{COD} g_{VSS}^{-1} d^{-1}) is a preliminary value for SAMA, based on historical data and the type of sources for the biosludge

sample. A gas depletion ΔP of 0.33 atm (250 mmHg) was set for a Δt of 0.21 days (5 h).

4.2.6. Kinetics model

The solid substrate (S) was represented by two fractions: a fraction of non-biodegradable material (X_{nb}) and a fraction of biodegradable material (X_b). First-order kinetics was considered for the substrate hydrolysis of the biodegradable fraction, considering $k_H(d^{-1})$ as the hydrolysis constant (Valentini et al., 1997). A value for k_H of 0.142 d^{-1} was assumed based on a determination of BMP conducted during period 1. Complete mixed behaviour was considered for the manure digester, with τ(d) as the hydraulic retention time. A microbial yield Y of 0.376 $g_{VSS\_X}\, g_{VSS\_S}^{-1}$ was considered, based on the value reported by Pavlostathis and Giraldo-Gomez (1991), 0.18 $g_{VSS\_X}\, g_{COD}^{-1}$, and a concentration of 2.09 $g_{COD}\, g_{VS\_Xb}^{-1}$ determined for the manure used as the substrate in the experiences. The decay of biomass was modelled as first-order kinetics with k_d of 0.01 d^{-1} as the coefficient. The dead biomass was assigned to the biodegradable fraction X_b.

In batch conditions, model equations were the following:

$$\frac{dX}{dt} = Y\, k_H X_b - k_d X \quad (4\text{-}3)$$

$$\frac{dX_b}{dt} = -k_H X_b + k_d X \quad (4\text{-}4)$$

$$\frac{dX_{nb}}{dt} = 0 \quad (4\text{-}5)$$

Whereas, in continuous conditions, the model could be expressed as:

$$\frac{dX}{dt} = \frac{X_{in} - X}{\tau} + Y\, k_H X_b - k_d X \quad (4\text{-}6)$$

$$\frac{dX_b}{dt} = \frac{X_{b,in} - X_b}{\tau} - k_H X_b + k_d X \quad (4\text{-}7)$$

$$\frac{dX_{nb}}{dt} = \frac{X_{nb,in} - X_{nb}}{\tau} \quad (4\text{-}8)$$

Where subscript 'in' indicates inlet conditions.

The model was numerically solved using SCILAB Software. The model solution allowed computing the concentrations of variables X, X_b, X_{nb} in the periods considered. The initial concentrations of X, X_b, and X_{nb} were 32.3, 10.5, and 5.7 g_{VS} L^{-1}, respectively, based on batch design and experimental data from Table 4-1, resulting in a known correction factor f_c of 0.67 at the beginning of the batch incubation.

For the CSTR digester, the inlet concentration of manure was 64 and 96 g_{VS_S} L^{-1} for period 1 and period 2, respectively. Then, $X_{b,in}$ and $X_{nb,in}$ were obtained for each period considering manure biodegradability from Table 4-1. The contribution of microorganisms from manure (X_{in}) was neglected for the CSTR digester modelling. The final values for X, X_b, X_{nb} resulting from modelling the 30-day-batch incubation were used as inputs to model period 1 of CSTR digester (involving 58 days), and analogously, for period 2 (88 days). The total content of VS (X_{tot}) was calculated for each period to estimate the corresponding correction factors f_c as the ratio between X and X_{tot}. Then, SHMA and SAMA results could be divided by f_c to obtain more representative values for microorganisms activities present in the sludge samples.

4.3. RESULTS AND DISCUSSION

4.3.1. Model simulation results

The initial and final concentrations of X, X_b, X_{nb}, and X_{tot} obtained in the model simulation of the batch incubation, and for the period 1 and period 2 of the CSTR can be seen in Table 4-2. Concentration profiles over time for the three stages can be observed in Figure 4-1. In batch model simulation, a moderate depletion in biomass concentration and also a decrease in biodegradable fraction were observed over the 30 days of incubation. The concentration of non-biodegradable material remained equal. Thus, total VS represented by X_{tot} decreased during batch incubation, obtaining a biomass correction factor of 0.79 at the end of the stage (Figure 4-1i and Table 4-2). Then, for the CSTR digester model simulation in period 1, an accumulation of non-biodegradable material, an increase in biodegradable fraction, a depletion in biomass concentration, and an increase in total VS along the stage were obtained, resulting in a correction factor of 0.30 (Figure 4-1ii and Table 4-2). Whereas in model simulation for period 2 of CSTR digester, biomass concentration had a net increase, also the total VS, obtaining a correction factor of 0.25 (Figure 4-1iii, Table 4-2).

Table 4-2. Biomass, biodegradable and non-biodegradable substrates, and total VS concentrations and correction factor calculated by model simulation for the different stages

ID	X (g/L)	X_b (g/L)	X_{nb} (g/L)	X_{tot} (g/L)	fc
Initial – Batch	32.3	10.5	5.7	48.5	0.67
Final – Batch	29.4	2.3	5.7	37.4	0.79
Final – Period 1	12.1	8.6	20.0	40.7	0.30
Final – Period 2	15.6	12.8	32.9	61.3	0.25

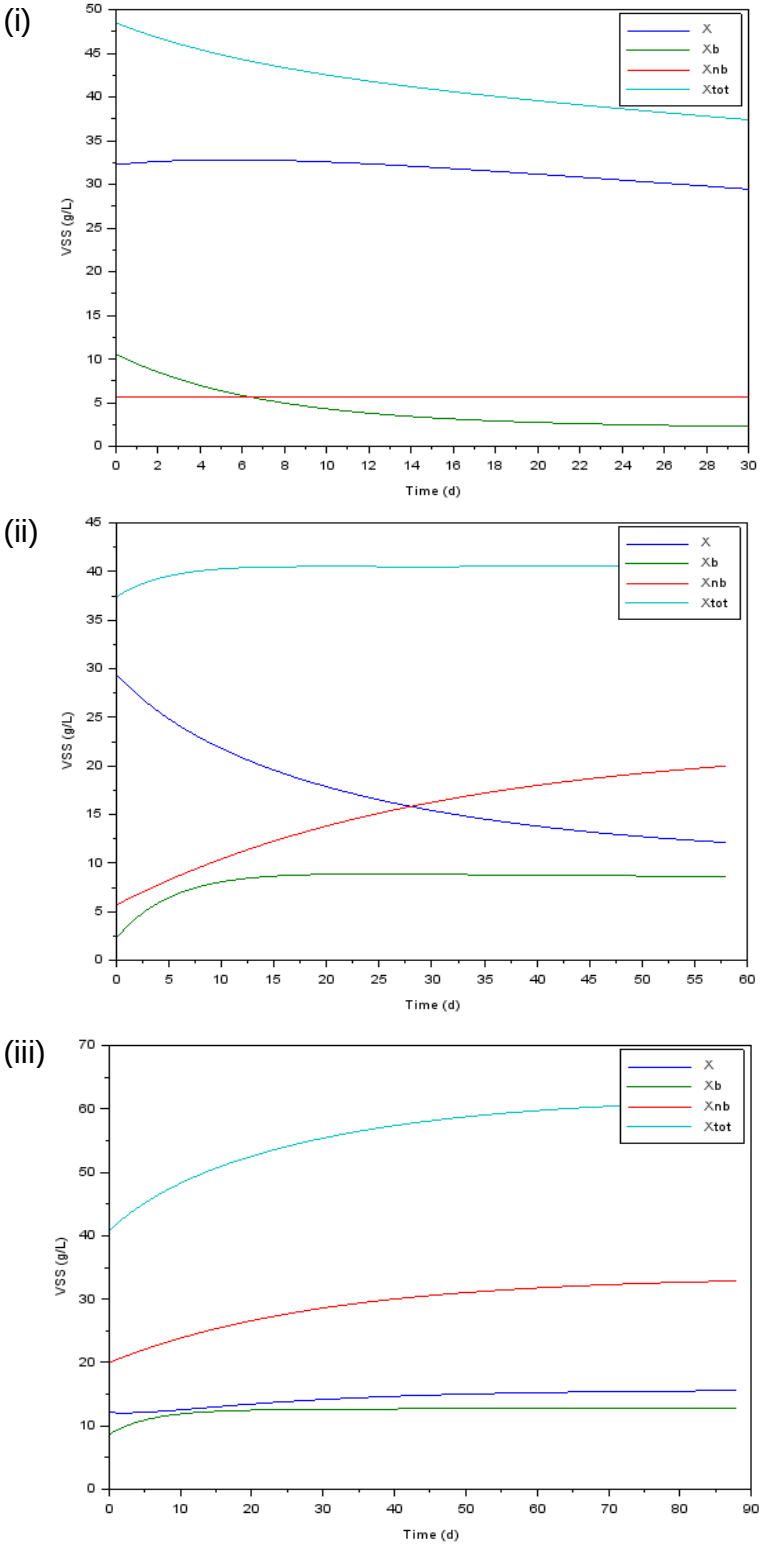

Figure 4-1. Concentration profiles over time obtained by model simulation for the different stages of the experiment: (i) Batch incubation (ii) CSTR – period 1 (iii) CSTR – period 2.

4.3.2. SAMA and SHMA tests

Results obtained from a direct application of SAMA and SHMA test protocols and activities values adjusted using f_c factors for each stage are shown in Table 4-3. When considering results from the direct application of SMA test protocols (raw SAMA and raw SHMA), depletion in the inoculum activities were observed after being incubated with manure. These could be first associated with the acclimatization of sludge to the new substrate and second with the low concentration of biodegradable material after 30 days. Then, during period 1, sludge was fed with 2.9 $g_{COD}\ L^{-1}d^{-1}$ of manure in CSTR digester, with a consequent methane production of 1.2±0.2 $g_{COD}\ L^{-1}\ d^{-1}$. An increase in both activities was expected due to changes in operational conditions and some acclimatization to the substrate. However, there was no significant difference between the raw SAMA for batch incubation and period 1, and a decrease was observed in the raw SHMA. Later, in period 2 an OLR of 4.4 $g_{COD}\ L^{-1}d^{-1}$ of manure was applied to CSTR digester producing 1.9±0.3 $g_{COD}\ L^{-1}\ d^{-1}$ of methane. Despite the fact that percentage of removed COD as methane remained similar to the one from period 1 with increasing OLR, decreases were registered in both raw activities. On the other hand, when analysing the case of beverage pilot-scale EGSB reactor, an increase in raw activities was observed, probably associated with the selection of the sludge through ascensional rate and the sludge adaptation to consume a simpler substrate, mainly based on carbohydrates.

Table 4-3. Methanogenic activity raw values from applying SAMA and SHMA protocols and adjusted values obtained using fc factors for different sludge samples: (0) Inoculum; (1) Batch incubation; (2) CSTR - period 1; (3) CSTR - period 2; and (4) EGSB. Confidence intervals for a confidence level of 95% reported

Sample ID	Raw SAMA ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	Adjusted SAMA ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	Raw SHMA ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	Adjusted SHMA ($g_{COD}\ g_{VSS}^{-1} d^{-1}$)	fc
0	0.100 ± 0.026	-	0.154 ± 0.004	-	-
1	0.052 ± 0.002	0.066 ± 0.003	0.103 ± 0.007	0.131 ± 0.009	0.79
2	0.052 ± 0.006	0.172 ± 0.021	0.072 ± 0.012	0.241 ± 0.040	0.30
3	0.045 ± 0.003	0.181 ± 0.011	0.042 ± 0.005	0.167 ± 0.019	0.25
4	0.267 ± 0.032	-	0.256 ± 0.020	-	-

While referring to values adjusted by correction factors, decrease in the activities were also seen for the inoculum at the end of the batch incubation. On the contrary, a change was observed in the trend of the adjusted values compared to the raw values, observing an increase in the activities for period 1 of CSTR digester with respect to the batch incubation. These evidenced an increasingly relevant effect of biomass dilution on activity values (with a correction factor of 0.30 compared to 0.79) and the usefulness of the correction factor for activity result adjustment and data interpretation. On the other hand, period 2 had a slightly lower correction factor than period 1. For this period, a decrease in SHMA was observed, whereas SAMA had no significant difference with the activity found for period 1. Also, it is relevant to notice that raw activity results undervalued the real activity of the biomass in the cases of batch incubation and for the CSTR digester.

4.3.3. Inoculum size estimation for SAMA and SHMA tests

For SMA tests, the aliquot of sludge V_B can be calculated by Eq. (4-1) or (4-2) considering the correction factor obtained through modelling. As an example, for an expected depletion of 120 mmHg in headspace pressure every 3 h, considering the value of SHMA for sample 0 as an estimation of $k_{m,h}$ (0.154 g_{COD} g_{VSS}^{-1} d^{-1}), the X obtained for sample 1 (37.4 g_{VS} L^{-1}), and a correction factor of 0.79, V_B was determined to analyse SHMA of sample 1, resulting in a concentration of 14 g_{VS} L^{-1} of the sample in each vial. The concentration of 14.8 g_{VS} L^{-1} dosed to vials (verified at the end of the test) produced a depletion of 100 mmHg every 3 h and a total number of 6 measures after 9 h, coherent with the adjusted SHMA obtained for sample 1 (0.131 g_{COD} g_{VSS}^{-1} d^{-1}). Analogously, the corresponding V_B was calculated at 37 mL per vial (14 g_{VS} L^{-1}) for the SAMA assay of sample 1, considering an increase of 250 mmHg every 5 h, an estimated $k_{m,a}$ of 0.100 g_{COD} g_{VSS}^{-1} d^{-1} from sample 0, and the correction factor 0.786. A concentration of 15.3 g_{VS} L^{-1} was verified in vials at the end of the test, observing an increase of about 150 mmHg every 5 h and obtaining 4 measures after 40 h, including two nights without measuring since there were hand-taken. This behaviour was consistent with the adjusted SAMA value obtained for sample 1. Greater concentrations were needed to quantify SAMA and SHMA for samples 2 and 3 from the CSTR digester, ascending to 30 or even 40 g_{VS} L^{-1} of sludge inside vials. These values are significantly higher than the concentrations usually recommended for the SAMA and SHMA tests, for

example, than those reported by Coates et al. (1996) from 1 to 8 g_{VSS} L^{-1}. Consequently, a more reasonable time-test was obtained, similar to those from wastewater systems, in contrast to the ones reported for solids that exceed 50 h for the SHMA test, as in the case of Coates et al. (1996).

4.4. CONCLUSIONS

A case of solid waste digestion was exposed from batch incubation to subsequent operation in a CSTR digester; the dilution effect of substrate and inert over biomass was studied through model simulation, obtaining a correction factor of 79% for batch incubation and 25 to 30% for the CSTR digester. These correction factors were used to adjust SAMA and SHMA from raw results to better represent methanogenic population activities. For the CSTR digester, tendencies changed after adjustment, evidencing the relevance of the application of this correction tool. Otherwise, raw activity results can underestimate the real activity of the biomass. The correction factors were used to perform more reliable calculations to determine inoculum size for SAMA and SHMA tests, obtaining higher values than the ones from wastewater systems protocols (up to 40 compared to 1 to 8 g_{VSS} L^{-1}) and similar time-test (10 to 12 h for SHMA, and about 40 h for SAMA), achieving a suitable test design.

REFERENCES OF CHAPTER 4

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J.L., Guwy, a. J., Kalyuzhnyi, S., Jenicek, P., Van Lier, J.B., 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: A proposed protocol for batch assays. Water Sci. Technol. 59, 927–934. https://doi.org/10.2166/wst.2009.040

Benzo, M., 2016. Aplicación de modelos cinéticos a la digestión anaerobia de estiércol vacuno. Universidad de la República - Facultad de Ingeniería.

Bi, S., Westerholm, M., Qiao, W., Xiong, L., Mahdy, A., Yin, D., Song, Y., Dong, R., 2020. Metabolic performance of anaerobic digestion of chicken manure under wet, high solid, and dry conditions. Bioresour. Technol. 296, 122342. https://doi.org/10.1016/j.biortech.2019.122342

Coates, J.D., Coughlan, M.F., Colleran, E., 1996. Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. J. Microbiol. Methods 26, 237–246. https://doi.org/10.1016/0167-7012(96)00915-3

Fotidis, I.A., Karakashev, D., Angelidaki, I., 2014. The dominant acetate degradation pathway/methanogenic composition in full-scale anaerobic digesters operating under different ammonia levels. Int. J. Environ. Sci. Technol. 11, 2087–2094. https://doi.org/10.1007/s13762-013-0407-9

Fotidis, I.A., Karakashev, D., Kotsopoulos, T.A., Martzopoulos, G.G., Angelidaki, I., 2013. Effect of ammonium and acetate on methanogenic pathway and methanogenic community composition. FEMS Microbiol. Ecol. 83. https://doi.org/10.1111/j.1574-6941.2012.01456.x

Liu, C., Li, H., Zhang, Y., Chen, Q., 2016. Characterization of methanogenic activity during high-solids anaerobic digestion of sewage sludge. Biochem. Eng. J. 109, 96–100. https://doi.org/10.1016/j.bej.2016.01.010

Pavlostathis, S.G., Giraldo-Gomez, E., 1991. Kinetics of anaerobic treatment. Water Sci. Technol. 24, 35–59.

Rice, E.W., Baird, R.B., Eaton, A.D. (Eds.), 2017. Standard Methods for the Examination of Water and Wastewater, 23rd ed. APHA, AWWA, WEF, Washington DC, USA.

Ripoll, E., López, I., Borzacconi, L., 2020. Hydrogenotrophic activity : A tool to evaluate the kinetics of methanogens. J. Environ. Manage. 270. https://doi.org/10.1016/j.jenvman.2020.110937

Soto, M., Mendez, R., Lema, J.M., 1993. Methanogenic and non- methanogenic activity tests: theoretical basis and experimental set up. Water Res. 27, 1361–1376.

Valentini, A., Garuti, G., Rozzi, A., Tilche, A., 1997. Anaerobic degradation kinetics of particulate organic matter: A new approach. Water Sci. Technol. 36, 239–246. https://doi.org/10.1016/S0273-1223(97)00528-3

Westerholm, M., Moestedt, J., Schnürer, A., 2016. Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance. Appl. Energy 179, 124–135. https://doi.org/10.1016/j.apenergy.2016.06.061

Yadvika, Yadav, A.K., Sreekrishnan, T.R., Satya, S., Kohli, S., 2006. A modified method for estimation of chemical oxygen demand for samples having high suspended solids. Bioresour. Technol. 97, 721–726. https://doi.org/10.1016/j.biortech.2005.04.013

Yenigün, O., Demirel, B., 2013. Ammonia inhibition in anaerobic digestion: A review. Process Biochem. 48, 901–911. https://doi.org/10.1016/j.procbio.2013.04.012

CHAPTER 5

FINAL CONSIDERATIONS

CHAPTER 5. FINAL CONSIDERATIONS

At the beginning of this thesis, the aim was to establish a basis for the design and selection of test conditions for SHMA determination in a wide variety of types of biosludge, based on both laboratory experiences and modelling. In this context, simple models were applied to analyze process kinetics: in Chapter 2, a zero-order model was considered for the quantification of the specific methanogenic activity; a literature revision about half-saturation constant for hydrogen was conducted to give sustain to zero-order condition hypothesis. Also, acid-base equilibrium equations and microbial growth kinetics were utilized to ponder the influence of CO_2 in the methodology of SHMA determination. Then, in Chapter 3, kinetics for the four reactions involved in the last step of the AD process were considered to estimate methanogen fractions in microbial population and study the contribution of each pathway in SMA tests. Finally, in Chapter 4, the evolution of biomass, degradable and non-degradable substrate was modeled for solid waste digestion during batch incubation and then in a CSTR digester. Biomass correction factors were estimated and used as correctors of the activity values to better reflect the microbial activity.

Standardization of SHMA and SAMA techniques is crucial to compare results between different reactors and laboratories. Interlaboratory experiences would enrich the discussion and provide a basis to analyse the influence of different variables and consider variations between available

equipment to achieve comparable conditions. To mention one of the relevant issues to be considered, serious studies must be done to analyse external mass transfer limitations and made different SHMA techniques comparable. Beyond the particular conclusions of each chapter that are summarized below, the entire work shows an approach methodology for the study of methanogenic routes through a cost-effective tool such as activity tests, making a critical analysis of their design parameters and limitations (Chapters 2 and 3) and trying to collaborate in solving drawbacks such as solid waste case (Chapter 4). In Chapter 2 of this work, a full calculation was presented and the basis was laid for a discussion of interlaboratory work and standardization of the SHMA technique. The equation proposed for size inoculum made SHMA determination possible within 7 to 10 h for both low activity and high-rate-reactor sourced biosludge, with negligible microbial growth. The zero-order model fitted adequately to data, and hypothesis simplifications applied for methane production calculation during the SHMA test introduced an error comparable to the experimental error. SHMA, together with SAMA, gives a better explanation of the performance of biosludge in reactors (represented by the parameter of specific methane production rate in the reactor, SMPR).

Regarding BES usage as an inhibitor during SMA tests, as presented in Chapter 3, IC50 values of 3 and 20 mM were observed for acetoclastic and hydrogenotrophic methanogens, respectively, in granular sludge samples exposed to BES. Homoacetogens contributed about 6% of the

value of SHMA obtained without inhibitor, whereas the SAO-HM pathway contributed about 23% of the methane produced from acetate during the SAMA test. Therefore, correction factors were introduced to adjust values from both SAMA and SHMA routine tests to represent methanogens activity. The specific biomass concentration was calculated based on kinetics data obtained. Contributions of methanogenic acetoclastic and hydrogenotrophic archaea were estimated in 0.8% and 3.3% of the VSS content of the biosludge sample, respectively.

Finally, a case of solid waste digestion was exposed in Chapter 4, from batch incubation to subsequent operation in a CSTR digester; the dilution effect of substrate and inert over biomass was studied through model simulation, obtaining a correction factor of 79% for batch incubation and 25 to 30% for the CSTR digester. These correction factors were used to recalculate SAMA and SHMA from raw results. For the CSTR digester, tendencies changed after adjustment, evidencing the relevance of the application of this correction tool. Otherwise, raw activity results can underestimate the real activity of the biomass. The correction factors were used to perform more reliable calculations to determine inoculum size for SAMA and SHMA tests, obtaining higher values than the ones from wastewater systems protocols (up to 40 compared to 1 to 8 g_{VSS} L^{-1}) and similar time-test (10 to 12 h for SHMA, and about 40 h for SAMA), achieving a suitable test design. Beyond the particular considerations of the case, the use of correction factors extracted from more or less simple modelling

represents an advance in the design of activity tests to deal with long test periods and significant microbial growth that hindered the comparison of results. Besides, this methodology uncovers the influence of biomass dilution that sometimes leads to erroneous conclusions regarding microorganism biological capacity and a false diagnosis of the system.

Regarding observations surrounding the thesis work, during literature review in search of kinetics constants, a worrisome fact came to light: the use of kinetic values out of context or miscited references, at different temperatures and for obviously different types of substrate or systems, whose sources were articles of the decades of the 70s and 80s, collected in reviews of the 90s. In Appendix 1, a sensitivity analysis was performed for $Y_{X/S}$ to weigh its effect on the error calculation associated with SHMA determination methodology. It might be advisable to make this tool more widely used for calculating kinetics to safeguard the differences between the original conditions considered for the kinetic constant and the case of study.

As mentioned in Chapter 2, further studies must be performed about the influence of temperature on SHMA and SAMA. Researchers report SMA in a wide variety of incubation temperatures, making results harder to be compared. More research on this issue could help to decide whether a standardized temperature must be defined and become widely used among the scientific community. It is needed to compare the activity tested in the

laboratory and the activity in the source reactor. Given the wide annual range of temperatures in Uruguay and the absence of isolation in most of the reactors marketed for this area, it is relevant to achieve a deeper understanding of the influence of temperature on methanogenic activities. This study can be enriching to predict behaviour and anticipate problems for reactors during winter based on data collected in summer. Additionally, SHMA technique should be adjusted for psychrophilic and thermophilic biosludge since kinetic parameters vary significantly due to changes in the microbial population.

After reviewing the literature, it was found as a common fact that most of the researchers still only including SAMA, if any activity, considering that this tool is enough to adequately describe AD systems, and attributing the activity value exclusively to acetoclastic methanogens. However, in Chapter 3 for the case studied, the SAO-HM pathway contributed to approximately 23% of the methane produced exclusively from acetate during the SAMA test. This result calls into question the iconic values used to describe the AD scheme in articles and textbooks from the days of the publication of Lawrence and McCarty (1969) to the present day that associates a 70:30 or 72:28 to acetoclastic and hydrogenotrophic pathways from a more substrate complex than acetate. Thus, more attention should be paid to the SAO-HM pathway, and the SAOB population should be more deeply study in terms of presence and kinetics in AD systems.

In addition to the research sector, methanogenic activities should be further promoted in the industrial sector as cost-effective diagnostic tools. With these SAMA and SHMA tests, it is possible to analyse the evolution of methanogenic activities over time and see whether one pathway develops more than another due to inhibition problems. Nowadays, there is a lack of knowledge, and sources are unnecessary spent to re-inoculate reactors with sludge that come from many kilometres and even from other countries due to problems that could have been anticipated or solved based on these simple tools.

GLOSSARY

ANAEROBIC BIOSLUDGE: Sludge containing anaerobic microorganisms responsible of organic matter degradation. It could be classified as granular (presence of granules) or flocculent biosludge (without organized structure), depending on its structure.

ANAEROBIC DIGESTER: Applied indistinctly for systems used in the anaerobic degradation of solid waste, wastewater, or energy crops. The term 'solids digester' is used when referring exclusively to solids digestion systems, where the most common model corresponds to a Continuous Stirred-Tank Reactor (CSTR).

ANAEROBIC DIGESTION: Biochemical process during which organic matter is decomposed by microorganisms in the absence of oxygen and transformed into biogas as the final product.

ANAEROBIC DIGESTION MODEL NO. 1: Kinetic model introduced by the IWA Task Group for Mathematical Modelling of Anaerobic Digestion Processes to simulate complex substrate degradation (Batstone et al., 2002).

ANAEROBIC REACTORS: Anaerobic systems for wastewater treatment. There are classified depending on the installed capacity in lab-scale, bench-scale, pilot-scale, and full-scale. Among high-rate reactors (in terms of wastewater inlet application), it can be named the Up-flow Anaerobic Sludge Blanket reactor (UASB), the Expanded Granular Sludge Bed reactor (EGSB), and the Internal Circulation reactor (IC); the last two have a superior capacity due to external or internal recirculation. On the other hand, anaerobic lagoons are counted as low-rate anaerobic reactors.

BIOGAS: Mixture of gases produced by the anaerobic degradation of organic matter primarily consisting of methane and carbon dioxide.

BIOGAS UPGRADING: Separation process of methane from carbon dioxide and other gases present in biogas to obtain biomethane (gas enriched in methane).

BIOMASS: Renewable organic material from living organisms. In this thesis, it is strictly applied to biosludge fraction corresponding to microorganisms.

CHEMICAL OXYGEN DEMAND (COD): Oxygen equivalents consumed in the chemical oxidation of organic matter by a strong oxidant (e.g., potassium dichromate).

HALF-MAXIMAL INHIBITORY CONCENTRATION (IC50): Quantity of an inhibitory substance, typically expressed as a molar concentration, needed to inhibit in vitro a given biological process or biological component by 50% (in this thesis applied to microorganisms involved in methanogenesis).

HOMOACETOGENESIS: Conversion reaction of H_2/CO_2 to acetate conducted by homoacetogenic bacteria.

INOCULUM: Biosludge employed for inoculation of digesters and vials in biological tests (e.g., Specific Methanogenic Activity).

METATRANSCRIPTOMIC APPROACH: A microbiological approach for microbial diversity and dynamics analysis based on the study of microbiota genomic content (more specifically, mRNA).

METHANOGENESIS: Anaerobic respiration conducted by methanogens that uses carbon as an electron acceptor and results in methane production.

ORGANIC LOADING RATE (OLR): Amount of organic matter per unit of volume or per grams of biomass and per unit of time, applied to an anaerobic digester.

SOLIDS RETENTION TIME (SRT): Average time that solid fraction spends inside the digester.

SPECIFIC METHANE PRODUCTION (SMP): Amount of methane expressed as grams of COD per gram of biomass produced in a certain period of time.

SPECIFIC METHANE PRODUCTION RATE (SMPR): SMP expressed per unit of time.

SPECIFIC METHANOGENIC ACTIVITY (SMA): Maximum production rate of methane from a substrate, expressed in grams of COD per gram of biomass, distinguishing between Specific Acetoclastic Methanogenic Activity (SAMA) and Specific Hydrogenotrophic Methanogenic Activity (SHMA), with acetate or H_2/CO_2 (or formate) as the substrate, respectively.

SYNTROPHIC ACETATE OXIDATION: Conversion reaction of acetate to H_2/CO_2 conducted by syntrophic acetate oxidizing bacteria (SAOB) in syntrophy with hydrogenotrophic methanogens to make reaction feasible under the conditions involved in the anaerobic digestion.

VOLATILE FATTY ACIDS (VFA): Short-chain fatty acids composed mainly of C2–C6 carboxylic acids produced in the anaerobic digestion process.

VOLATILE SOLIDS (VS): Amount of volatile matter present in a sample; commonly used to quantify biomass in biosludge samples from solids digester.

VOLATILE SUSPENDED SOLIDS (VSS): Amount of volatile matter present in the solid fraction separated by centrifugation or filtration from a measured volume of solution; commonly used to quantify biomass in biosludge samples from wastewater systems.

APPENDIX 1

HYDROGENOTROPHIC ACTIVITY: A TOOL TO EVALUATE KINETICS OF METHANOGENS

Full article from Journal of Environmental Management (2020)

Journal of Environmental Management 270 (2020) 110937

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: http://www.elsevier.com/locate/jenvman

Research article

Hydrogenotrophic activity: A tool to evaluate the kinetics of methanogens

Evangelina Ripoll *, Iván López, Liliana Borzacconi

Facultad de Ingeniería, Universidad de la República, Julio Herrera y Reissig 565, Montevideo, 11300, Uruguay

ARTICLE INFO

Keywords:
Anaerobic digestion
Biodegradation kinetics
Hydrogenotrophic methanogenesis
Specific methanogenic activity
Waste digestion

ABSTRACT

Anaerobic-digestion-based technology is key to achieving sustainable water management and resource recovery. It is essential to understand the material flux and kinetics involved in methanogenesis to optimize the organic matter removal and methane production. In this sense, specific methanogenic activity is a cost-effective tool to characterize the biological activity of anaerobic biosludge, to monitor the performance of reactors, and study the kinetics of acetate and H_2 conversion to methane. Established protocols are applied for the acetoclastic activity test. However, hydrogenotrophic activity assay remains less widespread and is not standardized. In this work, the assay design for hydrogenotrophic activity is discussed and full calculation is presented, based on the kinetics for the H_2/CO_2 conversion to methane. An equation to calculate the inoculum size is proposed, suitable for a wide variety of types of biosludge: from a wastewater treatment plant to solid digesters, from a high-rate reactor to lagoons. The applied zero-order model fitted adequately to data for pilot-scale and full-scale anaerobic reactors: the p-values from the ANOVA F-test were below 1E-03; standard deviations for triplicate experiments were between 3 and 12%, coherent with the values found in the literature. Microbial growth during the test was negligible, below 1.2% of the biomass dosed in the vial. As a complement, acetoclastic activity was determined for each sample. The use of both acetoclastic and hydrogenotrophic activity is relevant for the study of the methanogenesis and gives a better characterization of the performance of the biosludge in anaerobic reactors rather than only using the specific acetoclastic methanogenic activity.

1. Introduction

Anaerobic digestion (AD) technology has a fundamental role in waste digestion and energy and material recovery (Kougias and Angelidaki, 2018). For decades, researchers have been studying the processes involved in the AD of organic substrates, in particular, the methanogenesis, considered to be the rate-limiting step when hydrolysis is favoured. Understanding the transformation steps that lead to methane production is crucial to optimize the operational conditions of anaerobic reactors for waste digestion and biogas production. Efforts have been made by several researchers to determine the relevance of the hydrogenotrophic and acetoclastic methanogenesis in different anaerobic systems, using tools such as DNA and mRNA quantification, radiolabelled molecules, and methanogenic activities. The use of microbiological tools could provide information on the microbial population composition and quantity and distinguish between active and non-active microorganisms depending on the technique applied (for example, mRNA quantification). However, these tools cannot determine how active microorganisms are. The knowledge of the kinetics involved is essential for understanding the dynamics of methanogenesis, and the tools selected for its study must consider this. The specific methanogenic activity (SMA) is one of these tools, defined as the maximum rate of methane produced from a substrate and expressed per weight of volatile suspended solids (VSS) as a rough estimation of the biomass (the term 'biomass' refers to the microorganisms present in biosludge).

In recent years, many studies using radiolabelled sodium acetate have shown that syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis is relevant for methane production in solid digesters operating under stressful conditions, such as high levels of total ammonia nitrogen, high levels of volatile fatty acids, extreme pH or elevated temperature (Bi et al., 2020; Fotidis et al., 2013; Ho et al., 2013; Jiang et al., 2018; Oosterkamp et al., 2019; Westerholm et al., 2016). In another vein, researchers have recently focused their attention on the upgrade of biogas to methane for fuel usage purposes by adding H_2 to convert the remaining CO_2 of the biogas into methane (Angelidaki et al., 2018). In this context, hydrogenotrophic methanogens have a crucial role in proper performance. Hence the utility of studying hydrogenotrophic methanogenesis in biogas production and upgrading

* Corresponding author.
E-mail address: eripoll@fing.edu.uy (E. Ripoll).

https://doi.org/10.1016/j.jenvman.2020.110937
Received 9 January 2020; Received in revised form 16 May 2020; Accepted 6 June 2020

systems. In this context, SMA test represents a cost-effective and quickest method to monitor the activity of pilot-scale and full-scale reactors for biogas production and upgrading. It can provide information on-site, where most times, other approaches are not readily available to operators of the AD systems or require significantly more experimental apparatus and expertise. The specific acetoclastic and hydrogenotrophic methanogenic activities (SAMA and SHMA respectively) indirectly measure the consumption rates of acetate and H_2, respectively. Thus, these two activity values can provide information about a system imbalance associated with methanogens inhibition (low activities) or help to find opportunities to increase the organic load rate applied to a reactor (high activities). Established protocols are followed by researchers to determine SAMA, using acetate as a substrate (Dolfing and Bloeman, 1985; Soto et al., 1993; van Loosdrecht et al., 2016). Also, some works are reporting SHMA (Bhattad et al., 2017; Gonzalez-Estrella et al., 2013; Hao et al., 2017; Keating et al., 2016; Liu et al., 2016; Regueiro et al., 2012). Nevertheless, there is a lack of standardization among the techniques used to determine SHMA. The novelty of this work mainly consists of outlining the full calculation structure to accomplish the main objective: re-design and select the test conditions for a wide variety of types of biosludge (from a wastewater treatment plant to solid digesters, from a high-rate reactor to lagoons) and lay a basis for a discussion of inter-laboratory work and standardization of the SHMA technique.

Coates et al. (1996) assayed a variety of test conditions for SHMA considering biosludge from a single source and obtained an experimental concentration range for the biosludge inoculum in the batch test. In the same work, when the design was applied to a biosludge sample from a solid digester with low activity, the assay took more than 70 h, which implied significant microbial growth, distorting the SHMA determination. The same issue is observed in the work of Bhattad et al. (2017), where test bottles containing a VSS concentration of $\leq$0.3 g L^{-1} were used, obtaining assays of about 50 to 100 h, and no adjustment made in the design for those samples for which lower activities were expected. One of the specific objectives of this work is to solve this drawback; thus, a calculation formula is proposed and tested to size the inoculum used in the SHMA assay based on the biosludge characteristics and operational data of the source reactor. In the present work, the basis for the design of the SHMA test is discussed, using the detection method proposed by Coates et al. (1996), but designing the test conditions considering the kinetics of the H_2/CO_2 conversion to methane. The second specific objective of this work is verifying the zero-order model fit to data, and the suitability of the hypothesis simplifications applied when the stoichiometric formula of the H_2/CO_2 conversion to methane is used to calculate the methane production during a SHMA test. The third specific objective is testing the conditions resulting from the SHMA assay design in a wide variety of samples. In this work, eight different biosludge samples collected from pilot-scale and full-scale anaerobic systems are analysed using the design discussed for the SHMA test. Additionally, the SAMA is assessed to complete the activity characterization in the studied systems. The fourth specific objective is to correlate the SAMA and SHMA of the biosludge to the performance of the source reactor. The results obtained in our laboratory for the SAMA and SHMA are compared one to each other and with other values found in the literature. Additionally, a discussion regarding the influence of the operational conditions on the activity values is introduced in this article.

2. Materials and methods

2.1. Detection method selection

Consider the stoichiometric formula for the H_2/CO_2 conversion into methane:

$$4\ H_{2\ (g)} + \ CO_{2\ (g)} \rightarrow CH_{4\ (g)} + 2H_2O_{\ (l)} \quad (1)$$

A decrease in the total number of moles associated with gaseous species is observed during the conversion, producing depletion in the headspace pressure when the reaction is carried out in a closed system. In the literature, two different strategies for hydrogenotrophic activity measurement have been reported. On one hand, Dolfing and Bloeman (1985) recommended a methodology based on the measurement of the pressure headspace combined with the composition analysis of the gas samples by gas chromatography (GC). When those conditions were tested, some drawbacks were identified: (i) Since headspace pressure decreased during the experience not only due to the H_2/CO_2 conversion to methane but also because of the gas sampling, the vials needed to be re-pressurized with H_2/CO_2, increasing the safety risks, the gas loss and the length of the assay period. (ii) During the first period of the test, it was found that the detection limit was close to the concentration values obtained for methane when analysing a gas sample using GC, increasing the relative experimental error associated with the first measures. On the other hand, Coates et al. (1996) proposed a methodology based on the measurement of headspace pressure. Pressure depletion was correlated with methane generation using the stoichiometric relation for the chemical conversion of H_2/CO_2 to methane expressed in Eq. (1).

The latter method was chosen for SHMA experiences since it was shown to be more practical, less time-consuming and safer than the method proposed by Dolfing and Bloeman (1985). Then, the moles of methane Δn_{CH4} (mol) produced from H_2/CO_2 during a certain interval of time Δt (days) could be calculated as follows:

$$\Delta n_{CH4} = \ -\Delta n_{gas}/4 = \ -\Delta P\ \ V_{hs}/(4\ \ R\ \ T) \quad (2)$$

where ΔP (atm) is the pressure depletion inside headspace; V_{hs} (L) is the headspace volume; R (L atm K^{-1} mol^{-1}) is the universal gas constant; and T (K) is the temperature.

2.2. Kinetic model

Considering the Monod kinetics for the microbial growth rate r_X (g_{VSS} L^{-1} d^{-1}):

$$r_X = \mu_m\ \ X\ \ S/(K_S + S) \quad (3)$$

where μ_m (d^{-1}) is the maximum specific growth rate; X (g_{VSS} L^{-1}) is the microbial concentration (biomass); S (g_{COD} L^{-1}) is the substrate concentration; and K_S (g_{COD} L^{-1}) is the half-saturation constant.

Then, the substrate consumption rate r_S (g_{COD} L^{-1} d^{-1}) is based on the microbial yield $Y_{X/S}$ (g_{VSS} g_{COD}^{-1}):

$$r_S = r_X/Y_{X/S} = \mu_m\ \ X\ \ S\ \ /[Y_{X/S}\ \ (K_S + S)] \quad (4)$$

Thus, the specific substrate consumption rate k (g_{COD} g_{VSS}^{-1} d^{-1}) can be expressed as:

$$k = r_S/X = r_X\ /(X\ \ Y_{X/S}) = \mu_m\ \ S/[Y_{X/S}\ \ (K_S + S)] \quad (5)$$

As a result, the expression for the maximum specific activity k_m (g_{COD} g_{VSS}^{-1} d^{-1}) corresponds to:

$$k_m = \mu_m/Y_{X/S} \quad (6)$$

To determine the maximum specific rate for substrate consumption, k_m, the design of the assay must accomplish the following conditions: (i) the concentration of the substrate must be significantly higher than the half-saturation constant ($S \gg K_S$), which means 'zero-order conditions'; and (ii) the microbial growth should be negligible ($X \sim X_0$; where '0' subscript indicates initial conditions). In the hydrogenotrophic activity test, H_2 represents the limiting reagent since CO_2 is in excess due to the use of sodium hydrogen carbonate as the system buffer. For more details, please refer to Section 2 of the Electronic Supplementary Material (ESM). The volatile suspended solid (VSS) content is considered a representation of the biomass present in the biosludge.

2.3. Inoculum size calculation

The calculation of the amount of biosludge is crucial for a proper design of the duration of the batch assay. The volume aliquot V_B (L) of biosludge added to vial is calculated as:

$$V_B = \Delta n_{CH4} \; C/(\Delta t \; X \; k_m) \quad (7)$$

where $C = 64\ g_{COD}\ mol^{-1}$ is the conversion factor from moles of methane to grams of COD; X ($g_{VSS}\ L^{-1}$) is initially quantified by the VSS determined for the original biosludge sample; and k_m ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) is a preliminary value for SHMA based on historical data and considering the source reactor for the particular biosludge sample.

Two assumptions are considered for Eq. (7): (i) the specific methane production rate during the batch test is constant and equal to k_m; (ii) the growth during the test is negligible ($X \sim X_0$). The first assumption is accomplished when zero-order conditions are ensured. The second assumption relies on the microbial growth rate for hydrogenotrophic methanogens and the time-length of the batch test, which is associated with the Δt (days) and the number of planned measures. Then, Δn_{CH4} (mol) can be substituted by the expression in Eq. (2), obtaining the following expression for V_B (L) calculation:

$$V_B = - \; \Delta P \; V_{hs} C/(4 \; R \; T \; \Delta t \; X \; k_m) \quad (8)$$

2.4. Incubation temperature selection

Activity tests should be carried out under conditions that closely mimic the environment of a given system, for example, temperature, pH and mixing (Angelidaki et al., 2007; Holliger et al., 2016). In the literature, it is possible to find the SMA tests for mesophilic biosludge at different temperatures: (i) 30 °C (Gonzalez-Estrella et al., 2013; Karri et al., 2006); (ii) 35 °C (Bhattad et al., 2017; Hao et al., 2017; Liu et al., 2016); and (iii) 37 °C (Keating et al., 2016; McKeown et al., 2009; Regueiro et al., 2012). Modelling based on the Arrhenius equation, and experiences using VFA or acetate as substrate found in the literature, support the idea of a 50% increase in the SMA at 35 °C compared to the SMA at 30 °C (Lin et al., 1987; Rittmann and McCarty, 2020; Souto et al., 2010). In mild weather countries, the energy obtained from biogas is not enough to heat full-scale reactors treating diluted wastewater, then its operational temperature rarely exceeds 30 °C. On the other hand, it is possible to heat systems treating concentrated wastewater or solid waste due to a positive energy balance and maintain the operational temperature close to the optimal temperature for the mesophilic microbial population (35 to 37 °C). Pilot-scale reactors were operated at 30 °C. Consequently, the incubation temperature was set at 30 °C in the experiments presented in this paper to establish correlations between the SHMA and SAMA activities and the performance of the reactors analysed.

2.5. SHMA assay setup

Biosludge samples from pilot-scale and full-scale anaerobic reactors were assayed. The SHMA tests were carried out in triplicate in SCHOTT® 250 mL glass bottles (henceforth referred to as 'vials', 308 mL of total volume). For each vial, the screw cap was equipped with a rubber O-ring to improve sealing and a screw nut, a rubber septum, a needle, and a three-way medical stopcock to allow initial gas flushing and pressure measurement. Vial sealing was previously tested by pressurizing each vial to 900 mmHg with N_2. Before the SHMA test, the biosludge sample was flushed with N_2 to displace O_2 inside a SCHOTT® vial (250 mL or 500 mL) and incubated in an air-thermostated chamber at 30 °C until the remaining substrate was oxidized. The vial was monitored until methane production was negligible: after 3 days for washed samples from wastewater reactors, at least 7 days for samples from solid digesters; then, the exhausted biosludge was used for the SAMA and SHMA assays. If organic matter present in the inoculum is not eliminated, live blanks using N_2/CO_2 should be run and methane production discounted from the production recorded for each test vial.

The VSS content was determined following a standardized gravimetric method, using a Shimadzu Libror model AEX-200B analytical balance with a resolution of 1E-04 g (Rice et al., 2017). This method was used to determine the VSS content in the biosludge sample before the test, and then in the vials at the end of the test. The biosludge aliquot V_B was calculated using Eq. (8), assuming a pressure depletion of 100 mmHg every 1 or 2 h to minimize the error associated with the pressure measurement. The aliquot of biosludge was dispensed and an aqueous solution of sodium hydrogen carbonate was dosed to reach 100 mL of total volume of liquid (V_L) and a pH of 7.8 to 8.0 in vials, before gas flushing with H_2/CO_2. The pH was expected to decrease after flushing because of the CO_2 dissolution. For initially neutral samples, a concentration of approximately 3 g $NaHCO_3\ L^{-1}$ was obtained inside the vials. A fourth vial was prepared under the same conditions as for the triplicate test samples to be sacrificed after gas flushing to verify that the pH was close to neutral when the acid-alkaline balance was restored. Next, the vials were pressurized at 900 mmHg (gauge pressure) with H_2/CO_2 (80/20) and depressurized three times to displace the oxygen from the headspace and for a further sealing test. After that, the vials were pressurized at 760 mmHg (gauge pressure) with H_2/CO_2 (80/20), start time was recorded and then vials were incubated in a New Brunswick™ Innova® model 2100 orbital platform shaker at 180 rpm, inside of an air-thermostated chamber at 30 °C.

During the SHMA test, the gas pressure in the headspace was measured using a Sper Scientific® model PS100-2BAR pressure transducer (maximum range: 1500 mmHg, resolution: 2 mmHg) every 1 or 2 h according to the design. When necessary, the interval of time elapsed between two consecutive measurements was re-adjusted after observing the biosludge performance during the first period of the test. At the end of the test, the pH was checked to determine whether it was in the optimal range for the analysed anaerobic system. Additionally, the VSS was determined at the end of the test for each vial (Rice et al., 2017). Gas samples were taken from vials headspace at the end of the test to analyse its composition using a Shimadzu® gas chromatograph model GC-2014 equipped with a Restek® ShinCarbon model ST 100/120 2 m 1 mm ID 1/16" OD Silco packed column.

2.6. SHMA calculation

The pressure depletion ΔP (atm) was calculated with the recorded values of the gauge pressure and applied to determine Δn_{CH4} (mol) using Eq. (2) for each interval of time Δt (days) between two consecutive measures. The cumulative value n_{CH4} (mol) could be calculated for the methane produced during a time elapsed t (days), and then, the cumulative specific methane production SMP ($g_{COD}\ g_{VSS}^{-1}$) could be determined as:

$$SMP = n_{CH4} \; C/X \; V_L \quad (9)$$

where $C = 64\ g_{COD}\ mol^{-1}$ is the conversion factor from moles of methane to grams of COD, as in Eq. (7); V_L (L) is the volume of liquid inside the test vial. For this calculation, the final VSS determined for each vial at the end of the test is considered a better estimation for X ($g_{VSS}\ L^{-1}$) than the initial VSS of the sample, since the aliquot of biosludge might not conserve the original concentration after being dosed in the vial, especially for granular biosludge, and the testing for VSS is destructive.

Eq. (2) is valid for a constant ratio of partial pressures for CO_2 and H_2 (p_{CO2}:p_{H2}) of 1:4 and a negligible methane dissolved fraction. Due to acid-base equilibria, the p_{CO2} is higher than predicted based on the stoichiometric formula (1), which implies a lower ΔP. Then, the methane production would be underestimated using Eq. (2). On the other hand, methane production is overestimated when assuming that carbon is exclusively converted to methane by not including the microbial yield $Y_{X/S}$ in Eq. (2). Since these effects are contraposed, the

compensation would depend on the particular assay conditions and the assumed value for $Y_{X/S}$. Performing a comparison between the moles of methane calculated using Eq. (2) and the theoretical moles obtained considering the COD removed for methane, based on the microbial yield $Y_{X/S}$, the relative error values were +1.3% ($Y_{X/S}$ of 0.04 $g_{COD}\ g_{COD}^{-1}$) to −4.1% ($Y_{X/S}$ of 0.08 $g_{COD}\ g_{COD}^{-1}$). Even with the high uncertainties regarding $Y_{X/S}$, the resulting relative error was less than 5% in absolute terms. The experimental error for the SHMA assay was estimated to ponder the order of magnitude of these values (for more details, please consult Section 2 of the ESM).

A plot of the SMP ($g_{COD}\ g_{VSS}^{-1}$) versus t (days) was constructed for the triplicate samples and a least-squares regression line was adjusted to data corresponding to each test vial and confidence interval was calculated for the hydrogenotrophic activity of each sample by using the Data Analysis ToolPak of Microsoft® Office Excel® 2016 Software. The ANOVA F-test was run to check the adequacy of zero-order model to fit data (see Subsection 2.2 Kinetic model) and the p-value determined for each test vial. The zero-order model was considered adequate when the p-value was lower than 1E-03. Then, the SHMA ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) was determined as the mean value of the slopes calculated for the trendlines corresponding to the triplicate experiments, whether p-values were lower than 1E-03. Data from vials with a p-value higher than 1E-3 was dismissed, and the source of discordance was studied.

2.7. SAMA assay setup

The SAMA tests were carried out in triplicate at 30 °C in SCHOTT® 100 mL glass bottles (137 mL of total volume) with an initial concentration of sodium acetate of 3 $g_{COD}\ L^{-1}$. The protocol was based on the methodology of headspace pressure measurement and determination of biogas composition proposed by Soto et al. (1993). These gas samples were analysed using a Shimadzu® gas chromatograph model GC-2014 equipped with a Restek® ShinCarbon model ST 100/120 2 m 1 mm ID 1/16" OD Silco packed column under the following conditions: temperature of injection port, packed column, and TCD were adjusted at 110 °C, current in TCD was set to 60 mA and argon N50 carrier was set at a flow rate of 21 mL min^{-1}.

3. Results and discussion

3.1. Verification of zero-order conditions for the SHMA assay

To verify the zero-order conditions for substrate consumption ($S \gg K_S$), the concentration of H_2 dissolved in the aqueous bulk S ($g_{COD}\ L^{-1}$) was calculated at the beginning and the end of the assay. Considering the coefficient for the solubilization of H_2 in pure water at 30 °C, 1.7E-02 $cm^3\ cm^{-3}\ atm^{-1}$, and the partial pressure for H_2 inside the headspace at the beginning of the test, 1.6 atm absolute (which corresponds to a gauge pressure of 760 mmHg for the gas mixture of H_2/CO_2 80/20), an S_0 of 2.0E-02 $g_{COD}\ L^{-1}$ was obtained. The SHMA assays were carried out until gauge pressure in headspace dropped to 200 mmHg. A percent of H_2 close to 60% was found inside headspace, which implied a concentration of dissolved H_2 of about 9.0E-03 $g_{COD}\ L^{-1}$.

Regarding K_S consideration, experimental values of K_S for H_2 consumption collected from the literature showed a wide variation, from 1.2E-07 to 2.1E-03 $g_{COD}\ L^{-1}$ (refer to Table A1 in Section 1 of the ESM). External and internal mass transfer limitations for H_2 could lead to an overestimation of K_S (Giraldo-Gomez et al., 1992). When considering the external mass transfer, Coates et al. (1996) and Dolfing and Bloeman (1985) suggested that the mass transfer of H_2 from the headspace to the liquid bulk could be rate-limiting, and they proposed that vigorous shaking could be applied during vial incubation as an improvement. Besides, the mass transfer from the liquid bulk to the biosludge surface was improved. In our laboratory, vials with a liquid to headspace volume ratio of 1:2 were incubated vertically in a New Brunswick™ Innova® model 2100 orbital shaker at 180 rpm; that was the maximum operative value without causing significant granule disruption in the biosludge samples assayed. When referring to the internal mass transfer, a dependence on the diffusion phenomenon was observed, related to the physical properties of the biosludge. On this issue, Dolfing (1985) found a positive correlation between the size of the granules and apparent K_S determined using formate or acetate. Coates et al. (1996) reported a K_S of 3.6E-04 $g_{COD}\ L^{-1}$ for granular biosludge at 37 °C. This value is one order of magnitude higher than the values for pure cultures and non-granular biosludge reported in the literature, even when considering different incubation temperatures. The value of K_S assumed in the ADM1 development, 2.5E-05 $g_{COD}\ L^{-1}$ at 35 °C in mesophilic high-rate systems (Batstone et al., 2002), is one order of magnitude lower than the value reported by Coates et al. (1996). Based on these considerations, the K_S value reported by Coates et al. (1996) was considered as a conservative value for granular sludge, when there was no external mass transfer limitation. The S values were two orders of magnitude higher than the K_S, considering a pressure depletion inside headspace from 760 to 200 mmHg (gauge pressure) during the assay. The test conditions ensured S values that were one order of magnitude higher, even compared with values of K_S that were affected by external mass transfer. When the influence of temperature over Ks was introduced, the results still indicating the accomplishment of the zero-order condition during the whole testing period; by performing a conservative calculation, a value of 1E-03 $g_{COD}\ L^{-1}$ for Ks at 30 °C was obtained (Donoso-Bravo et al., 2009; Lawrence and McCarty, 1969). On the other hand, Robinson and Tiedje (1982) obtained an K_S of 9.1E-05 $g_{COD}\ L^{-1}$ for biosludge from a solid digestor at 30 °C, one order lower than the value obtained by Coates et al. (1996) for granular sludge, probably due to the difference in the structure of biosludge. Thus, it can be deduced the accomplishment of the zero-order conditions during the SHMA test from 760 to 200 mmHg (gauge pressure) for solid digesters biosludge (valid for non-granular sludge).

3.2. Verification of zero-order conditions for the SAMA assay

At the beginning of the SAMA batch test, the concentration of sodium acetate was 3 $g_{COD}\ L^{-1}$ inside each vial. By the end of the test, the concentration of acetate in each vial was higher than 1 $g_{COD}\ L^{-1}$. Thus, the kinetics of Monod for microbial growth at zero-order conditions were accomplished when a half-saturation constant for acetate of 0.05–0.30 $g_{COD}\ L^{-1}$ was considered for mesophilic acetoclastic *archaea* (Batstone et al., 2002), obtaining the maximum specific activity for the acetoclastic methanogenesis (see Eqs. (3)–(6) applied to this step).

3.3. Microbial growth

Based on the design proposed for the SHMA assay, a range of headspace pressures from 760 to 200 mmHg and an interval of time between measurements of 1.5 or 2 h were suitable for full data recording; the time elapsed to fulfil the test was 8–10 h. In most cases, the aliquot V_B contained from 500 to 800 mg of VSS depending on the biosludge characteristics. Microbial growth during the test was estimated in 0.8 to 1.2% of the initial VSS dosed in vials when considering an $Y_{X/S}$ of 0.06 $g_{COD}\ g_{COD}^{-1}$ for the hydrogenotrophic population (Batstone et al., 2002); therefore, the effect of microbial growth over VSS was negligible when comparing to the experimental error for the SHMA assay (for more details about experimental error see Section 2 of the ESM). Also, the cell decay is negligible, since it is normally considered to be 10% of the cell growth (from ADM1) (Batstone et al., 2002).

3.4. Experimental and literature values for SAMA and SHMA

The temperature inside the reactor, the type of substrate and the organic load rate OLR ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) applied, and the presence of inhibitors are among the factors that shape the microbial population, affecting the relative abundance and activity of the acetoclastic and

hydrogenotrophic microorganisms. In this work, biosludge samples from eight different anaerobic reactors were analysed to determine their SAMA and SHMA. For the SHMA assays, plots showing the specific methane production accumulated SMP ($g_{COD}\ g_{VSS}^{-1}$) over time t (days) for the different biosludge samples are presented in Fig. 1 and Fig. 2. Standard deviations associated with triplicate measurements were between 3 and 10% for SHMA and between 3 and 12% for SAMA. Whether one slope calculated from these graphs is significantly higher and has no concordance with the other two, the corresponding vial is under suspicion of gas loss. Therefore, it is advisable to test the sealing of the suspect vial to rule out this problem. When adjusting data graphed in Figs. 1 and 2 by least-squares regression, the R^2 values obtained were between 0.9924 and 0.9999, except for the vial 3 of sample (h) (R^2 of 0.9594). The ANOVA F-test was performed for data from each vial. The p-values of the F-test were lower than 1E-03 (from 4E-06 to 9E-04), except for the vial 3 of sample (h) (p-value of 0.02). These provide evidence of an adequate fit to the zero-order kinetic model, accordingly with verification performed previously based on the K_S and S magnitudes (see Subsection 3.1 Verification of zero-order conditions for the SHMA assay). The VSS concentration inside the vial 3 of the sample (h) doubles the VSS concentration inside the other two vials (vial 1 and 2). The last two measures were under 200 mmHg, and the zero-order model was unaccomplished. Then, only the slopes from vials 1 and 2 were considered in the calculation of SHMA for sample (h). The confidence intervals were between ±2.0% and ±8.2% for samples (a) to (g) for a confidence level α of 95%; on the other hand, sample (h) had a confidence interval of ±16.6%. The error associated with the application of Eq. (2) for methane production calculation was on the same order of magnitude as the experimental error involved in the SHMA assay (refer to Subsection 2.6 of the present text and Section 2 of the ESM).

The results for these methanogenic activity tests, as well as the organic loading rate OLR and the specific methane production rate in each reactor SMPr ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$), when available, are presented in Table 1. The OLR per gram of VSS (henceforth 'OLR') is used instead of per volume since it allows for distinguishing between reactors with the same working volume but with different amounts of biomass. The SMPr was calculated considering the methane production and the VSS in the reactor. Additionally, the SMPr could be determined using the specific OLR, the efficiency of COD removal observed in the reactor and assuming the value of microbial yield of 0.10 $g_{COD}\ g_{COD}^{-1}$ for complex substrates and the COD:VSS ratio of 1.42 $g_{COD}\ g_{VSS}^{-1}$ for microbial cells (from ADM1) (Batstone et al., 2002).

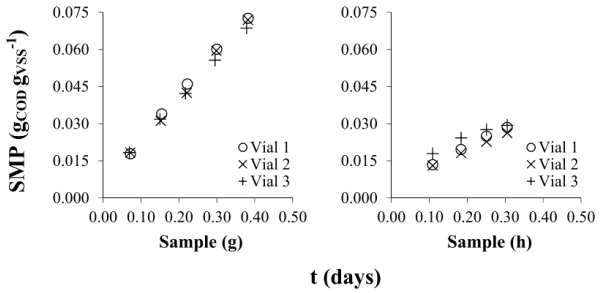

Fig. 2. Specific Methane Production (SMP) accumulated during the SHMA assay for biosludge with low activity: (g) Slaughterhouse lagoon; and (h) Manure bench-scale CSTR.

In Table 1, samples indicated with (a) to (f) correspond to granular biosludge; sample (g) corresponds to a flocculent sludge from an anaerobic lagoon and sample (h) to a solids digester. For samples (b), (e) and (h) shown in Table 1, the SMPr ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) observed in the reactor was higher than the corresponding value for SAMA ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$), even though activities were measured at optimal conditions. The former evidenced that the SAMA gave insufficient information about the biological characteristics of the biosludge to explain the performance observed in the reactor; hence, the relevance of including both methanogenic activities SAMA and SHMA in the study to consider the acetoclastic and hydrogenotrophic methanogenesis. When comparing the SAMA and SHMA ($g_{COD}\ g_{VSS}^{-1}\ d^{-1}$) from Table 1, for samples (c) and (f),

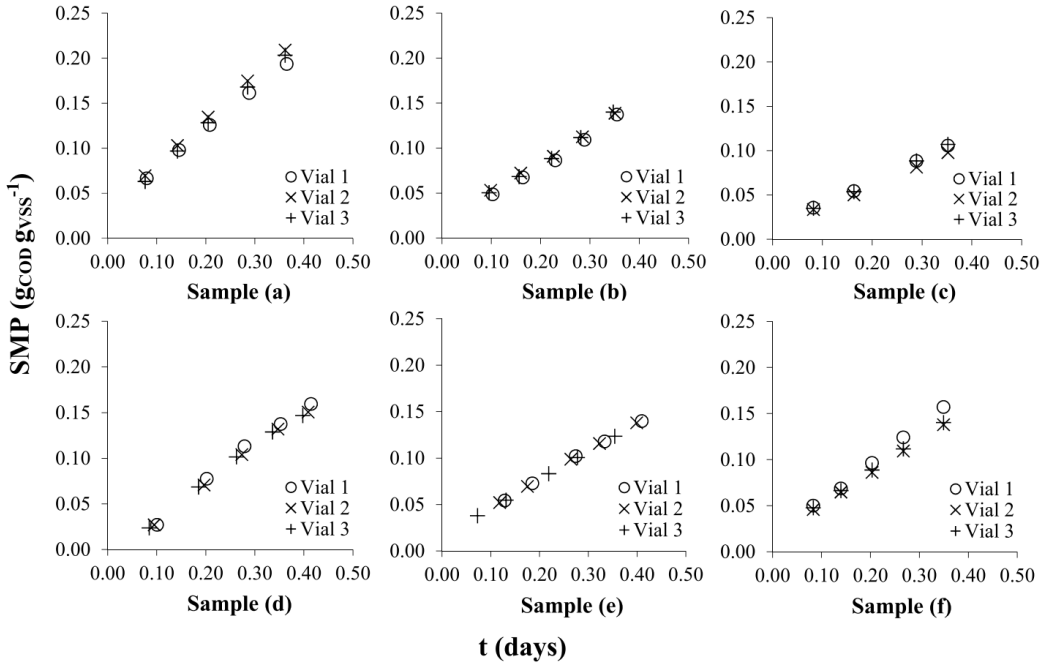

Fig. 1. Specific Methane Production (SMP) accumulated during the SHMA assay: (a) Dairy full-scale UASB; (b) Dairy pilot-scale EGSB; (c) Beverage pilot-scale EGSB; (d) Brewery full-scale IC; (e) Beverage bench-scale EGSB; and (f) Protein bench-scale EGSB.

Table 1
SAMA and SHMA at 30 °C obtained for anaerobic biosludge from different sources and operational data. The standard deviation is reported between brackets for the triplicate test samples, except for sample (h) marked with (*) that corresponds to duplicates.

Biosludge sample ID	SAMA (g_{COD} g_{VSS}^{-1} d^{-1})	SHMA (g_{COD} g_{VSS}^{-1} d^{-1})	OLR (g_{COD} g_{VSS}^{-1} d^{-1})	SMPr (g_{COD} g_{VSS}^{-1} d^{-1})
(a) Dairy full-scale UASB	0.22 (0.02)	0.48 (0.03)	0.18 (0.03)	0.14 (0.03)
(b) Dairy pilot-scale EGSB	0.28 (0.01)	0.35 (0.01)	0.48 (0.05)	0.41 (0.06)
(c) Beverage pilot-scale EGSB	0.27 (0.01)	0.26 (0.01)	0.19 (0.03)	0.15 (0.03)
(d) Brewery full-scale IC	0.34 (0.04)	0.41 (0.01)	0.26 (0.04)	0.19 (0.04)
(e) Beverage bench-scale EGSB	0.27 (0.01)	0.31 (0.01)	0.35 (0.03)	0.30 (0.03)
(f) Protein bench-scale EGSB	0.54 (0.02)	0.48 (0.03)	0.47 (0.05)	0.39 (0.05)
(g) Slaughterhouse lagoon	0.091 (0.003)	0.159 (0.007)	< 0.20	< 0.15
(h) Manure bench-scale CSTR	0.031 (0.003)	0.072 (0.007)(*)	0.08 (0.01)	0.05 (0.01)

the SHMA values were lower than those for SAMA, representing 85 to 93% of SAMA. Even in these samples it is still relevant to study both activities. In the other cases, the SHMA was higher than the SAMA; for example, SHMA was double the SAMA for samples (a) and (h). Due to the source of sample (g), an anaerobic lagoon, the biosludge had a higher age than the samples from the high-rate reactors, and the OLR applied is lower. The higher concentration of inert organic material dilutes the microbial fraction in the total VSS, producing lower activities (both SAMA and SHMA) than the activities observed for the high-rate wastewater reactors, as in samples (a) to (f) of Table 1. Additionally, this effect can also be seen by comparing the slopes of the graphs presented in Figs. 1 and 2.

In Table 2, activity values from the literature are summed. Standard deviations obtained by Bhattad et al. (2017), Gonzalez-Estrella et al. (2013), Hao et al. (2017), Keating et al. (2016), and McKeown et al. (2009) are similar to those obtained in our laboratory for SAMA and SHMA. However, in the other cases shown in Table 2, values for the standard deviation of approximately 20% are observed. The assay temperature must be considered when comparing the activity values from Tables 1 and 2. When the incubation temperature is raised in a suitable range for the microbial population, the specific substrate consumption rate is expected to increase. In most cases of Table 2, SHMA and SAMA were carried out at 37 °C, leading to an increase in the biosludge activity when compared to the values obtained at 30 °C. The kinetics variation for SAMA and SHMA with temperature could differ between each other, producing changes in the SHMA:SAMA ratio when the temperature increased from 30 to 37 °C.

Analyzing the activity values presented in Table 2, it can be noted that there are cases where the SHMA was lower than the SAMA, from 39 to 80% of SAMA (Liu et al., 2016; McKeown et al., 2009). However, there are examples of the SHMA being higher than the SAMA in Table 2, in some cases by one order of magnitude (Gonzalez-Estrella et al., 2013; Molina et al., 2008; Regueiro et al., 2012).

When considering biosludge from solids digesters, the microbial fraction in the total VSS is diluted by the presence of inert materials and remaining substrate; thus, in Table 1, sample (h) shows lower methanogenic activities than samples (a) to (f), in some cases by one order of magnitude. SAMA and SHMA for sample (h) are of the same order of magnitude as the values reported by Liu et al. (2016) as seen in Table 2. It is also important to note that the retention time for the biosludge and the OLR in solid digesters are markedly different from those in wastewater reactors. Therefore, data from solid digesters and wastewater reactors should be analysed separately. During the design of the SHMA test for samples (g) and (h), the aliquot of biosludge V_B determined using Eq. (8) implied concentrations of VSS in vials of 16 g_{VSS} L^{-1} and 35 g_{VSS} L^{-1} respectively, significantly higher than the ones suggested by Coates et al. (1996) (1.7 to 8 g_{VSS} L^{-1}). Based on this design, the time elapsed in the SHMA assay of these samples was 7 to 10 h.

Table 2
SAMA and SHMA values for mesophilic anaerobic systems found in the literature. Standard deviation is reported between brackets for the triplicate test samples. References: [1] Molina et al. (2008), [2] McKeown et al. (2009), [3] Regueiro et al. (2012), [4] Gonzalez-Estrella et al. (2013), [5] Liu et al. (2016), [6] Keating et al. (2016), [7] Bhattad et al. (2017), [8] Hao et al. (2017). References of the protocol applied for SHMA: (I) Coates et al. (1996), (II) Soto et al. (1993), (III) Internal.

Biosludge Sample	Type of waste	T (°C)	SAMA (g_{COD} $g_{VSS}^{-1}d^{-1}$)	SHMA (g_{COD} $g_{VSS}^{-1}d^{-1}$)	Reference
Lab-scale UASB	Ethanol-based	37	0.44 (0.10)	1.10 (0.20)	[1](III)
Lab-scale UASB	Carbohydrate	37	0.72 (0.08)	1.87 (0.04)	
Lab-scale UASB	Protein-based	37	0.57 (0.06)	1.87 (0.36)	
Full-scale IC	Alcohol	37	0.223 (0.003)	0.146 (0.006)	[2](I)
Lab-scale EGSB.AF	VFA	37	1.06 (0.08)	0.85 (0.03)	
Full-scale CSTR	Sewage sludge	37	0.15 (0.01)	0.55 (0.15)	[3](II)
Full-scale UASB	Brewery	37	0.33 (0.05)	0.79 (0.04)	
Full-scale CSTR	Dairy/fish	37	0.29 (0.02)	0.84 (0.08)	
Lab-scale CSTR	Glycerine/pig manure	37	0.01 (0.00)	0.37 (0.07)	
Full-scale CSTR	Sugar process	37	0.23 (0.02)	0.45 (0.06)	
Full-scale CSTR	Yeast process	37	0.05 (0.02)	0.83 (0.08)	
Full-scale UASB	Brewery	30	0.32 (0.03)	0.57 (0.03)	[4](III)
Lab-scale CSTR high TS	WWTP Sludge	35	0.027 (0.001)	0.018 (0.001)	[5](III)
Lab-scale CSTR low TS	WWTP Sludge	35	0.054 (0.001)	0.021 (0.004)	
Lab-scale hybrid FF	Sewage-based	37	0.50 (0.07)	0.91 (0.10)	[6](I)
Lab-scale CSTR	Not-fat-milk	35	0.21 (0.01)	0.72 (0.07)	[7](I)
Full-scale CSTR	WWTP Sludge	35	0.014 (0.005)	0.045 (0.002)	[8](I)

3.5. Future research

For the SHMA calculation, the conversion of H_2/CO_2 to methane was supposed to be carried out by hydrogenotrophic archaea. The contribution of homoacetogenic population was considered negligible (Coates et al., 1996). This assumption must be verified. Selective inhibitors could be incorporated into batch activity tests to puzzle down the different steps involved in methanogenesis, including acetoclastic and hydrogenotrophic methanogenesis, homoacetogenesis, and syntrophic oxidation of acetate. Similarly, syntrophic acetate oxidation must be considered when assessing SAMA. The standardization of this type of technique (SHMA and SAMA) is crucial to compare results between different reactors and laboratories. Inter-laboratory experiences would enrich the discussion and provide a basis to analyse the influence of different variables and consider variations between available equipment to achieve comparable conditions. As an example, external mass transfer limitations should be avoided or made comparable. Additionally, further studies must be performed to determine the influence of temperature on

the SHMA and SAMA. This information would provide a basis to weight this influence and decide whether a standardized temperature must be defined and become widely used among the scientific community. Also, it is necessary to adjust the SHMA technique for biosludge from psychrophilic and thermophilic reactors, since the kinetic parameters could vary significantly with changes in the temperature, mainly associated with changes in the microbial population.

4. Conclusions

This work presented full calculation and laid a basis for a discussion of inter-laboratory work and a standardization of the SHMA technique. The equation proposed to size inoculum made the SHMA determination possible for low activity biosludge within 7 to 10 h, as well as for biosludge from high-rate reactors, with negligible microbial growth. The zero-order model showed an adequate fit to data, and the hypothesis simplifications used for the calculation of methane production during the SHMA test introduced an error comparable to the experimental error. The SHMA, together with SAMA, gives a better explanation to the performance of the biosludge in the reactors (represented by the parameter of specific methane production in the reactor, SMPr).

Funding

Authors want to thank to the funding agency CSIC (Comisión Sectorial de Investigación Científica) for financing of experimental work through the R&D Groups program (#175725).

Declaration of competing interest

There are no conflicts to declare.

CRediT authorship contribution statement

Iván López: Methodology, Formal analysis, Writing - review & editing. **Liliana Borzacconi:** Conceptualization, Supervision, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2020.110937.

References

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, L., Guwy, A., Jenicek, P., Kalyuzhnui, S., van Lier, J., 2007. Anaerobic Biodegradation, Activity and Inhibition (ABAI) Task Group Meeting. In: Prague. Kgs. Lyngby. Institute of Environment & Resources, Technical University of Denmark, 9th to 10th October 2006.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: current status and perspectives. Biotechnol. Adv. 36, 452–466. https://doi.org/10.1016/j.biotechadv.2018.01.011.

Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S.V., Pavlostathis, S.G., Rozzi, a., Sanders, W.T., Siegrist, H., Vavilin, V.a., 2002. The IWA anaerobic digestion model No 1 (ADM1). Water Sci. Technol. 45, 65–73.

Bhattad, U., Venkiteshwaran, K., Cherukuri, K., Maki, J.S., Zitomer, D.H., 2017. Activity of methanogenic biomass after heat and freeze drying in air. Environ. Sci. Water Res. Technol. 3, 462–471. https://doi.org/10.1039/c7ew00049a.

Bi, S., Westerholm, M., Qiao, W., Xiong, L., Mahdy, A., Yin, D., Song, Y., Dong, R., 2020. Metabolic performance of anaerobic digestion of chicken manure under wet, high solid, and dry conditions. Bioresour. Technol. 296, 122342. https://doi.org/10.1016/j.biortech.2019.122342.

Coates, J.D., Coughlan, M.F., Colleran, E., 1996. Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. J. Microbiol. Methods 26, 237–246. https://doi.org/10.1016/0167-7012(96)00915-3.

Dolfing, J., 1985. Kinetics of methane formation by granular sludge at low substrate concentrations - the influence of mass transfer limitation. Appl. Microbiol. Biotechnol. 22, 77–81. https://doi.org/10.1007/BF00252160.

Dolfing, J., Bloeman, W.G.B.M., 1985. Acitivity measurements as a tool to characterize the microbial composition of methanogenic environments. J. Microbiol. Methods 4, 1–12. https://doi.org/10.1016/0167-7012(85)90002-8.

Donoso-Bravo, a., Retamal, C., Carballa, M., Ruiz-Filippi, G., Chamy, R., 2009. Influence of temperature on the hydrolysis, acidogenesis and methanogenesis in mesophilic anaerobic digestion: parameter identification and modeling application. Water Sci. Technol. 60, 9–17. https://doi.org/10.2166/wst.2009.316.

Fotidis, I.A., Karakashev, D., Kotsopoulos, T.A., Martzopoulos, G.G., Angelidaki, I., 2013. Effect of ammonium and acetate on methanogenic pathway and methanogenic community composition. FEMS Microbiol. Ecol. 83, 38–48. https://doi.org/10.1111/j.1574-6941.2012.01456.x.

Giraldo-Gomez, E., Goodwin, S., Switzenbaum, M.S., 1992. Influence of mass transfer limitations on determination of the half saturation constant for hydrogen uptake in a mixed-culture CH4-producing enrichment. Biotechnol. Bioeng. 40, 768–776. https://doi.org/10.1002/bit.260400704.

Gonzalez-Estrella, J., Sierra-Alvarez, R., Field, J.A., 2013. Toxicity assessment of inorganic nanoparticles to acetoclastic and hydrogenotrophic methanogenic activity in anaerobic granular sludge. J. Hazard Mater. 260, 278–285. https://doi.org/10.1016/j.jhazmat.2013.05.029.

Hao, X., Liu, R., Loosdrecht, M. Van, Cao, D., 2017. Batch influences of exogenous hydrogen on both acidogenesis and methanogenesis of excess sludge. Chem. Eng. J. https://doi.org/10.1016/j.cej.2017.02.093.

Ho, D.P., Jensen, P.D., Batstone, D.J., 2013. Methanosarcinaceae and acetate-oxidizing pathways dominate in high-rate thermophilic anaerobic digestion of waste-activated sludge. Appl. Environ. Microbiol. 79, 6491–6500. https://doi.org/10.1128/AEM.01730-13.

Holliger, C., Alves, M., Andrade, D., Angelidaki, I., Astals, S., Baier, U., Bougrier, C., Buffière, P., Carballa, M., De Wilde, V., Ebertseder, F., Fernández, B., Ficara, E., Fotidis, I., Frigon, J.C., De Laclos, H.F., Ghasimi, D.S.M., Hack, G., Hartel, M., Heerenklage, J., Horvath, I.S., Jenicek, P., Koch, K., Krautwald, J., Lizasoain, J., Liu, J., Mosberger, L., Nistor, M., Oechsner, H., Oliveira, J.V., Paterson, M., Pauss, A., Pommier, S., Porqueddu, I., Raposo, F., Ribeiro, T., Pfund, F.R., Strömberg, S., Torrijos, M., Van Eekert, M., Van Lier, J., Wedwitschka, H., Wierinck, I., 2016. Towards a standardization of biomethane potential tests. Water Sci. Technol. 74, 2515–2522. https://doi.org/10.2166/wst.2016.336.

Jiang, Y., Banks, C., Zhang, Y., Heaven, S., Longhurst, P., 2018. Quantifying the percentage of methane formation via acetoclastic and syntrophic acetate oxidation pathways in anaerobic digesters. Waste Manag. 71, 749–756. https://doi.org/10.1016/j.wasman.2017.04.005.

Karri, S., Sierra-Alvarez, R., Field, J.A., 2006. Toxicity of copper to acetoclastic and hydrogenotrophic activities of methanogens and sulfate reducers in anaerobic sludge. Chemosphere 62, 121–127. https://doi.org/10.1016/j.chemosphere.2005.04.016.

Keating, C., Chin, J.P., Hughes, D., Manesiotis, P., Cysneiros, D., Mahony, T., Smith, C.J., Mcgrath, J.W., Flaherty, V.O., 2016. Biological phosphorus removal during high-rate, low-temperature, anaerobic digestion of wastewater. Front. Microbiol. 7, 1–14. https://doi.org/10.3389/fmicb.2016.00226.

Kougias, P.G., Angelidaki, I., 2018. Biogas and its opportunities—a review. Front. Environ. Sci. Eng. 12, 14. https://doi.org/10.1007/s11783-018-1037-8.

Lawrence, A.W., McCarty, P.L., 1969. Kinetics of methane fermentation in anaerobic treatment. J. Water Pollut. Control Fed. 41, R1–R17.

Lin, C.Y., Noike, T., Sato, K., Matsumoto, J., 1987. Temperature characteristics of the methanogenesis process in anaerobic digestion. Water Sci. Technol. 19, 299–300. https://doi.org/10.2166/wst.1987.0210.

Liu, C., Li, H., Zhang, Y., Chen, Q., 2016. Characterization of methanogenic activity during high-solids anaerobic digestion of sewage sludge. Biochem. Eng. J. 109, 96–100. https://doi.org/10.1016/j.bej.2016.01.010.

McKeown, R.M., Scully, C., Mahony, T., Collins, G., O'Flaherty, V., 2009. Long-term (1243 days), low-temperature (4–15 8C), anaerobic biotreatment of acidified wastewaters. Bioprocess performance and physiological characteristics 43, 1611–1620. https://doi.org/10.1016/j.watres.2009.01.015.

Molina, F., García, C., Roca, E., Lema, J.M., 2008. Characterization of anaerobic granular sludge developed in UASB reactors that treat ethanol, carbohydrates and hydrolyzed protein based wastewaters. Water Sci. Technol. 57, 837–842. https://doi.org/10.2166/wst.2008.067.

Oosterkamp, M.J., Bauer, S., Ibáñez, A.B., Méndez-García, C., Hong, P.Y., Cann, I., Mackie, R.I., 2019. Identification of methanogenesis and syntrophy as important microbial metabolic processes for optimal thermophilic anaerobic digestion of energy cane thin stillage. Bioresour. Technol. Reports 7, 100254. https://doi.org/10.1016/j.biteb.2019.100254.

Regueiro, L., Veiga, P., Figueroa, M., Alonso-Gutierrez, J., Stams, A.J.M., Lema, J.M., Carballa, M., 2012. Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters. Microbiol. Res. 167, 581–589. https://doi.org/10.1016/j.micres.2012.06.002.

Rice, E.W., Baird, R.B., Eaton, A.D., APHA, AWWA, 2017. Standard Methods for the Examination of Water and Wastewater, 23rd ed. WEF, Washington DC, USA.

Rittmann, B.E., McCarty, P.L., 2020. Environmental Biotechnology: Principles and Applications, second ed. McGraw-Hill, New York.

Robinson, J.A., Tiedje, J.M., 1982. Kinetics of hydrogen consumption by rumen fluid, anaerobic digestor sludge, and sediment. Appl. Environ. Microbiol. 44, 1374–1384. https://doi.org/10.1128/aem.71.11.7483-7492.2005.

Soto, M., Mendez, R., Lema, J.M., 1993. Methanogenic and non- methanogenic activity tests: theoretical basis and experimental set up. Water Res. 27, 1361–1376.

APPENDIX 2

HYDROGENOTROPHIC ACTIVITY: A TOOL TO EVALUATE KINETICS OF METHANOGENS

Electronic Supplementary Material

ELECTRONIC SUPPLEMENTARY MATERIAL

1. Values of the half-saturation constant from the literature

Description of sample	T (°C)	Substrate added	K_S (g_{COD} L^{-1})	Reference
Methanobacterium ruminantium	39	Formate	1.6E-05	(Hungate et al. 1970)
Digested biosludge from municipal waste treatment	33	Acetate and H_2/CO_2	1.3E-03*	(Kaspar and Wuhrmann 1978)
Rumen fluid (without dilution)	39	H_2/CO_2	2.1E-03	(Robinson and Tiedje 1982)
20-fold-diluted rumen fluid (average)	39		9.3E-05	
Digestor sludge (average)	30		9.1E-05	
Methanobrevibacter arboriphilus AZ	35	H_2/CO_2	1.1E-04	(Kristjansson et al. 1982)
Methanobacterium formicicum	37	Formate or H_2/CO_2	9.6E-05	(Schauer et al. 1982)
Methanospirillum PM 1	37	H_2/CO_2	4.0E-05	(Robinson and Tiedje 1984)
Methanospirillum PM 2	37		6.6E-05	
Methanospirillum hungatei JF-1	37		8.0E-05	
Methanosarcina barkeri MS	37		2.1E-04	
Ruminococcus albus and Methanobrevibacter smithii	37	H_2/CO_2	1.8E-05	(Pavlostathis et al. 1990)
Digester biosludge treating synthetic waste	35	Propionate	1.2E-07	(Giraldo Gomez et al., 1992)
Granular anaerobic biosludge	37	H_2/CO_2	3.6E-04	(Coates et al. 1996)
Methanogenic strain MSB	30	H_2/CO_2	1.9E-05*	(Kotsyurbenko et al. 2001)
Methanogenic strain MSP	30		2.3E-05*	
Anaerobic Sewage Biosludge Digestor	35	Formate	1.0E-06	(Siegrist et al. 2002)
Methanobrevibacter arboriphilus DH1	37	Formate	9.6E-06	(Junicke et al. 2015)

Table A.1. Half-saturation constant (Ks) for the hydrogenotrophic methanogenesis from the literature. Note: (*) Partial pressure was converted to gCOD L-1 using the coefficient of Bunsen for pure water at T (°C) (Gordon et al. 1977)

For K_S determination, some authors used propionate or formate as the exogenous substrate and source of H_2 intending to avoid a mass transfer limitation for H_2 (Giraldo Gomez et al., 1992; Hungate et al., 1970; Junicke et al., 2015; Siegrist et al., 2002). Nevertheless, there are still two orders of magnitude of difference between some of these values, as shown in Table A.1, from 1.2E-07 to 9.6E-05 g_{COD} L^{-1}. On the other hand, Schauer et al. (1982) developed batch tests gassing with H_2 in the presence or absence of formate and concluded that formate did not significantly affect the K_S for H_2 uptake. Additionally, Dolfing and Bloeman (1985) found activities of the same order of magnitude when using either formate or H_2/CO_2. Consequently, the addition of another exogenous substrate than H_2 did not yield deviations in the K_S or SHMA values from the ones obtained for H_2 when zero-order conditions were ensured. In this sense, no strategic advantage was found for the use of formate or propionate. It is also important to note that the higher values observed in Table A.1 (1.3E-03 and 2.1E-03 g_{COD} L^{-1}) were identified by the respective authors as being affected by external mass transfer limitations (Kaspar and Wuhrmann 1978; Robinson and Tiedje 1982).

2. Methane calculation: Influence of the dissolved CO_2 and the microbial growth

The stoichiometric equation for the chemical conversion of H_2 and CO_2 into methane is:

$$4\,H_{2\,(g)} + CO_{2(g)} \rightarrow CH_{4\,(g)} + 2H_2O_{(l)} \qquad (A.1)$$

Based on Eq. (A.1), in the gas phase, the increase in methane moles (Δn_{CH4}) is equivalent to a fourth of decrease in the total number of moles (Δn_{gas}). Therefore, assuming ideal behavior, an increase in the partial pressure of methane (Δp_{CH4}) could be correlated with a decrease in total pressure (ΔP) inside the vial headspace as follows:

$$\Delta n_{CH4} = -\Delta n_{gas}/4 \;\text{ then, }\; \Delta p_{CH_4} = -\Delta P/4 \qquad (A.2)$$

However, during the SHMA assay, since it is a multi-phase system, there are mass transfer phenomena involved between the gas and the liquid phases, as well as microbiological processes.

Hydrogen gas is poorly soluble into the water; however, carbon dioxide is easily dissolved. The dissolution of CO_2 is associated with acid-base equilibria in the liquid bulk, implying a depletion in the partial pressure of CO_2 that is not strictly stoichiometric to that observed in H_2 due to the chemical reaction in (A.1). Additionally, the microbial yield must be incorporated to correct the stoichiometry to consider the fraction of CO_2 consumed as a carbon source for new cells. But these two effects are opposed; further calculations are needed to study whether they can cancel each other.

For further calculations, the following assumptions are considered: (i) mass transfer phenomena between phases and acid-base reactions that have reached equilibrium (faster than biological processes); (ii) mass transfer resistance is negligible; (iii) methane and new cells are the final products of reaction, and $Y_{X/S}$ represents the microbial yield; (iv) the fraction of H_2 dissolved in the liquid phase is negligible (less than 1%); and (v) the original sample of biosludge is neutral and its contribution to the total concentration of ionic species in the vial is negligible when compared to CO_2 dissolved and $NaHCO_3$ added as a buffer.

First, the fraction of CO_2 dissolved in water could be represented by $CO_{2\,(aq)}$ which is in equilibrium with H_2CO_3. Considering the Henry's law and $H_2CO_3^*$ as the sum of both species $CO_{2\,(aq)}$ and H_2CO_3, the following equation is obtained:

$$CO_{2\,(g)} + H_2O_{(l)} \rightleftharpoons H_2CO_3^* \qquad H_2CO_3^* = K_H p_{CO2} \qquad (A.3)$$

Second, the following acid-base equilibriums are established in the liquid phase:

$$H_2CO_3^* \rightleftharpoons H^+ + HCO_3^- \qquad K_1 = \frac{[H^+][HCO_3^-]}{[H_2CO_3^*]} \qquad (A.4)$$

$$HCO_3^- \rightleftharpoons H^+ + CO_3^{=} \qquad K_2 = \frac{[H^+][CO_3^{=}]}{[HCO_3^-]} \qquad (A.5)$$

$$H_2O \rightleftharpoons H^+ + OH^- \qquad K_w = [H^+][OH^-] \qquad (A.6)$$

Third, Eq. (A.7) ensures electroneutrality for the considered ionic species; due to the

addition of 3 g L^{-1} $NaHCO_3$ as a buffer, the concentration of Na^+ is known:

$$[H^+] + [Na^+] = [HCO_3^-] + 2[CO_3^=] + [OH^-] \text{ where } [Na^+] = 0.0357 \text{ M} \quad (A.7)$$

The moles of dissolved oxidized carbon (n_{Cd}) correspond to:

$$n_{Cd} = n_{H2CO3*} + n_{HCO3-} + n_{CO3=} \quad (A.8)$$

Then, the total moles of oxidized carbon (n_{Ct}) can be expressed as:

$$n_{Ct} = n_{Cd} + n_{CO2(g)} \quad (A.9)$$

For a depletion in p_{CO2} due to reaction (A.1), the concentration of $H_2CO_3^*$ decreases according to Eq. (A.3), the pH increases, and the other dissolved species of oxidized carbon also decreases, according to the acid-base equilibria, in Eqs. (A.4) to (A.7). Thus, as the substrates are consumed, the ratio p_{CO2}:p_{H2} is not maintained at 1:4. Assuming a certain p_{CO2}, the concentration of $H_2CO_3^*$ could be calculated using Eq. (A.3) and K_H at the working temperature (30 °C in this case) for CO_2 in water. Based on the determined system of Eqs. (A.4) to (A.7), the concentration for all the species of oxidized carbon associated with p_{CO2} can be calculated at a certain time t. Therefore, the variation of the total moles of oxidized carbon could be estimated during the time elapsed in the SHMA test.

Additionally, the variation of moles of H_2 can be calculated considering the ratio of 1:4 between the moles of H_2 and the moles of total carbon from the formula presented in (A.1), as follows:

$$\Delta n_{H2} = 4\Delta n_{Ct} \quad (A.10)$$

Next, the moles of methane produced could be obtained considering the fraction of carbon utilized for methane production and the fraction for new cell formation, as expressed in Eq. (A.11).

$$\Delta n_{CH4} = -(1 - Y_{X/S})\Delta n_{Ct} \quad (A.11)$$

Additionally, a fraction of the produced methane remains dissolved; this could be considered applying the Henry's law.

For a typical SHMA assay, starting from a total pressure (P) of 2 atm and until approximately 1 atm (absolute), the partial pressure variation over time for the gaseous species is presented in Figure A.1, considering a value of $Y_{X/S}$ of 0.06 g_{COD} g_{COD}^{-1} for the hydrogenotrophic methanogens.

APPENDIX 3

A CASE OF STUDY BASED ON ACTIVITY TESTS

XIII Latin American Workshop and Symposium on Anaerobic Digestion - DAAL XIII - Medellín, 2018 (Oral Presentation)

EGSB biosludge and reactor dynamics under different operational conditions

E. Ripoll, C. Callejas, I. López and L. Borzacconi

Institute of Chemical Engineering, Faculty of Engineering, UdelaR, Uruguay.
(E-mail : *eripoll@fing.edu.uy ; bioproa@fing.edu.uy*)

Abstract
In order to correlate operational conditions with reactor dynamics, two EGSB reactors (8 L) were inoculated using granular anaerobic sludge from a dairy plant. One was fed with carbohydrates (R1), whereas the other was fed with protein and carbohydrates (R2). Both were operated in stepwise specific OLR increments: (1) 0.2; (2) 0.4; (3) 0.3 gCOD/gVSS/d. Data was collected to characterize reactor performance, granules structure, methanogenic activity (SAMA; SHMA) and bacterial community structure. Significant changes were observed in bacterial community related to biosludge adaptation exposed to different substrates. VFA accumulation, the lack of enough alkalinity and pH conditions determined changes in COD removal efficiency, bacteria community structure, granules structure and methanogenic activity in R1 during stage 2. However, R2 showed a good performance, well-formed granules and methanogenic activity increased at the same OLR.

Keywords
EGSB reactor; specific methanogenic activity; bacterial community structure

INTRODUCTION

Success in the operation of anaerobic reactors is based on the deep knowledge of processes involved. Dynamics responses to changes in operational conditions and substrate characteristics must be analysed in relation to microorganism community. The objective of this work is to correlate operational conditions as OLR, substrate, pH and alkalinity with reactor dynamics, reflected by COD removal and biogas production, microorganism community structure and methanogenic activity.

MATERIALS AND METHODS

Two EGSB reactors (8 L) were inoculated using granular anaerobic sludge from a dairy plant. Reactor 1 (R1) was fed with carbohydrates; whereas Reactor 2 (R2) was fed with protein and carbohydrates 1:1 in COD. Both were operated in stepwise specific OLR increments in three stages: (1) 0.2; (2) 0.4; (3) 0.3 gCOD/gVSS/d. Following data was collected: inlet flow rate; inlet and outlet COD, pH, alkalinity and VFA; biogas production; diameter and 10x granule images; VSS, specific acetoclastic and hydrogenotrophic methanogenic activity (SAMA; SHMA) (Ripoll et al., 2015). Sludge samples were collected to study microbial composition and dynamics by 16S rRNA gene sequencing analysis. V4 region analysis of raw data performed with QIIME software (Caporaso et al., 2010), 80.000 sequences per sample. OTUs were created de novo (97% identity criteria).

RESULTS AND DISCUSSION

During the first stage of operation, significant changes in bacterial community structure were observed in both reactors. After one month of operation at OLR of 0.2 gCOD/gVSS/d (R1.1; R2.1) were distant from inoculum (R1.0). This result may reflect biomass adaptation to the new substrates (Figure 1). After two months, bacterial community structures in both reactors diverged (R1.2; R2.2). This result evidences the influence of different substrates on the biosludge over time. In addition, methanogenic activities of biosludge from both reactors changed slightly during this stage: SAMA 0.32 (0.04); SHMA 0.27 (0.02) gCOD/gVSS/d for inoculum; Table 1 and Table 2 for reactors. Both reactors achieved good performance in terms of COD removal (Table 1; Table 2).

During the second stage in R1, a tenfold increase in VFA concentration was observed (from 0.1 to 1 g/L HAc, 0.01 to 0.1 g/L HProp) when OLR was duplicated (from 0.2 to 0.4 gCOD/gVSS/d). A reduction in biogas production and COD removal efficiency was registered (Table 1). pH inside

reactor resulted between 6.0 to 6.5 due to the lack of enough alkalinity to neutralize VFA generated. A high percentage of granules showed deterioration on their outer shell, most of them with a hollow core exposed to liquid. Remarkable changes in bacterial community were seen after 2 and 3 months at stage 2 (R1.3; R1.4), which could be linked to an increase in *Spirochaetes* phylum abundance (Figure 1). Moreover, acetoclastic methanogenic population was affected, since SAMA decreased when OLR was increased (Table 1). However, SHMA increased, suggesting more resistance of hydrogenotrophic archaea, but not enough to maintain its performance. At the time, SAMA and SHMA increased in R2, which maintained its performance, with good buffer capacity due to substrate characteristics and well-formed granules (Table 2). The abundance of *Spirochaetes* increased comparing to inoculum. Also, *Synergistetes* was another important phylum (R2.3; R2.4; Figure 1).

In the third stage, OLR was decreased to 0.3 gCOD/gVSS/d in both reactors. After alkalinity adjust, R1 recovered its COD removal efficiency. SAMA increased, evidencing acetoclastic methanogens recovering from VFA inhibition, at the time SHMA remained unchanged (Table 1). However, SAMA decreased in R2 as OLR was decreased, but it maintained a good performance (Table 2). An increase in *Firmicutes* abundance was observed in both reactors when comparing to stage 2 (R1.5; R2.5 day 182; Figure 1). Then, both reactors were stopped in day 182 and sampled after 50 days. Results shows a re-structuration of bacteria community in response to the lack of feeding (R1.6; R2.6; Figure 1).

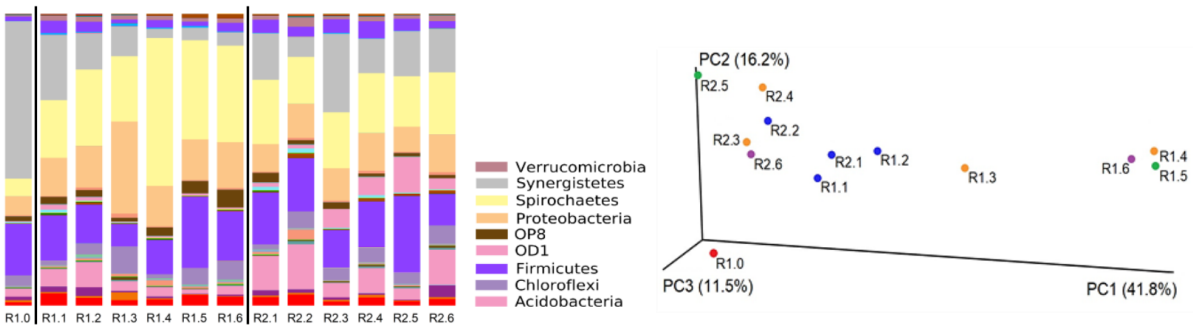

Figure 1. Bacterial structure over time in R1 and R2. Beta diversity analysis. PCoA-Unifrac

Table 1. Operational data of Reactor 1 (mean and standard deviation).

	Days	OLR (gCOD/gVSS/d)		Inlet COD (gCOD/L)		η COD removal (%)		SAMA (gCOD/gVSS/d)		SHMA (gCOD/gVSS/d)	
1	0-56	0.21	(0.04)	1.5	(0.1)	93	(2)	0.22	(0.01)	0.28	(0.03)
2	57-139	0.42	(0.04)	3.2	(0.3)	63	(8)	0.17	(0.01)	0.38	(0.01)
3	140-182	0.32	(0.03)	2.8	(0.2)	91	(8)	0.32	(0.02)	0.38	(0.04)

Table 2. Operational data of Reactor 2 (mean and standard deviation)

Stage	Days	OLR (gCOD/gVSS/d)		Inlet COD (gCOD/L)		η COD removal (%)		SAMA (gCOD/gVSS/d)		SHMA (gCOD/gVSS/d)	
1	0-56	0.21	(0.07)	1.4	(0.3)	88	(4)	0.28	(0.02)	0.32	(0.02)
2	57-139	0.38	(0.07)	2.8	(0.3)	87	(6)	0.54	(0.04)	0.44	(0.05)
3	140-182	0.33	(0.04)	2.5	(0.3)	93	(2)	0.46	(0.05)	0.46	(0.07)

CONCLUSIONS

Operational condition in EGSB reactors, as substrate, OLR, pH and alkalinity, could not only have significant effects over COD removal and biogas production, but also over granules physical structure, bacterial community structure and methanogenic population. SAMA and SHMA can both increase when the OLR is raised if a proper buffer capacity is ensured (e.g. protein-based wastewater).

REFERENCES

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., ... & Huttley, G. A. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature methods, 7(5), 335.

Ripoll, E., Callejas, C., Borzacconi, L. 2015 Need to standardize a technique for hydrogenotrophic methanogenic activity measurement: a proposal. *World Congress on Anaerobic Digestion, AD14*, Viña del Mar, Chile

APPENDIX 4

ACTIVITY TESTS WITH METHANOGENESIS INHIBITOR

16th World Congress on Anaerobic Digestion - ad16
Delft, 2019 (Poster Presentation)

Incidence of acetate oxidation and homoacetogenesis on methane formation: kinetic assessment using specific activities

I. López, E. Ripoll and L. Borzacconi

Facultad de Ingeniería, Universidad de la República, Uruguay
*Corresponding author. E-mail: ivanl@fing.edu.uy

Abstract: By using sodium 2-Bromoethanesulfonate (BES) as an enzymatic inhibitor of archaea during the acetoclastic and the hydrogenotrophic specific methanogenic activity tests (SAMA and SHMA), the methanogenic pathways can be decoupled from the syntrophic acetate oxidation and homoacetogenesis. In this work, IC50 thresholds of BES are confirmed and estimated values for the methanogenic population fractions in total volatile solids are found. As a remarkable result, even without hydrogen addition, 24 % of the methane is formed through the syntrophic acetate oxidation plus the hydrogenotrophic route.

Keywords: Acetate oxidation; Homoacetogenesis; Specific Methanogenic Activity

INTRODUCTION

The last steps in anaerobic digestion of organic matter, the acetoclastic and hydrogenotrophic methanogenesis, are well known. These steps have been frequently considered as rate-limiting due to the slow-growing rate of methanogens and their inhibition issues. For a long time, acetate oxidation and homoacetogenesis were considered irrelevant concerning their influence on methane formed by acetoclastic or hydrogenotrophic pathways. Nevertheless, these routes have recently been studied in greater depth and it was found that under inhibitory conditions, methane conversion seems to be mainly performed by hydrogenotrophic microorganisms. In that situation, syntrophic acetate-oxidizing bacteria (SAOB) could consume acetate accumulated because of the inhibition of acetoclastic methanogens. Therefore, since methane formation is usually identified as the controlling step, it is important to quantify the methane produced by the different routes indicated above and study the role of SAOB and homoacetogenic bacteria under different operational conditions. In Fig. 1, the following reactions are considered: r_1, methane formation from acetate; r_2, methane formation from H_2/CO_2; r_3, acetate oxidation to H_2/CO_2; r_4, acetate formation from H_2/CO_2.

A first step forward understanding the relevance of acetoclastic and hydrogenotrophic microorganisms involved in methane formation is to measure their specific activities (SAMA and SHMA) associated to r_1 and r_2 respectively (Soto *et al*., 1993; Coates *et al*., 1996). Nevertheless, as r_3 and r_4 could be also present, a fraction of acetate could be converted into H_2/CO_2 and vice versa, during the activity tests. Consequently, the methane produced in SAMA and SHMA tests could be the addition of the methane produced by both reactions, r_1 and r_2. In this context, decoupling the four steps is necessary to determine the importance of these reactions in the methane formation under different conditions. To disengage these reactions, selective inhibitors must be used. By using sodium 2-Bromoethanesulfonate (BES) (Xu et al., 2010), an enzymatic inhibitor of archaea, in low concentrations (1mM) the route r_1 would be inhibited, whereas with high BES concentrations (50mM) also r_2 would be inhibited. On the other hand, as r_3 and r_4 are performed by bacteria and not by archaea, these reactions would not be inhibited.

MATERIALS AND METHODS

The Specific Acetoclastic Methanogenic Activity test (SAMA) (Soto *et al*., 1993) and Specific Hydrogenotrophic Methanogenic Activity test (SHMA) (Ripoll *et al*, 2015) were performed using different levels of BES.

RESULTS AND CONCLUSIONS

In SHMA tests, without acetate addition and working at high hydrogen concentrations, r_3 would not be favoured and it could be neglected. If there is some contribution to methane formation via r_1, it is due to acetate formed by r_4. The results obtained for SHMA tests showed a lineal drop when BES concentration was increased and an IC50 value of 21 mM was observed. This behaviour agrees with the literature that

reports complete inhibition at 50 mM of BES. The value of 0.04 $g_{COD}\ L^{-1}\ d^{-1}$ observed at BES concentrations higher than 50 mM could be explain due to hydrogen consumed by r_4 (about 9 % of the value obtained for SHMA test performed without BES addition).

When using a value of $k_{m,hm} = 25\ d^{-1}$ for pure cultures (Gujer & Zehnder, 1983), the experimental value of hydrogen consumption corresponding to 0.239 $g_{COD}\ d^{-1}$ and a volume of V = 0.101 L, a value of X_{hm} = 0.086 $g_{COD}\ L^{-1}$ is obtained. Considering that the amount of solid in the vial was 4.97 $g_{VSS}\ L^{-1}$, equivalent to 7.06 $g_{COD}\ L^{-1}$, the hydrogenotrophic population represents the 1.2 % of the solids content.

In SAMA tests (high acetate and low hydrogen concentrations), r_4 could be assumed negligible. Nevertheless, methane production could occur by r_1 or by r_3+r_2. In fact, the experiences showed different behaviour at low and high BES concentrations. At low concentration (lower than 5mM), r_2 inhibition is negligible and the drop in SAMA values could be explained by r_1 inhibition. At high concentrations, r_1 is considered completely inhibited and the drop in SAMA values is due to the progressively inhibition of r_2 that affects the route r_3+r_2 (linear behaviour is observed).

From the experience, an IC50 value of 1.3 mM was found for acetoclastic methanogens, in accordance to literature (Xu *et al*., 2010). Without BES addition, the value determined in SAMA test would be associated to the sum of route r_1 plus route $r_3 + r_2$. From Fig. 2, the percentage of the activity due to r_3+r_2 could be estimated using an extrapolation of the lineal part of the curve at high concentrations over the axis of the ordinates. As a result, it is obtained that 24 % of methane comes from the syntrophic pathway even without hydrogen addition.

From SAMA test without BES, an experimental methane production of 0.0782 $g_{COD}\ d^{-1}$ was determined. Using literature values of $k_{m,am} = 8\ d^{-1}$, $Y_{am} = 0.05\ g_{COD}\ g_{COD}^{-1}$, $Y_{hm} = 0.06\ g_{COD}\ g_{COD}^{-1}$ (Batstone et al., 2002), $Y_{ao} = 0.10\ g_{COD}\ g_{COD}^{-1}$ (Rivera-Salvador et al., 2014) and a volume of 0.101 L, a value of X_{am} = 0.0796 $g_{COD}\ L^{-1}$ was obtained. Since the solids content in the vial was 3.76 $g_{VSS}\ L^{-1}$, equivalent to 5.34 $g_{COD}\ L^{-1}$, the acetoclastic biomass represents the 1.5 % of the solids content. Then, both methanogenic populations represent 2.7 % of the total biomass, in accordance to literature (Soto *et al*., 1993).

REFERENCES

Batstone, D., Keller, J., Angelidaki, I., Kalyuzhnyi, S., Pavlostathis, S., Rozzi, A., Sanders, W., Siegriest, H., Vavilin, V. (2002) Anaerobic Digestion Model N°1, IWA Scientific and Technical Report N° 13, IWA Publishing, ISBN 1 900222 78 7.

Coates, J. D., Coughlanb, M. F., & Colleran, E. (1996). Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. *Journal of Microbiological Methods*, *26*, 237–246.

Gujer, W. & Zehnder, A. J. B., (1983). Conversion Processes in Anaerobic Digestion. *Water, Science and Technology*, *15(November), 127–167*.

Ripoll, E., Callejas, C., Borzacconi, L. (2015) Need to standardize a technique for measuring the activity of hydrogenotrophic microorganisms: a proposal, XIV World Congress on Anaerobic Digestion, Viña del Mar, Chile.

Rivera-Salvador, V., López-Cruz, I. L., Espinosa-Solares, T., Aranda-Barradas, J. S., Huber, D. H., Sharma, D., & Toledo, J. U. (2014). Application of Anaerobic Digestion Model No. 1 to describe the syntrophic acetate oxidation of poultry litter in thermophilic anaerobic digestion. Bioresource Technology, 167(1), 495–502.

Soto, M., Méndez, R., & Lema, J. M. (1993). Methanogenic and non-methanogenic activity tests. Theoretical basis and experimental set up. Water Research, 27(8), 1361–1376.

Xu, K., Liu, H., & Chen, J. (2010). Effect of classic methanogenic inhibitors on the quantity and diversity of archaeal community and the reductive homoacetogenic activity during the process of anaerobic sludge digestion. *Bioresource Technology*, *101*(8), 2600–2607.

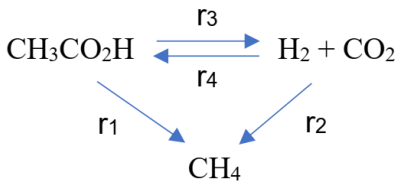

Figure 1. Reactions involved in methane production

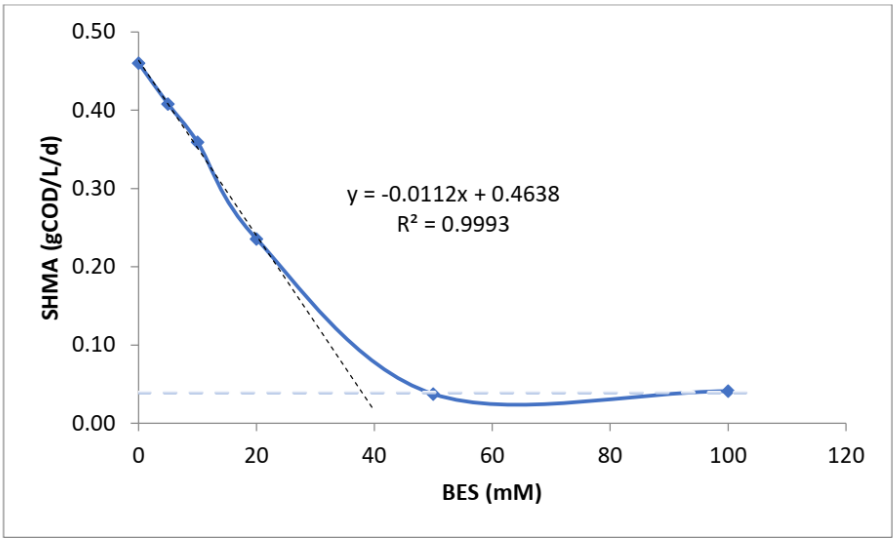

Figure 2. BES inhibition in Specific Hydrogenotrophic Methanogenic Activity test

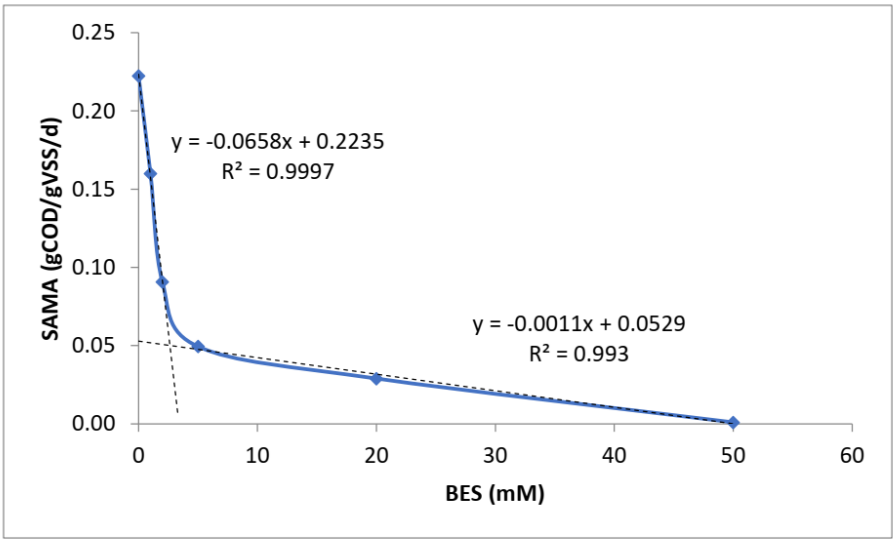

Figure 3. BES inhibition in Specific Acetoclastic Methanogenic Activity test

Table 1. Kinetic equations, with high hydrogen concentrations:

$$r_2 = k_{m,hm} X_{hm} \frac{S_{h2}}{K_{s,hm} + S_{h2}} \cong k_{m,hm} X_{hm}$$

$$r_4 = k_{m,ha} X_{ha} \frac{S_{h2}}{K_{s,ha} + S_{h2}} \cong k_{m,ha} X_{ha}$$

Mass balance in batch conditions: $-V \frac{dS_{h2}}{dt} = \left(k_{m.hm} X_{hm} + k_{m,ha} X_{ha}\right) V$

From Fig.2 $\frac{k_{m.ha} X_{ha}}{k_{m,hm} X_{hm}} = \frac{0.04}{0.46 - 0.04}$ por tanto $k_{m,ha} X_{ha} = 0.091 k_{m,hm} X_{hm}$

$$X_{hm} = \frac{0.909 \, {}^{dS_{h2}}/_{dt}}{k_{m,hm}}$$

Table 2. Kinetic equations, with high acetate concentrations:

$$r_1 = k_{m,am} X_{am} \frac{S_{ac}}{K_{s,am} + S_{am}} \cong k_{m,am} X_{am}$$

$$r_3 = k_{m,ao} X_{ao} \frac{S_{ac}}{K_{s,ao} + S_{ac}} \cong k_{m,ao} X_{ao}$$

Mass balance in batch conditions:

$$V \frac{dS_{CH4}}{dt} = \left[(1 - Y_{am}) k_{m.am} X_{am} + (1 - Y_{hm})(1 - Y_{ao}) k_{m,ao} X_{ao}\right] V$$

From Fig.3 $\frac{k_{m.ao} X_{ao}}{k_{m,am} X_{am}} = \frac{0.053}{0.222 - 0.053}$ else $k_{m,ao} X_{ao} = 0.314 k_{m,am} X_{am}$

APPENDIX 5

ADJUSTMENT OF HYDROGENOTROPHIC ACTIVITY APPLIED TO SLUDGE FROM SOLID DIGESTER

XII Latin American Workshop and Symposium on Anaerobic Digestion, Cuzco, 2016 (Oral presentation)

Adjustment of hydrogenotrophic methanogenic activity test applied to sludge from anaerobic solid digester

E. Ripoll, I. López and L. Borzacconi

Institute of Chemical Engineering, Faculty of Engineering, UdelaR, Uruguay
(E-mail: *eripoll@fing.edu.uy; ivanl@fing.edu.uy; lilianab@fing.edu.uy*)

Abstract
The specific hydrogenotrophic methanogenic activity (SHMA) is a valuable tool to characterize the sludge from anaerobic digesters. However, when considering the determination of SHMA for samples from solid waste digesters, the presence of solid substrate dilutes the biomass, distorting the experimental results and longing the measurement periods. To consider this effect, a dilution factor was evaluated from simulations and used to adjust the experimental values of SHMA, determined for samples from a manure digester. This factor can also be applied in the estimation of the sludge volume used in the SHMA test.

Keywords
Hydrogenotrophic methanogenic activity; solid waste

INTRODUCTION

Methanogenic activity is considered a valuable and simple tool that provides complementary information to microbiological techniques to characterize the biological conditions of anaerobic sludge operating in a certain system (Dolfing and Bloemen, 1985). Also, it might be another interesting piece in the puzzle in order to shed light to the different pathways involved in substrate degradation under certain conditions. The specific acetoclastic methanogenic activity (SAMA) has an established method of determination whereas the protocol for the specific hydrogenotrophic methanogenic activity (SHMA) still have some drawback to be solved and it is not standardized yet. Coates *et al* (1996) developed a method for SHMA determination based on monitoring the gas pressure depletion in vial headspace and the stoichiometric relation of the conversion of H_2/CO_2 to methane.

An adapted protocol was designed based on this methodology (Ripoll *et al*, 2014). A formula to calculate the size of inoculum was proposed considering the selected conditions for the test (temperature and headspace volume) and the characteristics of the sludge: the volatile suspended solids content (VSS) and an estimated value for SHMA. As a result, a greater volume of sludge is added as inoculum for the test when the sludge has low VSS or is presumed to have low SHMA. This protocol was adjusted testing sludge samples from different reactors treating wastewater. However, SHMA protocol needs to be adjusted to analyze samples from solid waste digester and the present work was developed in that context. In the case of liquid substrates, it could be useful to include an exhausting stage or wash the sludge beforehand to reduce the remaining substrate to the minimum. However, when considering samples from solid digesters, substrate cannot be separated from biomass (microorganisms), so the biomass is diluted by the presence of solid substrate. This might have two practical consequences: i) a greater volume of sample must be added to assess SHMA in a reasonable period. ii) Since the total VS of the sample represent both biomass and substrate and SHMA is usually expressed per gVS of sample, the activity of the biomass is undervalued.

The purpose of this work is to verify and quantify the incidence of the solid substrate dilution of the biomass. Also improve the methodology followed to determine SHMA of samples from solid waste digester, in particular the sludge volume estimation used in the test.

MATERIALS AND METHODS

Inoculum source

Sludge from a slaughterhouse anaerobic lagoon was provided to inoculate two different systems: a pilot-scale EGSB reactor to treat wastewater from beverage industry and a bench-scale CSTR digester to treat manure from dairy industry. The inoculum was not granular indeed but had some incipient granules. A sample was taken to analyze the SHMA of the inoculum (sample labeled as 'sample I', t=0).

Beverage pilot-scale EGSB reactor

An EGSB reactor of 15 m^3 treating beverage wastewater was sampled once during its start-up for SHMA analysis ('sample W'). At that time, the OLR was 5 $kgCOD/m^3/d$ (0.3 kgCOD/kgVSS/d).

Manure bench-scale digester

A mixture of inoculum and manure (2.0 $gVS_{inoculum}/gVS_{substrate}$) was incubated in batch conditions for 30 days, to adapt the inoculum to consume that substrate. Manure characterization can be seen in Table 1. Then, the digester (3.0 L of working volume) was started at an OLR of 3.0 gCOD/L/d and a SRT of 30 days (period 1), obtaining a COD removal efficiency of 54 %. Next, the OLR was increased to 4.5 gCOD/L/d (period 2), achieving a COD removal efficiency of 51 %. Details about the operation of the digester (identified as 'Reactor 2') can be consulted in Benzo *et al* (2016). Four samples were taken for SHMA analysis: one at the end of batch ('sample B', t=30 d), the second at the end of period 1 after 2 SRT ('sample P1', t= 103 d), the third after 2 SRT in the period 2 ('sample P2-1', t=165 d) and the last one after 3 SRT in the period 2 ('sample P2-2', t=196 d).

Table 1. Manure characterization. Analytical methods: APHA, 1998 (SV), Yadvika *et al*., 2006 (total COD), Angelidaki *et al*, 2009 (BMP and biodegradability). Mean and standard deviation for each data are presented.

VS (mg/g)	COD (mg_{COD}/mg_{VS})	BMP (mL_{CH4}/g_{VS})	Biodegradability (%)
139 ± 3	1.36 ± 0.06	307± 9	65 ± 2

SHMA test

The SHMA tests were carried out by triplicate using an adapted protocol (Ripoll et al, 2015). This protocol is based on monitoring the depletion of gas pressure in vial headspace (P_{hs}), using a 0-2 barg pressure transducer, and calculations considering the stoichiometric relations (Coates *et al*, 1996). Before SHMA tests, samples I and W were exhausted during 3 days at 30 ºC whereas samples P1, P2-1 and P2-2 were left 6 days at 37 ºC. Sample B was not exhausted. SCHOTT® 250mL flasks (308 mL of total volume) were used as test vials in SHMA assay, with a headspace volume of 208 mL. Vials containing inoculum were gassed with H_2/CO_2 (80/20) to 760 mmHg over atmospheric pressure and incubated at 30 ºC in an orbital shaker (150 rpm). Measures of P_{hs} were taken every 2 hours.

Inoculum size. The formula included in the original SHMA protocol was adapted to consider the dilution of the biomass with the solid substrate by using a dilution factor f_{dil} (equation 1):

$$V_{sample}\,(L) = \frac{64\,\frac{g_{COD\ CH4}}{mol_{CH4}} * \Delta P\ (atm) * V_{hs}\,(L) * 0.25\,\frac{mol_{CH4}}{mol_{gas}}}{\Delta t(d) * T(K) * R\left(\frac{L.atm}{K.mol_{gas}}\right) * SHMA\left(\frac{g_{COD\ CH4}}{d.g_{VSS\ biomass}}\right) * VSS_{sample}\left(\frac{g}{L}\right) * f_{dil}} \quad \text{(Eq. 1)}$$

where V_{sample} is the sample aliquot volume (mL) that needs to be added to the vial, ΔP the desirable depletion of headspace pressure between measures, V_{hs} the headspace volume, Δt the time elapsed between measures, T the temperature, an estimated value of SHMA for the inoculum and VSS the volatile suspended solid content (APHA, 1998) of the sludge sample. R is the universal gas constant.

Based on the V_{sample} calculated, the VSS of the sample and the total volume of liquid considered in vial, the concentration of VSS added the vial can be determined.

Kinetics model

The solid substrate is represented by a fraction of non-biodegradable material (X_{nb}) and a fraction of biodegradable material (X_b). First-order kinetics is considered for the substrate hydrolysis of the biodegradable fraction with k_H as a hydrolysis constant (Valentini *et al*, 1997). A value for k_H was determined based on a BMP assay conducted during period 1, k_H = 0.142 d^{-1}. Complete mixed behavior is considered for the digester, with τ as hydraulic retention time. The biomass yield (Y) is assumed as the ratio of the biomass growth to substrate consumption:

Y = (0.18 gVSS/gCOD) * (1.36 gCOD/gVS manure) (Pavlostathis and Giraldo-Gomez, 1991)

The decay of biomass (X) is modeled as first-order kinetics with k_d as the coefficient, k_d=0.01 d^{-1}. The dead biomass is assigned as biodegradable substrate. X, X_b, X_{nb} are expressed in gVSS/L. The total content of VSS is calculated (X_{tot}) in order to estimate the dilution factor as the ratio between X and X_{tot}.

In batch conditions model equations are:

$$\frac{dX}{dt} = Yk_H X_b - k_d X$$

$$\frac{dX_b}{dt} = -k_H X_b + k_d X$$

$$\frac{dX_{nb}}{dt} = 0$$

In continuous conditions the model is:

$$\frac{dX}{dt} = \frac{X_{in} - X}{\tau} + Yk_H X_b - k_d X$$

$$\frac{dX_b}{dt} = \frac{X_{b,in} - X_b}{\tau} - k_H X_b + k_d X$$

$$\frac{dX_{nb}}{dt} = \frac{X_{nb,in} - X_{nb}}{\tau}$$

Where subscript 'in' indicates inlet conditions.

The model is numerically solved using SCILAB Software. The solution of model allows computing the concentrations of variables in the periods considered. Then, the fraction of biomass in total VS (dilution factor) can be calculated.

RESULTS AND DISCUSSION

The experimental results are presented in Table 2. It is important to note that experiences for sample I, B, P1 and P2-2 were conducted with higher VS concentrations that the ones conventionally reported (i.e. 1 – 8 g/L, Coates *et al*, 1996), involving shorter periods of test, about 10 – 12 h.

A decrease in SHMA and SAMA values was observed in the samples from digester (B, P1, P2) respect to inoculum (I). On the contrary, SAMA and SHMA of sample from the wastewater reactor (W) increased respect to inoculum. These results are coherent with the hypothesis of solid substrate dilution in the digester, aspect that does not occurred when considering a wastewater reactor. In the last case, the SHMA increased could be explained by the acclimation and selection of the sludge. In the sample P2-1, the amount of sludge used was insufficient to accurately determine SHMA, since the depletion of P_{hs} was comparable to the errors produced by the measure capture (Figure 1, Table 2). In addition, as the assay involved a larger period of time than the one observed in the other cases, the microbial growth cannot be considered negligible, observing a slope change (Figure 1). For this reason, a second determination with higher sludge content (P2-2) was successfully conducted (Figure 2).

Figure 3 shows the results of model simulation, considering the experimental conditions. For the batch experience, the initial conditions were: X = 32.3 g/L, X_b = 10.5 g/L, X_{nb} = 5.7 g/L. The initial conditions of Period 1 were the final conditions determined from batch modeling. The Period 2 simulation started at the final values of the Period 1.

The values for the dilution factor calculations are obtained as a result of the simulations (Table 3). It can be observed that the SHMA adjustment associated to the dilution of biomass with solid substrate is more than relevant. The decrease of the adjusted SHMA observed in batch experience could be explained by the decay of the hydrogenotrophic microorganisms because of no feeding of substrate. However, in the continuous fed operation an important increase in the adjusted SHMA was observed, comparable with the

one observed in the case of sample from wastewater reactor (W).

It is important to point out that the dilution of the biomass with solid substrate must be considered in the determination of the inoculum size for SHMA test. Knowing the inoculum activity, the dilution factor estimated from modeling or experimental determination can be used for determining the sludge volume necessary to perform the SHMA test, according to equation 1.

Table 2. Data obtained for samples I, B, P1, P2-1, P2-2 and W: the VS content of each sample (APHA, 1998), SAMA (Soto et al, 1993), the VSS of sample added to each vial for SHMA test, the depletion of Phs observed during SHMA test considering an interval of 2h, and the SHMA value obtained. Mean and standard deviation are informed.

Sample ID	Sample VSS (g/L)	SAMA (gCOD/gVS/d)	VSS added (g/L)	P_{hs} depletion (mmHg/2h)	SHMA (gCOD/gVS/d)
I	38.7 ± 0.5	0.100 ± 0.006	16.2–16.3	113–125	0.153 ± 0.002
B	37.1 ± 0.9	0.052 ± 0.001	14.6–15.3	65–84	0.101 ± 0.002
P1	35.9 ± 0.1	0.031 ± 0.003	13.7–13.8	40–51	0.071 ± 0.007
P2-1	46.3 ± 0.5	n/a	23.7–23.9	3–20(*)	n/a
P2-2	49 ± 1	n/a	42–49	85–111(**)	0.041 ± 0.004
W	14.5 ± 0.1	0.25 ± 0.01	5.8–6.0	57–87	0.26 ± 0.1

The P_{hs} depletion reported corresponds to: (*) the first 7h of assay of the sample P2-1 and (**) the vials 1 and 2, each one with 49 gVS/L of sample P2-2. Vial 3 with 42 gVS/L showed a P_{hs} depletion of 60-79 mmHg every 2h.

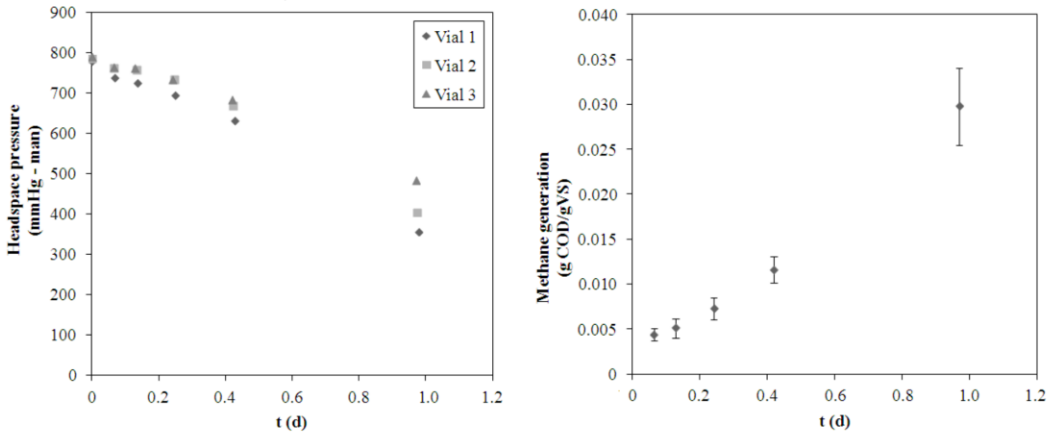

Figure 1. Headspace depletion during SHMA assay and methane generation per VS gram for sample P2-1

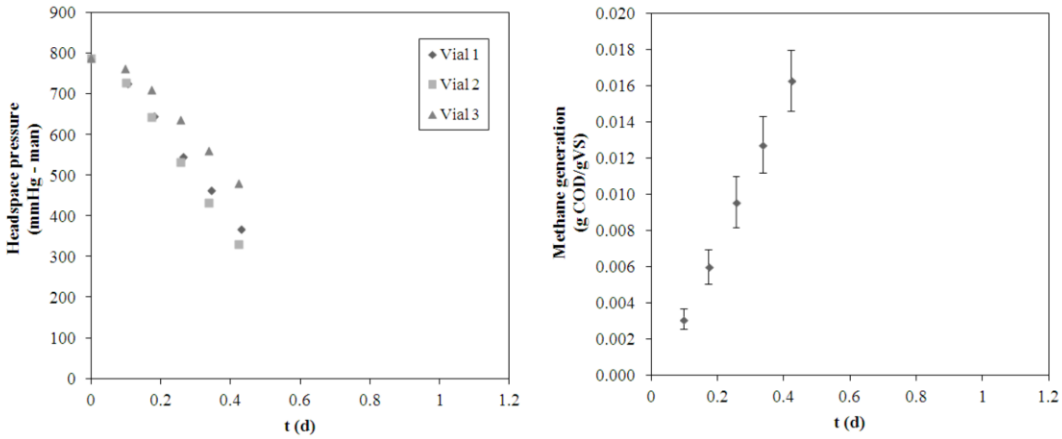

Figure 2. Headspace depletion during SHMA assay and methane generation per VS gram for sample P2-2

Table 3. SHMA measured, dilution factor and corrected values.

Sample	I	B	P1	P2	W
Measured SHMA (gCOD/gVSS/d)	0.153	0.101	0.071	0.041	0.26
Dilution factor	-	0.78	0.23	0.18	-
Adjusted SHMA (gCOD/gVSS/d)	-	0.129	0.309	0.228	-

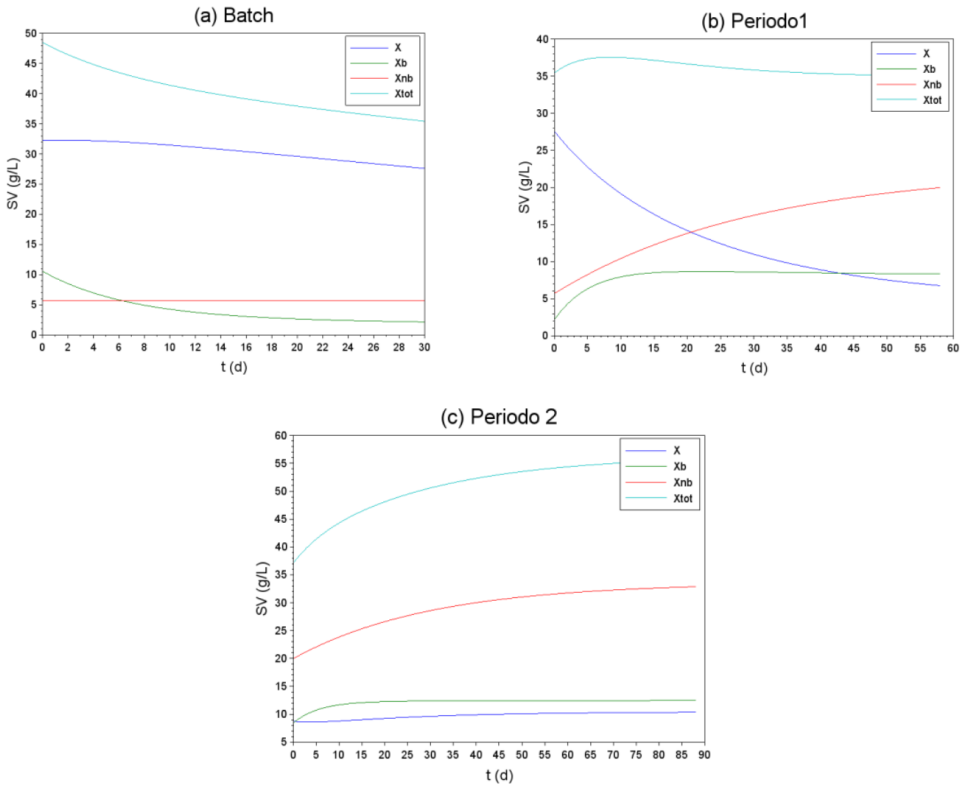

Figure 3. Results of the model simulation: (a) Batch conditions; (b) P1 conditions; (c) P2 conditions

CONCLUSIONS

The effect of the dilution of biomass with solid substrate on methanogenic activities is extremely important. If this effect is not considered, the activity test results can undervalue the real activity of the biomass.

A dilution factor that represents the fraction of biomass in total VS can be calculated from modeling or experimental data and allows an adjustment of the test results.

The dilution factor can also be used to perform more reliable calculations in order to determine a suitable sludge volume to carry on the SHMA test.

REFERENCES

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J.L., Guwy, A.J., Kalyuzhnyi, S., Jenicek, P., Van Lier, J.B. (2009) Defining the biomethane potential (BMP) of solid organic wastes an energy crops: a proposed protocol for batch assays. *Water Sci. Technol.* **59**(5), 927-933.

APHA, AWWA and WEF (1998) Standard Methods for the Examination of Water and Wastewater. 20th Ed. Washington DC, USA.

Benzo, M., Passeggi, M., Borzacconi, L. (2014) Anaerobic digestion of cattle manure: effect of total solids concentration and substrate to inoculum ratio. *XI DAAL*, La Habana, Cuba.

Benzo, M., Passeggi, M., Borzacconi, L. (2016) Start-up of anaerobic cattle manure digesters: success and failure. Research paper submitted to *XII DAAL*, Cusco, Perú.

Coates, J.D., Coughlan, M.F., Colleran, E. (1996) Simple method for the measurement of the hydrogenotrophic methanogenic activity. *J. Microbiol. Meth.* **26**(3), 237-246.

Dolfing, J., Bloemen, W. (1985) Activity measurements as a tool to characterize the microbial composition of methanogenic environments. J. Microbiol. Meth. **4**, 1 – 12.

Pavlostathis, S. G. and Giraldo-Gomez, E. (1991) Kinetics of anaerobic treatment. *Wat. Sci. Tech.* **24** (8), 35-59.

Ripoll, E., Callejas, C., Borzacconi, L. (2015) Need To Standardize A Technique For Hydrogenotrophic Methanogenic Activity Measurement: A Proposal. *World Congress on Anaerobic Digestion, AD14*, Viña del Mar, Chile.

Soto, M., Méndez, R., Lema, J.M. (1993) Methanogenic and non-methanogenic activity tests. Theoretical basis and experimental set up. *Wat. Res.* **27**(8), 1361-1376.

Valentini, A., Gilbert, G., Rozzi, A., Tilche, A. (1997) Anaerobic degradation kinetic of particulate organic matter: a new approach. *Wat. Sci. Tech.* **36**, 239-246.

Yadvika, A.K.Y, Sreekrishnan, T.R., Satya, S., Kohli, S. (2006) A modified method for estimation of chemical oxygen demand for samples having high suspended solids. *Bioresource Technol.* **97**, 721-726.