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## Experimental coalescence of microbial communities in anaerobic digesters

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## Résumé de la thèse

La digestion anaérobie est un procédé biologique effectué par un réseau complexe et synergique de communautés microbiennes permettant la dégradation de matière organiques comme les déchets agricoles ou les effluents de station d'épuration en biogaz, un gaz valorisable en énergie. Les mécanismes influençant les communautés microbiennes au cœur de ce procédé mais aussi dans la nature restent incompris du fait de la faible compréhension de leur dynamique. Les objectifs de ce projet visent à donc développer un système de digestion anaérobie permettant de mieux comprendre la dynamique de l'assemblage des communautés microbiennes. Ainsi un nouveau système de réacteurs en continu dont les fonctions d'alimentation de soutirage et de dégazage sont automatisées a été développé. L'automatisation et le multiplexage des réacteurs permettent la manipulation de 30 réacteurs en continu en parallèle. Outre l'automatisation ce système, de nombreux paramètres sont flexibles comme le taux de charge (une fois par minute jusqu'à une condition batch), le volume de réacteur (50 à 200mL), la température (ambiante – 55°C), mais aussi l'utilisation du système en aérobie ou l'implémentation d'autres outils comme des LEDs pour les cultures phototrophes. Capable de quantifier précisément la performance d'un écosystème méthanogène, ce système nous a permis de tester la structure et la performance d'écosystèmes méthanogènes mis en mélanges et testés de façon individuelle. En mélangeant des écosystèmes méthanogènes différents, la diversité des *Archées* a augmenté transitoirement. Une corrélation est d'ailleurs observée entre la diversité de ces communautés mélangées et leur performance méthanogène, seulement la performance des communautés individuelles est plus forte à même niveau de diversité. L'assemblage de certaines communautés mélangées a pourtant permis une meilleure production de méthane que les communautés individuelles, ce qui suggère le développement d'interactions spécifiques de ces communautés. De façon nouvelle par rapport à la littérature, la majorité des communautés bactériennes individuelles sont retrouvées dans les communautés mélangées. Soit contrairement à la sélection d'une communauté plus adaptée ou plus fonctionnelle, ici la majorité des communautés se sont implantées. Ces expériences suggèrent qu'un paramètre tel que la fonctionnalité d'un bioprocédé peut-être amélioré par bioaugmentation.

## Thesis summary

Anaerobic digestion is a biological process carried out by a complex and synergistic network of microbial communities allowing the degradation of organic matter such as agricultural waste or effluents from wastewater treatment plants, into biogas, a gas recoverable into energy. The mechanisms influencing microbial communities at the heart of this process but also in nature remain misunderstood because of a low understanding of their dynamics. The objectives of this project are therefore to develop an anaerobic digestion system to better understand the dynamics of microbial community assembly. Thus, a new continuous reactor process has been developed with automated feeding, biomass wasting and degassing functions. Automation and multiplexing of reactors allows for the continuous parallel manipulation of 30 reactors in parallel. In addition to the automation, many parameters are versatile, such as the substrate loading (once a minute up to batch conditions), the reactor volume (50 to 200 mL), the temperature (room to 55°C), but also the use of the aerobic system or the implementation of other tools such as LEDs for phototrophic cultures. Capable of accurately quantifying the performance of a methanogenic ecosystem, this system has enabled us to test the structure and the performance of five different methanogenic ecosystems that have been mixed and tested individually. By mixing different methanogenic ecosystems the *Archaea* diversity has increased transiently. Besides, a correlation is observed between the diversity of mixed communities and their methanogenic performance; yet the individual communities have a better functioning at the same level of diversity. Interestingly, the mixture of some communities has allowed for better methane production than individual communities, suggesting the development of specific interactions in these communities. In a novel way compared to the literature and that the majority of individual bacterial communities are found in mixed communities. Contrary to the selection a more adapted or functional community, here the majority of communities have settled. These experiments suggest that a parameter such as the functionality of a bioprocess can be improved by bioaugmentation.

*“Don’t adventures ever have an end?*

*I suppose not.*

*Someone else always has to carry on the story.”*

J.R.R. Tolkien

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# Résumé étendu

Les microorganismes Archées et Bactéries colonisent tous les biotopes sur Terre et rendent de nombreux services écosystémiques comme les cycles du Carbone et de l'Azote, grâce par exemple au recyclage de la matière organique ou à la transformation de l'azote atmosphérique en forme assimilable par les plantes. Les microorganismes assurent des fonctions essentielles comme la digestion dans les tubes digestifs des animaux, mais aussi dans des procédés biotechnologiques d'intérêt pour l'Homme. Les fermentations alimentaires et les procédés de traitement et de valorisation des déchets et effluents (station d'épuration des eaux usées, compostage, méthanisation, etc.) en font partie.

En particulier, la méthanisation (ou digestion anaérobie) convertit le carbone contenu dans les déchets constitués de matière organique complexe (effluents industriels, résidus issus de l'agriculture et de l'élevage, ordures ménagères, etc.) en biogaz, composé majoritairement de gaz carbonique et de méthane. Ce méthane peut ensuite être valorisé énergétiquement par injection dans le réseau de gaz naturel, utilisé comme biogaz carburant, ou bien produire de l'électricité et de la chaleur par cogénération. Dans le cadre du paquet « Énergie Climat », la France s'est vue fixer par l'Union Européenne l'objectif de 23 % d'énergie renouvelable dans sa consommation d'énergie finale d'ici 2020. Le développement de la méthanisation va contribuer à atteindre cet objectif. Le gouvernement français a d'ailleurs lancé en 2013 le plan « Energie Méthanisation Autonomie Azote » (EMAA) visant à implanter 1000 méthaniseurs à la ferme en 2020.

Dans le cadre de cette thèse en écologie microbienne des bioprocédés, les communautés microbiennes au cœur du processus de méthanisation ont été

étudiées et en particulier les moyens de pilotage de cette ressource microbienne.

Les communautés microbiennes accomplissant la digestion anaérobie sont apportées naturellement par les effluents et déchets servant de substrats à la méthanisation. Les méthaniseurs sont donc inoculés naturellement lors du démarrage de l'installation et les communautés microbiennes établies sont soumises en permanence à l'arrivée de nouveaux microorganismes provenant des déchets alimentant le méthaniseur. De nombreuses études ont observé le comportement très dynamique de ces communautés microbiennes au cours du temps, indépendamment du fonctionnement (Fernandez et al., 2000; Zumstein et al., 2000). Il est donc important de comprendre les mécanismes et les facteurs influençant la dynamique des diverses populations microbiennes lors de la méthanisation (Shade et al., 2012; Vanwonterghem et al., 2014a; Zhou et al., 2013). Or comprendre la dynamique des communautés impliquées apporterait un avantage certain pour le procédé. La prédiction du comportement de ces communautés microbiennes face à des perturbations permettrait une meilleure gestion de la ressource microbienne afin de prévenir ou rétablir des dysfonctionnements éventuels. Le pilotage d'un procédé de méthanisation pourrait passer par de la bioaugmentation ou de la biorestauration, à l'image de l'ingestion de probiotiques ou de prébiotiques chez l'Homme pour rétablir ou prévenir une dysbiose de la microflore intestinale. Différents types d'expériences ont été mis en œuvre pour comprendre les mécanismes d'assemblage des communautés microbiennes méthanogènes par bioaugmentation (Bouchez et al., 2000; Venkiteshwaran et al., 2016) ou par le mélange de plusieurs communautés (Sierocinski et al., 2017). Une récente théorie d'assemblage des communautés, appelée coalescence, propose que les communautés mélangées agissent comme des entités indépendantes et

que l'évolution de ces communautés aboutisse à la dominance d'une seule (Rillig et al., 2015; Tikhonov, 2016). Cette théorie a d'ailleurs été vérifiée expérimentalement en réacteurs batch, où les espèces les plus performantes des inocula utilisés dans les mélanges dominaient en fin d'expérience (Sierocinski et al., 2017). Dans cette dernière étude, plus les communautés mélangées étaient nombreuses, plus la production de biogaz était importante car la probabilité de tirer des espèces performantes augmentait avec le nombre d'inocula mélangés au départ. Il reste cependant de nombreux points à éclaircir, comme la rémanence du phénomène observé (en batch) ou bien la généricité des résultats (obtenus sur substrat simple seulement).

L'étude plus approfondie des phénomènes d'assemblage est donc stratégique pour piloter la ressource microbienne en méthanisation. Les objectifs de ces travaux de thèse sont donc d'affiner notre compréhension des effets provoqués par l'assemblage des communautés microbiennes méthanogènes, à la fois sur les interactions microbiennes et sur la performance du procédé.

Pour conserver un écosystème méthanogène à l'équilibre et ne conserver que les espèces qui participent au fonctionnement, l'utilisation de réacteurs en continu est pertinente car cela permet de lessiver les microorganismes inactifs. Les procédés en continu requièrent néanmoins des compétences, des ressources humaines et matérielles plus importantes que des expériences en batch (durée plus longue, gestion de l'alimentation/soutirage, etc.). Dans le but de multiplier les conditions et d'éviter la conservation des communautés qui diminue la performance des communautés (Hagen et al., 2015; Kerckhof et al., 2014), il a fallu au préalable mettre au point et construire un dispositif constitué de plusieurs réacteurs en continu qui soit le plus automatisé possible. Des systèmes commerciaux avec des caractéristiques proches sont disponibles, mais à des

coûts prohibitifs et dont les configurations sont très peu flexibles. Nous avons ainsi développé notre système de réacteurs en continu en minimisant les coûts et en optimisation son automatisation.

Le LAMACs (Lab-scale Automated and Multiplexed Anaerobic Chemostat system) est un dispositif expérimental de réacteurs anaérobies développés durant cette thèse grâce à l'expertise technique et scientifique du laboratoire. Le LAMACs permet donc l'utilisation simultanée de 30 chémostats (5 modules de 6 réacteurs) en continu dont l'alimentation, le soutirage et le dégazage sont automatisés. Chaque réacteur peut être opéré indépendamment, excepté pour la température et l'agitation qui sont fixées pour un module entier. Le volume utile peut être compris entre 50 et 200 mL ce qui permet l'échantillonnage d'un volume compatible avec des analyses de biologie moléculaire. Le mode de fonctionnement du réacteur est aussi facilement converti en batch, en fed-batch ou bien continu en adaptant la programmation des pompes péristaltiques, avec un taux de dilution maximal du réacteur de moins de 20 minutes, compatible avec la croissance d'*Escherichia coli.*

La fiabilité technique du LAMACs a été testée et validée en conditions réelles. En particulier, l'homogénéité et la stabilité de la température au sein des modules, ainsi que la précision du débit de biogaz par la mesure de pression ont été évaluées.

Par ailleurs, 12 réacteurs ont été opérés en mode continu pendant neuf semaines. La performance des réplicats techniques est restée similaire durant toute la durée de l'expérience. Les trois écosystèmes de départ ont réagi aux variations d'alimentation imposées de telle manière à ce que des différences entre écosystèmes soient observées mais pas entre réplicats ; ainsi une vraie différence de performance d'écosystèmes a été observée.

Nous avons donc réussi à relever un défi technique qui comble un manque à l'échelle des procédés puisque le design des expériences est souvent conditionné par des contingences techniques, aboutissant généralement à restreindre le nombre de réacteurs employés, ce qui a pour conséquence de limiter le pouvoir statistique des résultats obtenus. Une des limites majeure du système est l'impossibilité d'alimenter avec des composés solides. Néanmoins, la flexibilité du LAMACs permet de diversifier les usages, adaptable très facilement à un procédé aérobie, ou à une inter-connexion en série des réacteurs pour mimer la topologie du tractus digestif, ou encore à l'implémentation d'autres accessoires comme des LEDs pour des cultures phototrophes. Les multiples possibilités d'application du LAMACs présentent donc un intérêt majeur pour des chercheurs de diverses disciplines: du génie des procédés à l'écologie microbienne. Le système fournit une solution de détection automatisée à haute résolution pour surveiller le fonctionnement des écosystèmes (Shade et al., 2009). Dans ce cadre, le LAMACs nous permet de franchir un pas vers la compréhension de la dynamique et du fonctionnement des communautés microbiennes complexes (Widder et al., 2016).

Le développement de ce système LAMACs nous a permis de tester les effets de la complexité du substrat et de la composition de l'assemblage des communautés microbiennes méthanogènes sur le devenir des communautés et l'incidence sur la performance de l'écosystème. Ainsi le mélange et la performance de cinq inocula méthanogènes ont été testés dans le processus de la digestion anaérobie dans 30 réacteurs parallèles pendant 12 semaines. L'hypothèse de départ était que les assemblages seraient plus performants que les inocula pris individuellement, et que cela se vérifierait d'autant plus avec une l'alimentation complexe par rapport à un substrat simple.

La complexité du substrat n'a pas influencé la performance des écosystèmes à l'équilibre. L'hypothèse du maintien d'un réseau métabolique plus complexe lors de la dégradation d'un substrat complexe ne s'est pas vérifiée ici, contrairement à des observations antérieures (Lu et al., 2013). Néanmoins, il est clairement apparu que les structures des communautés bactériennes étaient fortement corrélées à la complexité du substrat utilisé, et que des communautés bactériennes structurellement différentes étaient comparables en termes de performance, due à une forte redondance fonctionnelle des populations bactériennes.

De façon générale, des comportements différents ont été observés entre les Bactéries et les Archées. Le mélange des inocula a induit une augmentation transitoire de la diversité des Archées mais pas pour les Bactéries. Cette diversité des Archées s'est réduite au cours du temps pour se stabiliser ensuite au même niveau que les communautés d'Archées qui n'avaient pas été mélangées. Une corrélation positive a été observée entre la diversité des Archées et la performance des écosystèmes pour les communautés mélangées, mais de façon intéressante, les communautés individuelles sont plus performantes que les communautés mélangées pour un niveau de diversité donné.

Les bactéries appartenant au phylum *Firmicutes* et à la classe des *Clostridia* étaient les plus abondantes quel que soit le substrat utilisé. Ces bactéries font partie du core microbiome des écosystèmes de méthanisation (Nelson et al., 2011; Sundberg et al., 2013). Les bactéries appartenant au phylum *Bacteroidetes* étaient sur-représentées dans les communautés alimentées avec des substrats complexes. Ces organismes appartenant au phylum *Bacteroidetes* ont montré qu'ils étaient capables de dégrader des sucres complexes par fermentation (Wexler, 2007).

Les communautés *Archées* issues des mélanges de plusieurs inocula gardent l'empreinte des communautés d'origine des différents inocula, même après deux mois de culture continue. L'ordre majoritaire des Archées est *Methanobacteriales*, correspondant à des méthanogènes hydrogénotrophes. De façon intéressante, la plupart des réacteurs alimentés avec du substrat complexe ont leur composition marquée par la présence de l'ordre des *Thermoplasmatales,* particulièrement dans les communautés issues de mélanges. Les Archées appartenant à cet ordre apparaissent dans des écosystèmes extrêmes et divers (Adam et al., 2017), ainsi que dans les digesteurs anaérobies en présence de méthanogènes hydrogénotrophes (Chouari et al., 2015).

En étudiant la performance des communautés mélangées par rapport à la performance des communautés individuelles correspondantes, il est apparu que certaines communautés mélangées avaient une production de méthane plus importante que la moyenne des individuelles. Il est possible que les assemblages obtenus par mélange d'écosystèmes aient provoqué la mise en place de nouvelles interactions qui auraient été bénéfiques au fonctionnement de l'écosystème.

Un des résultats les plus surprenants tirés de cette expérience est que la trace des communautés bactériennes individuelles utilisées pour les mélanges se retrouve après séquençage dans les communautés mélangées après deux mois de culture continue. Il y a eu coalescence et incorporation d'éléments issus de plusieurs communautés de départ pour former un nouvel assemblage. En moyenne, trois quarts des communautés mélangées initialement sont retrouvées en fin d'expérience et il apparait que la coalescence était plus marquée dans les mélanges alimentés avec le substrat complexe. Ici encore, les communautés des Archées ont un comportement différent et il semble que la coalescence soit moindre et que ces

communautés provenant de mélanges se soient spécialisées. Dans cette expérience, le phénomène de coalescence des communautés était très marqué, et pourrait venir du fait que les communautés individuelles utilisés au départ venaient d'environnements similaires. Nous aurions assemblé des communautés proches entre elles et déjà fonctionnellement redondantes. Cette hypothèse pourrait aussi expliquer pourquoi l'assemblage n'a pas permis une meilleure utilisation des ressources pour la performance (aussi appelé effet de complémentarité) comme il a été observé dans d'autres études à l'échelle de populations (Bell et al., 2005; Langenheder et al., 2010).

La coalescence des communautés implique que la fonctionnalité d'un écosystème peut potentiellement être compensée par un autre, ce qui peut être considéré comme une stratégie de bioaugmentation à l'échelle de l'écosystème. Le dispositif expérimental LAMACs offre de nombreuses possibilités de composition d'assemblages pour mieux comprendre à l'avenir les facteurs qui favorisent l'implantation de nouvelles fonctionnalités lors d'essais de bioaugmentation.

La combinaison de plusieurs communautés en mélange peut donc être bénéfique au fonctionnement de l'écosystème. Il serait intéressant de pouvoir qualifier le rôle de chaque élément et de quantifier leur importance pour le fonctionnement. L'application d'un modèle combinatoire pourrait nous aider à évaluer les communautés bénéfiques et défavorables dans un mélange en termes de paramètres fonctionnels définis (Jaillard et al., 2014). Les propriétés bénéfiques individuelles des communautés dans les mélanges sont actuellement à l'essai avec ce modèle.

Pour aller plus loin dans les travaux de coalescence, il serait intéressant de tester la force des interactions présentes dans les communautés mélanges en assemblant par exemple des communautés 'jeunes' à des mélanges de communautés plus 'matures'. Cette proposition est renforcée par

l'observation de l'importance de l'historique d'assemblage des communautés sur les performances d'écosystèmes variés (Fukami, 2015; Rummens et al., 2018). L'hypothèse de travail est que les communautés 'matures' auraient plus de difficultés à interagir avec de nouvelles espèces et à les intégrer dans le réseau existant. À l'inverse, l'invasion de communautés 'jeunes' par des espèces exogènes serait plus aisée.

Ces travaux de thèse ont donc permis l'élaboration d'un système de réacteurs multiplexés automatisés, qui ont déjà été repris au laboratoire pour des applications à visées applicatives et fondamentales. Le système a aussi permis la traduction expérimentale de questions de recherche sur l'assemblage des communautés qui auraient été difficiles à mener sans ce dispositif.

# Table of contents

**RESUME DE LA THESE** ---- I

**RESUME ETENDU** ---- IV

**TABLE OF CONTENTS** ---- XIX

**LIST OF FIGURES** ---- XXI

**LIST OF TABLES** ---- XXIV

**1 INTRODUCTION** ---- 27

**2 LITERATURE REVIEW** ---- 32

2.1 MICROBIAL COMMUNITIES IN THE ANAEROBIC DIGESTION PROCESS ---- 35

*2.1.1 The different steps of anaerobic digestion* ---- *35*

*2.1.2 Abiotic parameters influencing anaerobic digestion* ---- *39*

2.2 MOLECULAR TOOL TO STUDY MICROBIAL COMMUNITIES IN THE ANAEROBIC DIGESTION PROCESS ---- 40

2.3 MICROBIAL DIVERSITY AND STRUCTURE ---- 43

*2.3.1 Concept of microbial diversity* ---- *43*

*2.3.2 Diversity measurements* ---- *45*

*2.3.3 Community distances* ---- *47*

*2.4* MICROBIAL INTERACTIONS ---- 48

2.5 MICROBIAL DIVERSITY AND ECOSYSTEMS FUNCTION ---- 51

*2.5.1 Relationship between microbial diversity and ecosystems function* ---- *51*

*2.5.2 Engineering microbial diversity-function experiments* ---- *56*

2.6 COMMUNITY ASSEMBLY ---- 61

**3 THESIS OBJECTIVES** ---- 65

**4 MATERIAL AND METHODS** ---- 70

4.1 INOCULUM AND SUBSTRATE CHOICE ---- 70

4.2 DESIGN OF LAMACS ---- 73

4.3 PRESSURE DATA ANALYSIS ---- 78

4.4 ANALYTICAL METHODS ---- 79

*4.4.1 Biochemical analyses* ---- *79*

*4.4.2 Biological analyses* ---- *80*

4.5 BIOMASS PREPARATION AND INOCULATION ---- 82

4.6 STATISTICAL ANALYSES ---- 82

4.7 SUMMARY OF EXPERIMENTS PERFORMED ---- 84

**5 MULTIPLEXED CHEMOSTAT SYSTEM FOR QUANTIFICATION OF BIODIVERSITY AND ECOSYSTEM FUNCTIONING IN ANAEROBIC DIGESTION ---- 86**

5.1 ABSTRACT ---- 87

5.2 INTRODUCTION ---- 88

5.3 RESULTS ---- 89

*5.3.1 Range of operating conditions of the multiplexed chemostats ---- 89*

*5.3.2 Sensitivity of performance measurement ---- 91*

*5.3.3 Long-term operation of the multiplexed chemostats ---- 93*

5.4 DISCUSSION ---- 95

5.5 ADDITIONAL DATA ---- 101

**6 SUBSTRATE AND INOCULUM CHOICE AFFECT ASSEMBLY OF FUNCTIONALLY REDUNDANT ANAEROBIC COMMUNITIES ---- 106**

6.1 INTRODUCTION ---- 106

6.2 RESULTS ---- 108

*6.2.1 Reactor operation ---- 108*

*6.2.2 Influence of substrate and community assembly on ecosystem functioning ---- 110*

*6.2.3 Relating microbial diversity to ecosystem functioning ---- 115*

*6.2.4 Relating microbial community structures to ecosystem functioning ---- 121*

*6.2.5 Coalescence of mixed communities ---- 129*

6.3 DISCUSSION ---- 135

6.4 CONCLUSION ---- 138

6.5 ADDITIONAL DATA ---- 139

**7 GENERAL CONCLUSION ---- 146**

**8 PERSPECTIVES ---- 150**

**9 REFERENCES ---- 156**

# List of Figures

FIGURE 1 SCHEMATIC VIEW OF THE ANAEROBIC DIGESTION DIFFERENT STEPS ..... 36

FIGURE 2 DIFFERENT MOLECULAR BIOLOGY TECHNIQUES TO ASSIGN MICROBIAL DIVERSITY FACETS (ESCALAS ET AL., 2013) ... **ERREUR ! SIGNET NON DEFINI.**

FIGURE 3 RANGE OF POSSIBLE INTERACTIONS BETWEEN TWO ORGANISMS (FAUST AND RAES, 2012) ..... 48

FIGURE 4 BLACK LINE: ALL SPECIES ARE COMPLETELY COMPLEMENTARY LIGHT GRAY LINE: SPECIES ARE FUNCTIONALLY REDUNDANT. DARK GRAY LINE: FEW SPECIES ARE ABLE TO THE MAXIMUM ECOSYSTEM FUNCTION (BELL ET AL., 2005)..... 53

FIGURE 5 SHORT AND LONG-TERM EFFECT OF BIODIVERSITY ON ECOSYSTEM FUNCTION (SALLES, 2015)..... 55

FIGURE 6 MICROBIAL COMMUNITIES ARE DISTINCT BETWEEN INOCULA BUT REPRODUCIBLE BETWEEN REPLICATES (PERROTTA ET AL., 2017) ..... 58

FIGURE 7 ECOSYSTEM PARAMETERS DRIVING RESISTANCE AND RESILIENCE OF MICROBIAL COMMUNITIES AFTER DISTURBANCE (SHADE ET AL., 2012)..... 63

FIGURE 8 SUGGESTED COMMUNITY ASSEMBLY EXPERIMENT OF TWO INDIVIDUAL COMMUNITIES AND THEIR MIX OVER TIME..... 67

FIGURE 9 GENERAL VIEW OF THE LAMACS AND OF COMPONENTS OF ONE CHEMOSTAT IN THE LAMACS MODULE. A) 3D VIEW OF A LAMACS MODULE, I.E., SIX CHEMOSTATS. THE ELECTRIC BOX CONTAINING CONTROLLING CARDS IS ON THE TOP OF THE MODULE AND ABOVE ANY SOURCE OF LIQUIDS TO PREVENT ELECTRIC FAILURE IN CASE OF LEAKAGE. A WATERPROOF STIRRING PLATE IS UNDERNEATH THE HEATING BLOCK CONTAINING THE SIX REACTOR VESSELS. THREE PERISTALTIC PUMPS ARE ALIGNED ABOVE EACH VESSEL. THE UPPER RANGE OF BAGS IS FOR BIOGAS COLLECTION; THE LOWER RANGE SERVES AS SUBSTRATE RESERVOIRS. THE DIMENSIONS OF ONE MODULE ARE 50 CM WIDTH × 52 CM LENGTH × 100 CM HEIGHT. B) SCHEMATIC VIEW OF A CHEMOSTAT DETAILING THE USE OF THE THREE-HOSE CONNECTOR. ONE PORT IS USED AS INLET FOR FEEDING, AND TWO PORTS AS OUTLET FOR BIOMASS WASTING AND DEGASSING. ONE PORT FOR MANUAL SAMPLING IS SEALED WITH A RUBBER STOPPER. A 0.45 µM PORE-SIZE FILTER IS PLACED IN THE FEEDLINE AHEAD OF THE REACTOR TO PREVENT CONTAMINATION OF THE MEDIUM..... 74

FIGURE 10 CALIBRATION OF ONE PERISTALTIC PUMP. THE CALIBRATION WAS MADE WITH MEASURING DIFFERENT VOLUMES OF WATER AFTER HAVING ROTATED THE PUMP ONE MINUTE AT DIFFERENT VELOCITIES..... 75

FIGURE 11 CALIBRATION OF ONE PRESSURE SENSOR. THE CALIBRATION WAS MADE WITH A PRESSURE IMPOSED MANUALLY, READ BY THE SOFTWARE AND CHECKED WITH A MANOMETER. ..... 77

FIGURE 12 STABLE TEMPERATURE OF THE LAMACS DURING OPERATION. AVERAGE TEMPERATURE AND STANDARD DEVIATION OVER THREE DAYS OF OPERATION AT THREE DIFFERENT POSITIONS IN THE HEATING BLOCK. DATA POINTS WERE RECORDED EVERY 15 MINUTES, WITH 280 TOTAL DATA POINTS..... 91

FIGURE 13 BIOGAS PRODUCTION OF FOUR ANAEROBIC DIGESTERS MEASURED BY THE LAMACS. A) ACTUAL ACCUMULATED BIOGAS OVER TIME (RED CURVES) AND LINEAR REGRESSIONS (BLUE LINES) B) BIOGAS PRODUCTION RATES DERIVED FROM LINEAR REGRESSIONS..... 92

FIGURE 14 DEVELOPMENT OF AVERAGE WEEKLY BIOGAS PRODUCTION OVER TIME FOR ANAEROBIC DIGESTERS WITH THREE DIFFERENT INOCULA. FOUR REPLICATED ANAEROBIC DIGESTERS WERE OPERATED PER CONDITION. WHITE BOXES STAND FOR THE INOC A CONDITION, LIGHT GRAY BOXES FOR INOC B AND DARK-GRAY BOXES FOR INOC C. THE KRUSKAL WALLIS TEST WAS SIGNIFICANT IN THE FIRST EIGHT WEEKS AND THE DUNN POST HOC TEST FOR PAIRWISE MULTIPLE COMPARISON DISPLAYS INOCULA DIFFERENCES WITH '*' SYMBOLS. IN WEEKS 4 TO 6, THE SIMPLE SUBSTRATE WAS USED INSTEAD OF THE COMPLEX SUBSTRATE. 93

Figure 15 Dynamics of biomass concentration for twelve anaerobic digesters over nine weeks. Biomass concentration is expressed as volatile solids. A color code was applied by inoculum origin; Light gray stands for INOC A replicates, dark-gray stands for INOC B replicates and black stands for INOC C replicates. During weeks 4 to 6, a simple substrate with halved loading rate in terms of COD was applied to the reactors. ..... 95
Figure 16 Volatile Fatty Acid concentrations over time in the twelve reactors. Lines in light gray stand for INOC A, dark-gray stand for INOC B, and black stand for INOC C. ..... 102
Figure 17 Picture of 5 LAMACs modules. ..... 102
Figure 18 Bacterial abundances in 30 continuous reactors over a period of twelve weeks. Experimental details are given in chapter 2 and data come from chapter 5). Orange lines stand for data from reactors fed with complex substrate, green for simple substrate and blue for intermediate substrate..... 103
Figure 19 Temporal developments of (a) mean daily???? biogas production over two week intervals and (b) mean biomass concentration at specific time points by substrate. Yellow, green and red bars and lines represent data from reactors fed with "simple", "intermediate" and "complex" substrates, respectively. Error bars represent standard deviations of measurements in 10 reactors (1 measurement per reactor). The grey line in (b) represents the theoretical washout curve of the initially present biomass in the absence of microbial growth. ..... 109
Figure 20 Temporal development of mean biogas production over two week intervals by community assembly (gray = individual communities, blue = mixed communities). Each bar represents the mean biogas production of fifteen reactors and its standard deviation. ..... 111
Figure 21 Ratio of methane production of the mixed communities over reference communities, i.e., the mean methane production of the corresponding individual communities by each substrate. The different colors represent the substrate: yellow for the simple substrate, green for the intermediate substrate, red for the complex substrate. Note that the y-axis is log2 transformed to visually treat ratios above and below 1 equally..... 114
Figure 22 Linear regressions of Bacteria and Archaea diversity calculated with Shannon diversity index along time and separated as a function of community assembly: in panel a-c) individual communities and panel b-d) mixed communities. At time 0, the diversity of the five inocula have been calculated and represented in dark points. For mixed communities, diversity is the average of the 4 inocula of the mixture. Along the experiment, each point is calculated from ten reactors fed with different substrate: yellow for simple substrate, green for intermediate substrate and red for complex substrate ..... 117
Figure 23 Methane production during steady state (weeks 8 to 12) as a function of archaeal diversity expressed as Shannon index (H). Grey symbols and lines represent the data points and the regression for individual communities; blue symbols and lines represent mixed communities. ..... 120
Figure 24 Ordinations of microbial communities with principal coordinate analysis (PCoA) from a distance matrix calculated with Jaccard distance: a) Bacteria b) Archaea. ..... 122
Figure 25 Similarity between mixed bacterial communities (columns) and the respective individual communities derived from the inocula (rows). The similarities are calculated based on the Jaccard distances (1-dissimilarity) and significant results are displayed in red. ..... 132

Figure 26 Similarity of archaeal communities (columns) with each used inocula in the different mixed communities (rows) distinguished per feeding substrate. The similarities are calculated based on the Jaccard distances (1-dissimlarity) and significant results are ..... 134
Figure 27 Monitoring along the experiment A) Volatile fatty acids concentrations B) pH. Each point is calculated from ten values. Colors stand for substrate used: yellow line for simple substrate, green line for intermediate substrate, red line for complex substrate ..... 139
Figure 28 Volatile fatty acids concentrations monitoring along the experiment as a function of substrate. Each mean is calculated from ten values..... 139
Figure 29 Percentage of COD balance calculated after two weeks. Each bar represent ten reactors fed with different substrates : yellow for simple substrate, green for intermediate substrate and red fo compex substrate..... 140
Figure 30 Percentage of COD breakdown in hydraulic retention time 1. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA ..... 140
Figure 31 Percentage of COD breakdown in hydraulic retention time 2. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA ..... 141
Figure 32 Percentage of COD breakdown in hydraulic retention time 3. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA ..... 141
Figure 33 Percentage of COD breakdown in hydraulic retention time 4. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA ..... 142
Figure 34 Percentage of COD breakdown in hydraulic retention time 5. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA ..... 142
Figure 35 Percentage of COD breakdown in hydraulic retention time 6. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA ..... 143
Figure 36 Environmental variables represented on bacterial data ordinations made with Jaccard distance index for the last six weeks of the experiment. The different substrate use is highlighted by the different forms and colors: in yellow diamonds the simple substrate, in green squares the intermediate substrate and in red circles the complex substrate..... 143
Figure 37 Environmental variables (p-value <0.01) represented on Archaea data ordinations made with Jaccard distance index for the last six weeks of the experiment. The shape and color of the plot represent the community assembly: grey triangle for the individual communities and blue circle for the mixed communities ..... 144
Figure 38 Different Archaea relative abundances Order for each inoculum per substrate in steady state conditions (weeks 8 to 12) ..... 144
Figure 39 Experiment testing the strength of different community assembly with different communities coming from different sources and the addition of community at different degrees of development..... 153

# List of Tables

Table 1 Transformation of final steps of the anaerobic digestion and the standard Gibbs free energy (Amani et al., 2010) ..... 38
Table 2 Type of substrate and concentration of the substrate ..... 71
Table 3 Substrate composition for the different experiments ..... 72
Table 4 Detailed prices of components required for the construction of one LAMACs module containing six reactors. ..... 78
Table 5 Summary of parameters used in experiments. ..... 84
Table 6 Linear regression of biogas accumulation over time. ..... 92
Table 7 Comparison of biogas production. Four replicated reactors were used. Mean biogas production rates are expressed over the organic loading rate (ml·g$COD^{-1}$). Kruskal Wallis tests ($Chi^2$) were performed. When the test was significant (p-value < 0.05), the Dunn post-hoc test was applied to account for multiple comparisons of independent samples. ..... 101
Table 8 Bacterial and archaeal diversities calculated as Shannon diversity were compared using t-test. The diversity of the inocula at 0 weeks was compared to the mixed and individual communities. ..... 118
Table 9 Linear regressions slope of Archaea and Bacteria diversity over time as a function of community assembly and substrate. Loss rates derived from linear regressions fitted to archaeal and bacterial diversity over time (Figure 22). ..... 118
Table 10 Linear regressions of archaeal diversity measured as Shannon index (*H*) and steady-state methane production (weeks 8 to 12). ..... 120
Table 11 Correlations of environmental data to microbial community distance data. Correlations were performed with the function 'envfit' of the vegan package and the matrix distance was calculated with the Jaccard index. These correlations were tested on all data from week 2 to 12, then on the six last weeks of the experiment. The parameter of the community assembly (mixed or individual communities) has been tested but is never significant. ..... 125
Table 12 Bacteria absolute abundances in steady state reactors represented for phylum total abundances superior to $10^{10}$ and class abundances superior to $10^7$ expressed in percentage of total abundance ..... 128

# Articles and communications

Peer-reviewed articles

Plouchart D, Milferstedt K, Guizard G, Latrille E, Hamelin J. Multiplexed chemostat system for quantification of biodiversity and ecosystem functioning in anaerobic digestion. PLoS ONE 13(3): e0193748. https://doi.org/10.1371/journal.pone.0193748

International communications

Plouchart D, Milferstedt K, Guizard G, Latrille E, Hamelin J Multiplexed chemostats: a smart way for testing ecological theory. *International Symposium on Microbial Ecology 16th* (ISME 16) 21–26 August 2016 Montreal Canada.

Hamelin, J., Plouchart, D., Guizard, G., Latrille, E., Milferstedt, K. (2017). *Replication of experiments in anaerobic digestion: simpler, greater and easier than you had ever hoped*. Presented at 15. IWA World Conference on Anaerobic Digestion (AD-15), Beijing, CHN (2017-11-17 - 2017-11-20). https://prodinra.inra.fr/record/427671

# Chapter 1

# Introduction

# 1 Introduction

For thousands of years, microorganisms have been exploited for food preservation, brewing and baking. The development of culture and analysis methods has allowed to understand their metabolism and to exploit their potential. Thus, many technologies use the properties of microorganisms for agricultural, health or environmental purposes. One challenge of the 21st century is the development of environmental biotechnologies for renewable energy supply or pollution removal, of which anaerobic digestion is one of the promising technologies. Anaerobic digestion takes advantage of natural microbial communities to degrade various wastes composed of complex organic matter into biogas, a valuable source of energy. Engineering the involving microbial communities could improve process performance (yield, stability, etc.).

Much research has been invested into engineering microorganisms to perform desired biotransformation. Nonetheless, mechanisms and factors affecting complex microbial community assembly and diversity in anaerobic digestion remain little understood (Carballa et al., 2015; Vanwonterghem et al., 2014a; Zhou et al., 2013). Improving the anaerobic digestion process is often studied by tuning abiotic parameters even though the inoculum source for anaerobic digestion was shown to be crucial for process performance (Perrotta et al., 2017; Raposo et al., 2011). It is also acknowledged that microbial communities in anaerobic digestion are highly dynamic irrespective of the functioning (Fernandez et al., 2000; Zumstein et al., 2000) and preservation of a microbial community in a stable and reproducible state for biodiversity-functioning experiment is challenging (Hagen et al., 2015; Kerckhof et al., 2014). It is then desirable to develop better microbial resource

management strategies in anaerobic digestion to control performance or to achieve a desired performance.

This manuscript begins with a literature review (chapter 2) which introduces anaerobic digestion with the main biochemical steps, as well as the importance of abiotic parameters to control the process (2.1). Molecular biology tools used to characterize the microbial communities are presented (2.2) with a particular emphasis on the different measurement of microbial diversity (2.3). Since a microbial community is not just defined by a list of species that build a whole, but also by interactions between members of the community, a summary of known interactions that could take place between microorganisms is provided (2.4). The knowledge about the link between ecosystem functioning and diversity is then presented, exemplified with studies dealing with microbial communities (2.5). Interactions can be defined at multiple levels and many events can influence community structure and dynamics. In the following chapter, these factors are presented and key theories are discussed (2.6).

The material and methods that were used during this work are presented in chapter 4 after the thesis objectives chapter 3.

The first result chapter (chapter 5) deals with the design and the set-up of a multiplexed chemostat that allows anaerobic digestion studies with 30 independent conditions in parallel. This work was submitted for publication and is accepted in the journal PLoS One.

The second result chapter (chapter6) describes the study of community coalescence, with the lasting effects on both performance and community structure. This experiment was developed in close cooperation with colleagues from the University of Exeter in the UK.

This manuscript ends with chapter 7 & 8 with general conclusions and perspectives of the work.

# Chapter 2

# Literature review

# 2 Literature review

Many of ecosystem services rely on biodiversity, as pollination, soil fertilization, regulation of climate by the carbon dioxide absorption, of trees, plants or phytoplankton, as *Emiliania huxleyi* and the cyanobacterium *Prochlorococcus* (Bagby and Chisholm, 2015; Kottmeier et al., 2016). However, scientific researchers currently discuss of an ongoing process toward a sixth mass extinction of species (Barnosky et al., 2011). According to the Millennium Ecosystem Assessment (UNO) of 2005, the current extinction rate is up to one thousand times higher than the fossil record and human activities. Over exploitation and pollution are the main causes. Aware of the global biodiversity loss, ecologists have shown the relationship between biodiversity and the ecosystem functioning (Baumann et al., 2013; Philippot et al., 2013). However, despite microbial communities ubiquity and importance to carry out biosphere activity (Falkowski et al., 2008), only partial understanding is established such as their organization, their stability and functions. To compensate for this lack of information, microbial ecology, synthetic biology and microbiology fields are on the edge of knowledge.

Microbial ecology is a fast-growing subject matter, since advances in high throughput sequencing (Branton et al., 2008; Caporaso et al., 2012; Ronaghi et al., 1998) and big data have made it possible to analyze microbial DNA at high precision. In fact, a complete inventory of the species present in a sample from any environment can be drawn up. These techniques changed our microscopic worldview by revealing the uncountable microbial ecosystem diversity and variety of their habitat (seas, soil or gut tracts). An estimated of the number of prokaryotes inhabiting the Earth is $10^{30}$ prokaryotes (Whitman et al., 1998), among those 1 trillion ($10^{12}$) microbial

species (Locey and Lennon, 2016) and less than one percent of microbial species have been identified..

Microbial diversity has been estimated and it appears that the majority are still unknown. (Curtis et al., 2006; Torsvik and Ovreas, 2002), Microbes are responsible for all kinds of desirable functions, as fermentation of various food (cocoa, cheese, yoghurt, wine, beer etc.), or during digestion in the gut, or again as the key providers for different forms of nitrogen compounds assimilable by plants. Most frequently, these functions are not done by one group of microbes but through the interactions between microbes in a microbial community. An example is wastewater treatment followed by anaerobic digestion with the production of a renewable source of energy in the form of methane (Verstraete et al., 2007). Microbial communities offer many possibilities for recycling waste and produce energy, such as biofuels, hydrogen production, high added value molecules or anaerobic digestion.

Engineering of microbial communities is then an outstanding scientific interest because their dynamics and complexity are still not well understood. Predicting and managing these microbial communities would make it possible to drive their functionality in specific cases such as preservation, performance and also in cases of bioaugmentation or bioremediation. To achieve this level of understanding, models and experiments testing parameters influencing the dynamics and assembly of these complex microbial communities are implemented. The community assembly can be driven by specific taxa (niche theory) and random taxa (neutral theory). The niche theory implies complementarity of species that would have a better uptake of resource when the community is more diverse. The neutral theory suggests the probability of better functioning increase with an increasing number of taxa. These effects can be both influenced by stochastic and deterministic processes depending on nutrients, disturbances, etc.

(Nemergut et al., 2014). Taking an interest in the community assembly is all the more interesting for understanding their function and development. For example, when different species have a similar functional role in an ecosystem, they can be considered as functionally redundant. A concrete example of functional redundancy can be seen in microbial communities in wastewater treatment plants (WWTP), which may be structurally different but carry out the degradation of organic matter (Valentin-Vargas et al., 2012). Yet in WWTP, wastewater continues to flow in, bringing in new taxa while the performance of ecosystem function is still carried out. Hence in WWTP the functional redundancy can possibly involve both neutral processes and niches complementarity for non-overlapping substrate specificities, as micropollutants (Saunders et al., 2015).

These findings show that having functionally redundant species may play an important role in ensuring ecosystem stability. Yet, it has not been demonstrated. To study the ecosystem stability, different types of experiments can be carried out to manipulate diversity and disturbance. Manipulating microbial diversity can be achieved by adding isolated species to a mixture (Bell et al., 2005), or conversely by dilutions (Roger et al., 2016), or by coalescence studies (Rillig et al., 2015). Coalescence studies assume that mixed communities behave as single entities and that the evolution of mixed communities leads to the dominance of one community (Tikhonov, 2016).

In the following chapters, microbial communities of the anaerobic digestion will be presented, as long as the different tools to perform microbial community studies in the frame of the anaerobic digestion process. Concepts of microbial diversity, interactions with different examples of these notions in the context of anaerobic digestion will be introduced. We will end the literature review with notions on the link between microbial community and

the ecosystem functioning and review the community assembly fundamentals.

## 2.1 Microbial communities in the anaerobic digestion process

Anaerobic digestion is the degradation process of organic matter into biogas, mainly methane and carbon dioxide. This process is carried out by complex microbial communities under anaerobic condition and naturally happens in marine sediments, hydrothermal sources, and digestive tracts of animals and many more environments where degradable organic matter is available in the absence of a major oxygen source.

Anaerobic digestion used in biotechnology under controlled conditions is used to generate biogas (Moletta, 2008) from various wastes: sewage sludge, energy crops, agricultural, industrial and distillery wastes, etc. Biogas can then be used in different forms: combustion for production of electricity and heat, as biofuel or injection into the natural gas grid after purification. In June 2016 in France, 463 anaerobic digestion units are installed and are equivalent to the power of 380MW (https://www.ecologique-solidaire.gouv.fr/biogaz). The objectives of Europe for 2020 are 625MW. These units are gradually establishing at different scales (farms and centralized) as the biotechnology becomes better understood and optimized.

### 2.1.1 The different steps of anaerobic digestion

The anaerobic digestion can be described in four main steps implying different microorganisms: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1).

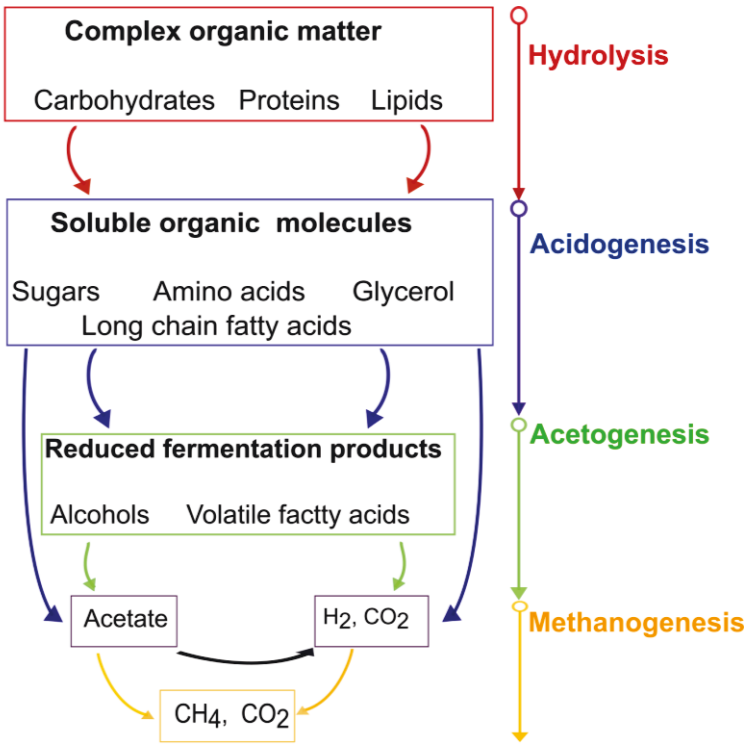


Figure 1 Schematic view of the anaerobic digestion different steps

The first step is hydrolysis involving various strict or facultative anaerobic bacteria as for example *Bacteroidetes, Firmicutes*, two phyla recording most of the identified species in some reactors (Venkiteshwaran et al., 2015). These bacteria produce exo-enzymes for polymer degradation (polysaccharides, protein and lipids) into oligomers and soluble monomers (glucose, glycerol, fatty acids, etc.).

Acidogenesis is the second step and involve fermenting strict anaerobic (*Clostridium* genus, etc.) and facultative anaerobic (*Streptococcus, Bacillus* genera, etc) bacteria. They degrade soluble monomers into alcohols and volatile fatty acids (acetic, propionic, butyric, valeric acids), organic acids (lactic, succinic, etc.), hydrogen and carbon dioxide.

The acetogenesis step converts volatile fatty acids and alcohols into acetate, carbon dioxide and hydrogen by two different ways. Homo-acetogenic bacteria transform organic molecules or hydrogen and carbon dioxide into acetate. Acetogenic bacteria transform volatile fatty acids into hydrogen and

carbon dioxide. The acetogenesis process is endergonic (Table 1) and can only occur in nature in syntrophy, in presence of other groups of microorganisms that render the reaction thermodynamically favorable. In anaerobic digestion, syntrophic bacteria (*Syntrophobacter, Syntrophomonas*, etc.) live in close association with methanogenic *Archaea*. This syntrophy enables the consumption of hydrogen that decrease the partial pressure of hydrogen, rendering the reaction exergonic and preventing hydrogen inhibition of syntrophes, as shown in Table 1.

Methanogenesis is the final step of conversion of acetogenesis products into biogas under strictly anaerobic conditions. Hydrogenotrophic methanogens (e.g., *Methanothermobacter*, *Methanoculleus*) use hydrogen and carbon dioxide in syntrophy with acetogenic bacteria, whereas acetoclastic archaeal methanogens (e.g., *Methanosarcinales)*, use the acetate. All known methanogens belong to the domain Archaea. Depending on the conditions, the production of methane accounts for the consumption of 70% of acetate and 30% of carbon dioxide and hydrogen (Venkiteshwaran et al., 2015). Generally, the average biogas composition is relative to the substrate and ranges between 60–70% methane and 30–40% carbon dioxide with possible traces of hydrogen and hydrogen sulfide.

In the *Archaea* domain, it was previously thought that only the phylum *Euryarchaeota* contains methanogens (*Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanocellales, Methanopyrales and Methanomassiliicoccales*), but a new phylum *Verstraetearchaeota* was recently discovered to have a gene for methylotrophic methanogenesis (Vanwonterghem et al., 2016). The methanogenesis step requires the enzyme methyl coenzyme M reductase encoded by the *mcrA* gene found on all genomes of methanogenic archaea (Luo et al., 2002). Expression of *mcrA* has been demonstrated to highlight the

active members of the methanogenic community in anaerobic digesters (Alvarado et al., 2014).

Table 1 Transformation of different steps of the anaerobic digestion and the standard Gibbs free energy (Amani et al., 2010)

| Transformation | Formula | $\Delta G^{0'}$ [kJ·mol$^{-1}$] at 25 °C |
|---|---|---|
| One example of acetogenetic transformation: butyrate to acetate[1] | $C_3H_7COO^- + 2\ H_2O \leftrightarrows 2\ CH_3COO^- + H^+ + 2\ H_2$ | 48 |
| Methanogenesis by hydrogenotrophic methanogens | $HCO_3^- + 4H_2 + H^+ \leftrightarrows CH_4 + 3H_2O$ | -139 |
| Syntrophic acetate oxidation by acetoclastic methanogens | $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$ | -31 |

[1]one example of various possible reactions

A number of publications aimed to characterize the microbiome in many anaerobic digesters to consider an essential core species for biogas production. While an important part of the microbiome is still unknown (Nelson et al., 2011; Rivière et al., 2009; Sundberg et al., 2013; Treu et al., 2016), the core of syntrophic bacteria and methanogens seems to be stable and resilient, potentially because of the constant feeding of the same substrate and the core key role in the anaerobic digestion (De Vrieze et al., 2016a; Treu et al., 2016; Werner et al., 2011). Conversely, the populations of fermenting bacteria seem different and therefore may be more functionally redundant.

Many factors influence the microbial communities of the anaerobic digestion process. According to Sundberg et., al., the temperature and the substrate type are the most influencing factors (Sundberg et al., 2013), but also the pH (Boaro et al., 2014), the retention time (Vanwonterghem et al., 2015), the organic loading rate (OLR) (Pholchan et al., 2010), the feeding regimes (Pholchan et al., 2010) play a role.

### 2.1.2 Abiotic parameters influencing anaerobic digestion

The performance of anaerobic digesters is determined by many abiotic parameters. In this part, the main parameters are discussed and have been taking into account for the development of the system used in our experiment. In addition, the parameters controlling the proper functioning of the digesters are listed and will be used to monitor the correct functioning conditions in the experiments carried out in the project.

Firstly, different types of bioreactors (continuously stirred tank – CSTR, sequential batch – SBR, plug flow, etc.) can carry out liquid or solid anaerobic digestion and have specific characteristics favoring different in the anaerobic food web or methane yield. CSTR do not produce as much methane as other reactor configurations (Bensmann et al., 2013). This CSTR configuration was anyway used during this work, with a constant dilution rate to select populations according to their growth rates for answering ecological question (chapter 6). Depending on the system temperature, three modes of production are defined: psychrophilic (15–25°C), mesophilic (25–45°C) and thermophilic (55–65°C). Multiple parameters can be also controlled, as the retention time and the organic loading rate (OLR), which is recommended to be low at the start of the experiment to avoid volatile fatty acids (VFA) accumulation. Finally, the substrate used for the process is crucial. Not all substrates are degraded at the same rate nor do they have the same methanogenic potential depending on their composition and complexity. For the moment, no clear recipe exists but two main properties are considered when feeding a digester: the methanogenic potential of a substrate and the ratio of organic matter and mineral elements. Substrate is a commonly studied parameter and its complexity is thought to increase the potential of metabolic pathways. In one study, the authors tested the methane performance and the community with three substrates of different

complexity in ascending order: xylan, cellulose and food waste. The authors found that the methane yield was higher with reactors fed with the more complex food waste (Lu et al., 2013) and explained these results with the higher number and a different structure of *Bacteria* and *Archaea* taxa observed in these reactors. In this work, we did not vary temperature as abiotic parameter but substrate load and composition instead (details in chapter 4.1).

The optimal C/N ratio is about 35 and deviation from this value may cause reduction of biogas production, digesters instability or failure. Wheat straw is for example often used as substrate in anaerobic digestion but due to the high C/N ratio (50-150), the methanogenic potential can be lower (Hagos et al., 2017). Strategy of co-digestion has for example been tested to adjust the ratio with a co-substrate of high nitrogen content such as cow manure (C/N: 16-25) (Hagos et al., 2017; Sun et al., 2015). The C/N ratios of the synthetic media used in the different experiments of the project were kept in the optimal range to avoid such problems.

Finally, different environmental conditions can be monitored to control the stable functioning of the anaerobic digestion process such as the pH range, which should be between 6.5 and 7.3 (Moletta, 2008) or as the non-accumulation of the volatile fatty acids (Su et al., 2015).

## 2.2 Molecular tool to study microbial communities in the anaerobic digestion process

Using miniaturized microcosm or laboratory-based microbial system allows for conscientious measurement of environmental parameters and microbial populations (Jessup et al., 2004). Furthermore, miniaturizing makes it possible to multiply replicates or design experiments with an increased statistical power (Lennon, 2011; Prosser, 2010). Miniaturized bioreactor

studies managed to perform anaerobic digestion in 10 mL scale (Kusterer et al., 2008; Schmideder et al., 2015), but this small size does not provide sufficient biological material for subsequent microbial community analyses. Although, 10 mL scale does not allow enough volume for sampling and ensuring representative biomass for molecular and technical analysis. Multiplying conditions in anaerobic digestion can be achieved relatively easily in batch condition. In batch reactors the substrate is gradually absorbed by the succeeding microbial populations over time until its consumption. Instead of following a final point reaction, continuous anaerobic digestion (e. g. in chemostats) allows for the constant addition of substrate, parallel to equal volume removing, thus ensuring stable environmental conditions after few retention times. The continuous process therefore provides monitoring the stability of anaerobic digestion process over time.

DNA sequencing stands for the gold standard to study microbial communities from natural or engineered environments, allowing characterization and quantification of the microorganisms present in virtually any sample. After genomic DNA extraction, sequencing can be performed directly without any PCR amplification step; we then speak of metagenomic. The metagenomic approach is resource intensive but gives access to the richest information with taxonomic and functional characterization of the community (Eloe-Fadrosh et al., 2016; Treu et al., 2016). A more parsimonious approach is to focus one marker gene with PCR amplification, the 16S ribosomal RNA gene being the most commonly targeted gene because it is conserved in the tree of life and is present in both archaeal and bacterial organisms (Rivière et al., 2009; Roux et al., 2011). The 16S rRNA gene sequence is composed of nine hypervariable regions (V1-V9) that differentiate species. The whole sequence of 16S rRNA gene have been

determined for a large number of microorganisms and are publicly available in databases, such as Greengenes, SILVA or Ribosomal Database Project (RDP). The sequencing of the 16S rRNA gene and comparison of the sequences obtained with those of the databases allow the sequences to be taxonomically affiliated with the closest microorganism in the database. However, some sequences remain unaffiliated since they are distantly related to known sequences, because not all the diversity of microorganisms has been recorded (Werner et al., 2012; Woese and Fox, 1977).

The hypervariable V4-V5 region of 16S rRNA gene is long enough to allow phylogenetic placement and is present in both archaeal and bacterial organisms. After sequencing analyses, taxonomic classification and microbial community structures can be established. Targeting this region is proved to be relevant in anaerobic digestion studies (Venkiteshwaran et al., 2016) and this hypervariable V4-V5 region of 16S rRNA gene will be used in the project.

Other molecular methods based on the extraction of RNA, metabolites or protein from a sample define specifically the activity of the microbial communities (Figure 2). These methods have been already used to describe the microbial activity of anaerobic digesters (Hanreich et al., 2013; Heyer et al., 2015). Combining identification and activity methods enabled to link the active microbes and their functionality and for example, methanogens have been showed to be highly metabolically active than represented (Hanreich et al., 2013).

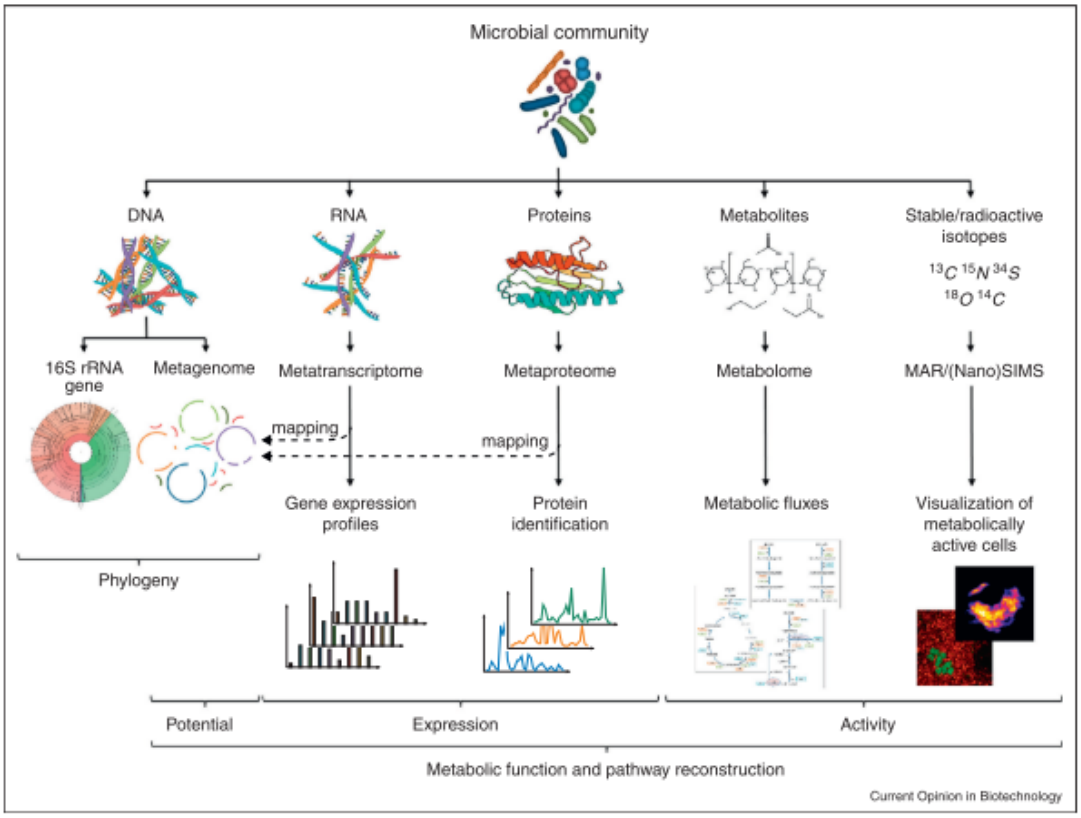


Figure 2 Molecular methods for determining phylogeny and functional diversity of microbial communities (Vanwonterghem et al., 2014b)

## 2.3 Microbial diversity and structure

### 2.3.1 Concept of microbial diversity

While biodiversity measurements can easily be based on the number of species for macro-organisms, the notion of species is ambiguous for *Bacteria* or *Archaea* because of constant mutations, homologous recombination and horizontal gene transfers (Konstantinidis and Tiedje, 2005; Stackebrandt et al., 2002). However, DNA sequencing has revolutionized how we study microbes. High-throughput sequencing analyses gives now access to 16S rRNA gene sequences and enable to distinguish Operational Taxonomic Units (OTU) where clustering sequences is usually set to 97% of similarity corresponding to an average species delineation threshold (Konstantinidis and Tiedje, 2005; Schloss and Handelsman, 2005), with notable exceptions in the threshold for some species (Nguyen et al., 2016).

Several facets of microbial diversity can be explored based on DNA sequences (Figure 3), as for example **nucleic acid fragments diversity**, which is related to the number of nucleic acid fragments within and between species, and reflects the potential activity of the species. This level of diversity can be measured with fingerprinting techniques (Haegeman et al., 2008; Leclerc et al., 2004). The **taxonomic diversity** takes into account the classification of sequences into different phylogenetic levels (domain, kingdom, phylum, class, order, family, genus, and species). This affiliation is done on the basis of genetic similarities of 16S rRNA gene sequences or specific marker genes. The **phylogenetic diversity** gives weight to the phylogenetic distances between sequences.

The **functional diversity** can be based on protein-coding genes and targets the functional potential of a sample. Functional genes may be more appropriate than the 16S rRNA gene when attempting to relate community structure and function of the ecosystem (Evans et al., 2017). This was already observed in the context of biohydrogen fermentation with the *hydA* gene encoding a sub-unit of the [Fe-Fe] hydrogenase (Quéméneur et al., 2011, 2010) or in the context of anaerobic digestion with the *mcrA* gene encoding the sub-unit of the methyl-coenzyme-M reductase found in methanogenic *Archaea* (Gagnon et al., 2011; Luton et al., 2002). Method based on RNA (Figure 2) as for example as RNA-seq, reflects the potential activity of the microbial communities and has already underlined different diversity results obtained between DNA and RNA specially with Archaea (De Vrieze et al., 2018)

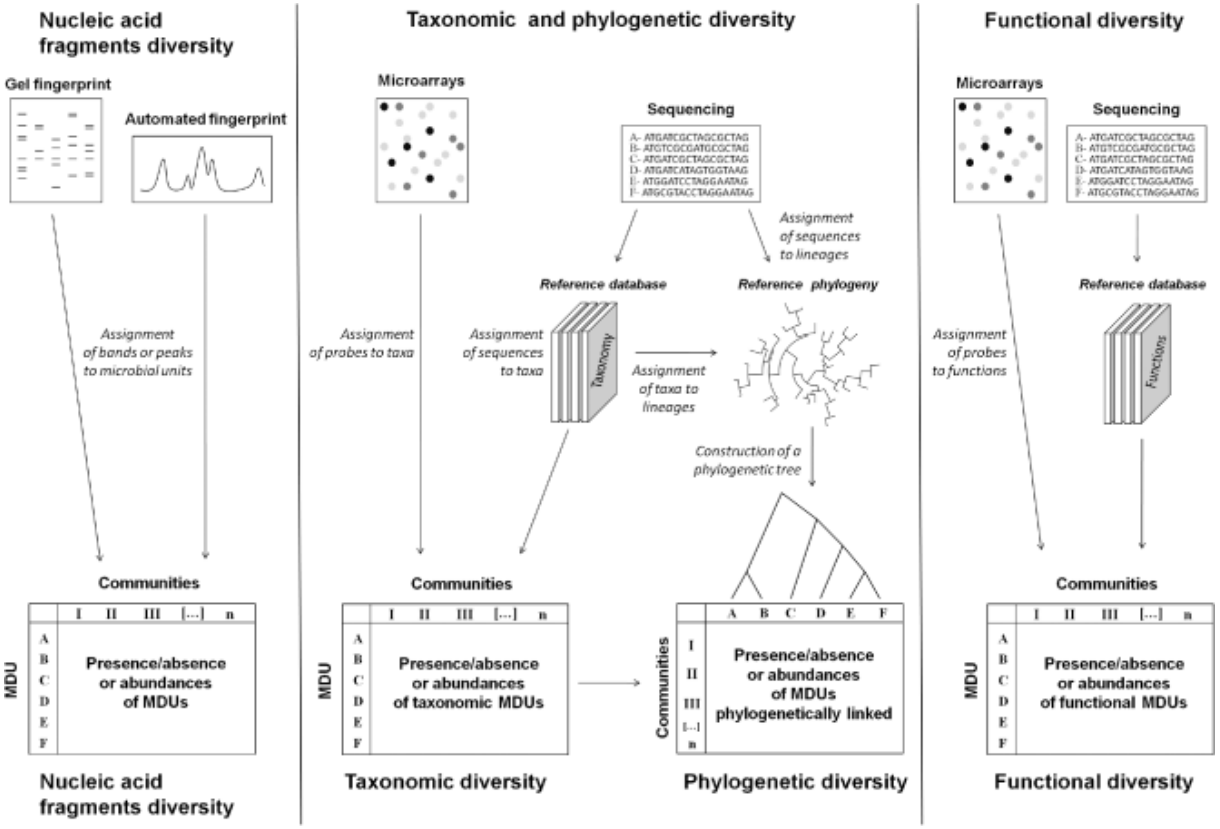


Figure 3 Different molecular biology techniques to assign microbial diversity facets (Escalas et al., 2013)

## 2.3.2 Diversity measurements

Diversity can be described by the Richness and the Evenness of species present in as sample. Richness refers to the number of species and is insensitive to species frequencies, whereas evenness refers to the species abundance distribution. These parameters are often found in complex microbial communities study and will be used to describe the *Bacteria* and *Archaea* communities in the community assembly experiment.

Several Richness estimators were developed based on sequence data ($S_{ACE}$, $S_{Chao1}$) and $S_{Chao1}$ is commonly used in microbiology (Chao, 1984; Kemp and Aller, 2004). This $S_{Chao1}$ estimator uses a non-parametric approach to extrapolate a rarefaction curve. It is based upon the number of rare OTUs found in a sample. The formula of $S_{Chao1}$ estimates is:

$$S_{Chao1} = S_{obs} + \left(\frac{([f]_1)^2}{2f_2}\right)$$

where $S_{obs}$ stands for the number of observed species, $f_1$ for the number of singleton OTU (single occurrence) and $f_2$ for the number of doubleton OTU (two occurrences). However, this $S_{Chao1}$ estimator heavily rely on the sampling effort and may be therefore only considerate as a lower bound estimation of the species Richness with low accuracy (Haegeman et al., 2013; Lemos et al., 2011).

On the opposite of the Richness estimation, the Simpson diversity index ($D$) (Simpson, 1949) is insensitive to the number of species but only on species frequencies. This is the reason why the Simpson diversity index is very easy to estimate even with low sampling effort and is very robust towards the different molecular methods used (Haegeman et al., 2014, 2013, 2008). The formula is:

$$D = 1 - \frac{\sum n(n-1)}{N(N-1)}$$

where N stands for the total number of individuals of all species and n stands for the number of individuals of each species. The probability varies between 0 and 1, low scores (close to 0) indicate low diversity and high scores (close to 1) high diversity.

The Shannon diversity index ($H'$) is a diversity measure based on entropy (Jost, 2006; Shannon, 1948). The Shannon diversity index is the most commonly found because of its unique ability to weigh OTUs by their frequency, without disproportionately favoring either rare or common OTUs. We used the Shannon diversity index in this work in chapter 0.

The formula is:

$$H' = -\sum_{i=1}^{S} p(i) \ln p(i)$$

where p($i$) stands for the proportion of the $i^{th}$ among the total number S of OTUs.

In anaerobic digestion, the diversity of *Archaea* is always much lower than the diversity of *Bacteria*, but vary widely from one study to another (Maspolim et al., 2015; Sun et al., 2015; Sundberg et al., 2013).

### 2.3.3 Community distances

The diversity measurements described in the previous section apply for a single community and are called α-diversity. The difference between two communities is called the β-diversity. A brief introduction on available tools to compare communities is presented.

All these methods rely on the study of the relationship between the different OTUs. Two general approaches are differentiated whether abundance is taking into account or not: (1) Quantitative measures work with the abundance, as weighted UniFrac (Lozupone et al., 2007) or Bray-Curtis distances. (2) Qualitative measures use the presence/absence, as for example as Unweighed UniFrac (Lozupone and Knight, 2005) or Sörensen index. Qualitative measures would be more appropriate for detecting differences in composition and the contribution of the different founding populations, whereas quantitative measure would be more appropriate for measuring differences in community structure and enable to highlight transient factor effects (Lozupone et al., 2007). In this work, quantitative measures of distances between communities were more appropriate.

## *2.4* Microbial interactions

Interactions between two individuals or populations can be described with their effect on growth of another individual or population: positive (commensalism, mutualism), negative (amensalism, parasitism or predation) or neutral (no effect). As represented in Figure 4 (Faust and Raes, 2012), the two-way interaction established between two partners depends on the sign of the unidirectional effects of each partner.

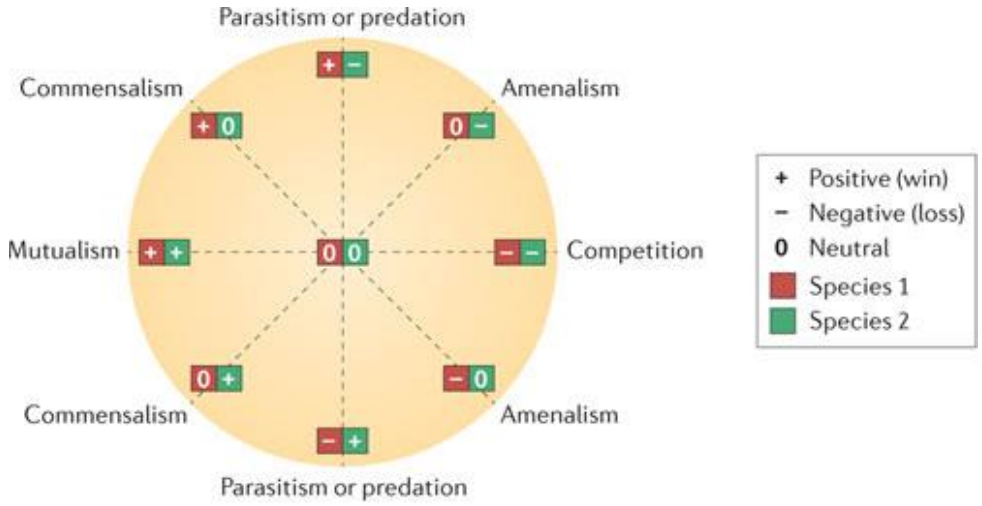


Figure 4 Range of possible interactions between two organisms (Faust and Raes, 2012)

In commensalism only one partner benefits without harming the other, such as in trophic chain, where the latter benefits from the excretion products of the former. In a mutualistic relationship, the entities equally benefit and the term syntrophy is employed when mutualistic interaction is necessary for the growth of both partners. For example, a syntrophic interaction is involved in the last step of the anaerobic foodweb, between the syntrophic Bacteria that produce hydrogen and the Archaea hydrogen consuming the hydrogen as explained in Table 1 before.

Amensalism is an interaction where one partner is harmed without the advantage of the other. This relation is often the consequence to a change of

the environment, toxins secretion, etc. For example in anaerobic digestion, when VFA production rate exceeds the VFA consumption rate, this imbalance results in the VFA accumulation and in the acidification of the process also called acidosis, a major cause of anaerobic digestion process failure (Akuzawa et al., 2011).

Two types of loose-win interactions exist: i) parasitism where bacteria use and harm the host to replicate, as for example as in the fermentative process where *Geobacter metallireducens* gains energy by parasitism of *Clostridium pasteurianum* (Moscoviz et al., 2017) and ii) predation is direct such as bacteriophages and bacterial prey in anaerobic digesters (Shapiro et al., 2010; Zhang et al., 2017).

Competition is a very common relationship where two partners compete for the same resource (nutrient, living space, etc.). This relation was first described by Gause (Gause, 1934). From this experiment, Gause derived the law of competitive exclusion where only one species can survive when several species compete for the resource. Competition is one of the main drivers for the dynamics of community composition (Freilich et al., 2011; Violle et al., 2010), but cannot explain why so many species can coexist in ecosystems with few available resources. This statement was popularized by Hutchinson in his famous paper on the Paradox of the plankton (Hutchinson, 1961).

The interactions can also depend on environmental parameters, such as temperature, nutrient availability, physico-chemical factors and with spatial or time scale. One interesting example of microbe interaction is quorum sensing. This property enables fast exchange of low-weight molecules to adopt new properties required for molecular communication, such as with human cells in the gut (Holm and Vikström, 2014), or for biofilm formation in reactors accomplishing anaerobic digestion (Langer et al., 2014).

For the time scale, successions of different microbial interactions have been shown in various forms, such as the anaerobic digestion microbial communities where acidogenic bacteria consume waste products from hydrolytic bacteria (even though the successive steps happen at the same time). Two factors are nevertheless limiting these dynamics: dispersal limitation and interactions. Another example of succession would be the cheese ripening where different yeasts compete with each other while yeast-bacteria interactions are important for colonization on the cheese surface (Mounier et al., 2008). In the latter example, non-trophic interactions were exemplified (Arditi et al., 2005). Similarly in anaerobic digestion, rare taxa could contribute to the anaerobic digestion process by secreting a growth factor or extra-cellular enzymes while other members of fermenting bacteria compete for the substrate degradation products. However, the successions of microbial communities did not interfere with the stable bioreactor performance (Fernández et al., 1999; Wu et al., 2016; Zumstein et al., 2000).

Community distance measures allow us to establish the structure of the different microbial communities as seen earlier. However, establishing interactions among complex microbial communities, such as anaerobic digestion, where many species are involved with cross-feeding and syntrophic interactions require developed tools. In order to assess the interactions, different methods use correlations to establish pairwise taxa co-occurrence networks (Faust et al., 2015; Weiss et al., 2016). However, these co-occurrence relationships are not always meaningful from an ecological point of view (Faust and Raes, 2012). The putative positive interactions when the abundances of two OTUs correlate could be in fact explained by other phenomena, such as cross-feeding, niche overlap or even the influence of a third organism driving both organisms, etc. As the range of

interactions is wide from cooperation to competition and everything in between, building interactions networks is subject to interpretation.

## 2.5 Microbial diversity and ecosystems function

### 2.5.1 Relationship between microbial diversity and ecosystems function

In ecology, species loss has been shown to be concomitant with impaired ecosystem functioning (Cardinale et al., 2012, 2011; Duffy et al., 2017). However, this link has not been clearly shown in microbial ecology (Graham et al., 2014; Prosser et al., 2007) despite gathered studies which have not succeeded in drawing patterns (Bier et al., 2015; Graham et al., 2016; Roger et al., 2016; Smith, 2007).

Some showed a positive link between microbial diversity with ecosystem function (Bell et al., 2005; Carballa et al., 2011; Ho et al., 2014; Langenheder et al., 2010; Salles et al., 2009; Werner et al., 2011), other a random relationship where no pattern was evident (Venkiteshwaran et al., 2017) or flat (Szabó et al., 2017) or curved in the form of a 'hump', where a maximum is reached before a decreasing effect or curved in U-shaped relationship (Horner-Devine et al., 2003). Others found a negative link (Philippot et al., 2013; Pholchan et al., 2013, 2010). However, in the studies where a lower diversity was observed, the ecosystem functioning was not impacted or less stable. These researches suggest, as highlighted in Shade et al., (2017) that: 'diversity is the question not the answer' (Shade, 2017). A clear example of this statement can be illustrated with patients affected by inflammatory bowel disease of *Clostridium difficile*, which is characterized by a very low diversity of the intestinal microbiome. To cure this dysbiose, fecal transplants have been used are promising, yet they do not always work and this phenomenon may be due to the gut microbial legacy. Postulating that

diversity itself would explain the success of these transplants would be then a misleading path.

An interesting study of Bell et al., (2005) considered the mechanisms between bacterial diversity and their functioning. Synthetic communities were designed with an increasing number of species from 1 to 42 and daily respiration rate were measured (Bell et al., 2005). To explain why the respiration rate increases with the diversity, they postulated the role of both niche complementarity and selection process. Selection is the assumption that more diversity increases the probability of containing successful organisms. These organisms would outperform others in terms of abundance and performance (either in negative or positive performance). On the other hand, complementarity assumes the niche differentiation for competitive organisms. In the study, the authors assumed as shown in Figure 5, that if all species are completely complementary, a constant positive relation would be observed between the ecosystem and the species richness (dark line). At the opposite, if the selection process drives the ecosystem functioning, process stabilization would be observed rapidly (light gray line). If both mechanisms of complementary and selection happen, system functioning would first increase and then stabilize (dark gray line). They in fact observed both phenomena of selection and niche complementarity, with a greater effect of complementarity. The authors explain these mechanisms mainly with

synergistic interactions and with a minor role of bacterial community composition.

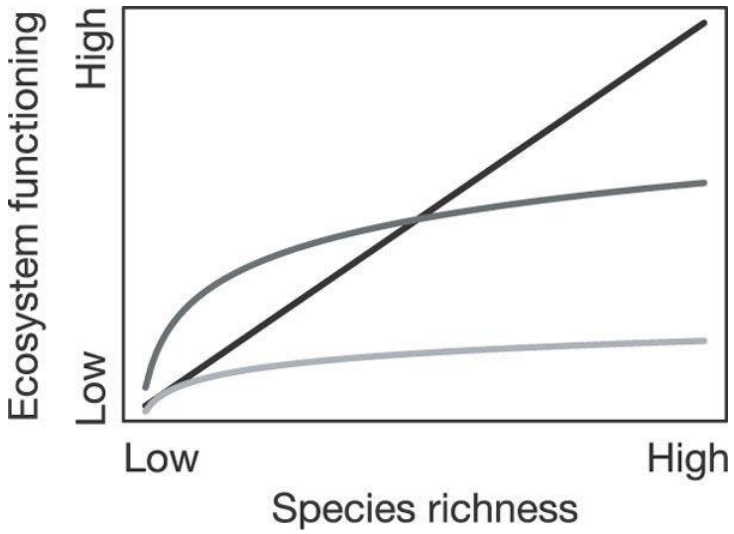


Figure 5 Black line: all species are completely complementary light gray line: Species are functionally redundant. Dark gray line: Few species are able to the maximum ecosystem function (Bell et al., 2005)

An ecological approach postulates the diversity-stability debate (McCann, 2000), where stability is defined based on either its dynamic stability or by its ability to face perturbations (resilience and resistance). Remarkably, an increase of diversity seem to have a strong influence on population variability (Yachi and Loreau, 1999).

This stability can be explained by three different ecological concepts. Firstly, the portfolio effect or the averaging effect refers to the diversification helping stabilize returns (Schindler et al., 2015). Secondly, the existence of multitude interactions between species could lead to a community less sensitive to the loss of one or more species (Hooper and Gordon, 2001). Finally, the insurance hypothesis states that ecosystem function is maintained with biodiversity during environmental change (Yachi and Loreau, 1999). This hypothesis does not mean that diversity increases stability, the stability rather depends on functional groups capable of responding.

In the microbial context, Konopka et al., (2014) support the diversity-stability debate by providing endogenous and exogenous explanatory mechanisms (Konopka et al., 2014). On the one hand, the exogenous mechanisms, such as the spatial niche partitioning would enable physical space for diversity. And, on the other hand, endogenous mechanisms provide: (i) high dynamic, such as phages dynamics, protest grazing, and rare biosphere, (Louca and Doebeli, 2017; Lynch and Neufeld, 2015; Saleem et al., 2013) and (ii) strong interactions in microbial communities, as for example syntrophic interactions in the anaerobic digestion As such, a strong network and a high intrinsic dynamic of microbial communities would favor a buffer effect face to a disturbance (Konopka et al., 2014; Loreau, 2001). High microbial diversity supports the portfolio effect hypothesis by the reservoir of ecotypes with broad ecological functions. Finally, the insurance hypothesis has been supported by different studies where stability increased with diversity, and no changes of stability were influenced by variation of diversity (Tardy et al., 2014).

In fact, microbiologists have found that diversity was not the metric changing the Biodiversity and Ecosystem Functioning (BEF) relationship, but they rather observed a **functional redundancy**, i.e. the change of species diversity. This microbial dynamic does not affect the system functioning thanks their ability to perform the same function (Allison and Martiny, 2008). Microbial communities are often assumed to be redundant owing to their high abundance, dispersal, their physiological versatility and their fast growth rate compared to plants or animals. These properties allowed microbial communities metabolic flexibility and physiological tolerance against disturbances. Therefore, if the microbial abundance drops due to environmental perturbations, microbial communities would adapt thanks to

the degree of overlap in functioning among different species, which assumes that redundant species occupy broad niches to coexist.

As shown in Figure 6 (Salles, 2015), the effect of biodiversity can be studied in the short (a) and long-term (b, c, d). In the short effect, the productivity increase, and as explained before, the community assembly is driven by selection and complementarity (Bell et al., 2005). In the long-term effect, functional redundancy after stress disturbance enables the stability, resilience and adaptation (b, c). In a study of Fernandez et al., (2000), eight bioreactors performing anaerobic digestion were studied after glucose shock. Two sets of four bioreactors were inoculated with digestates fed with glucose for 200 days (HS) and 60 days (LS) respectively. Reactors where digestate was operated for 200 days remain functionally stable with high community diversity, whereas the other one decreased in performance and the microbial community compositions were resilient. In conclusion, the community with functional stability had a higher diversity after perturbation and it has been shown that this diversity enables the functional stability (Fernandez et al., 2000).

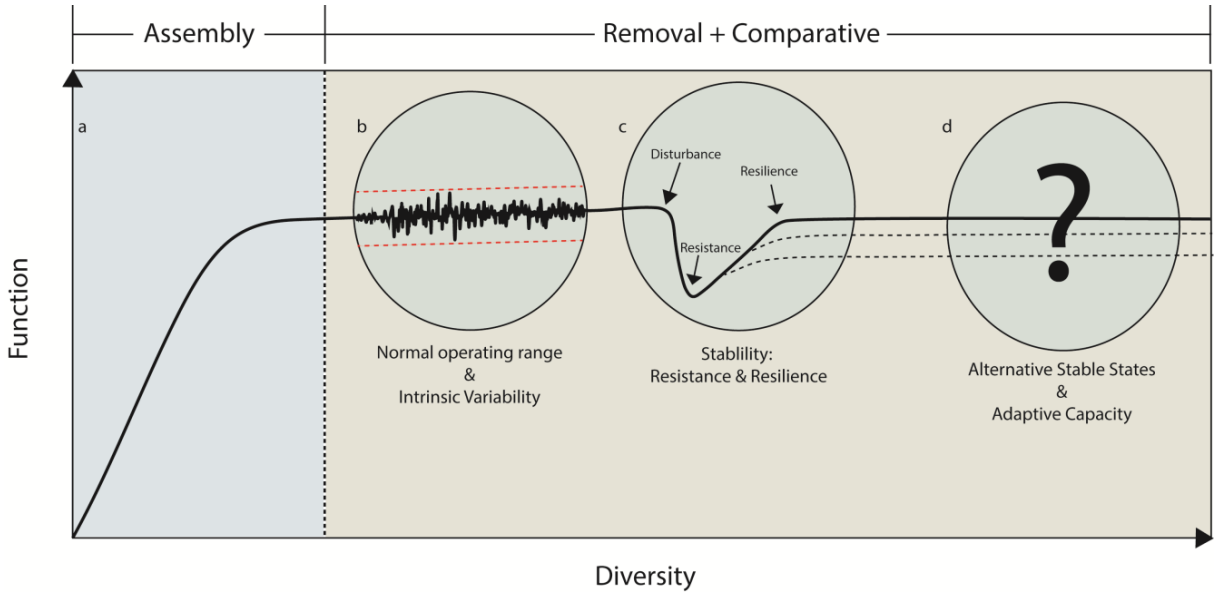


Figure 6 Short and long-term effect of biodiversity on ecosystem function (Salles, 2015)

However, over time, buffer community capacity to face disturbances is challenged and strong perturbations can affect the ecosystem stability (Boaro et al., 2014; Vuono et al., 2014). The extent of functional redundancy is case specific and a specific community would not have the same functional process in another ecosystem (Fetzer et al., 2015) nor a different nutrient resource (Pholchan et al., 2013) or multifunctionality (Roger et al., 2016).

In summary, the relationship between diversity and ecosystem function can be explained by three mechanisms: neutral assembly theory (Knelman and Nemergut, 2014; Nemergut et al., 2014), network buffering and functional redundancy, i.e. interactions and niche complementarity.

### 2.5.2 Engineering microbial diversity-function experiments

As described earlier, anaerobic digestion involves complex and diverse microbial communities in a trophic chain with interactions, notably syntrophic interactions between syntrophs and archaeal communities. The proper functioning of anaerobic digestion depends on many environmental factors (temperature, pH, volatile fatty acids, etc.) but also on the microbial community diversity. And, applying constraints proved to be effective in changing and improving the balance of the system. Understanding and handling these constraints, or levers, can improve the system in a more favorable state and would enable to understand the underlying mechanisms of the diversity-function relationship. Therefore, in the following paragraphs, we will first discuss which parameters are important for handling complex microbial communities such as anaerobic digestion communities and in a second step we will see how to manipulate diversity to study diversity-function relationships.

The importance of the initial inoculum has been underlined not quite so often in the literature. In fact, the initial community structures are shaped with the

different abiotic parameters and the divergence between communities can decrease over time. In some studies, microbial communities structures dynamic was found to be driven by deterministic patterns (Lin et al., 2017; Vanwonterghem et al., 2014a). Other studies operated different inocula and showed the importance of the initial inoculum for the operational stability (in methane production) and/or the digester resistance to disturbance (De Vrieze et al., 2014; Perrotta et al., 2017; Raposo et al., 2011). Despite similar operational conditions, evenness, diversity, phylogenetic structure and even product, microbial composition were clearly distinct between these inocula (Perrotta et al., 2017). Furthermore, the microbial structures were reproducible between inocula, as shown Figure 7 where the heat map represents the relative abundance of OTUs between the three inocula in triplicate.

There is a lot of white space here because there is a non-floating figure below. This happens also quite a lot in the printed version of the thesis (see page 27 or page 35, 69, 71 …). I do not know how to manage this well in Word. One way of doing it is – as a last step when the content is settled – to move text around manually. There must be a way in Word to do this nicer, I assume (long live LaTeX!).

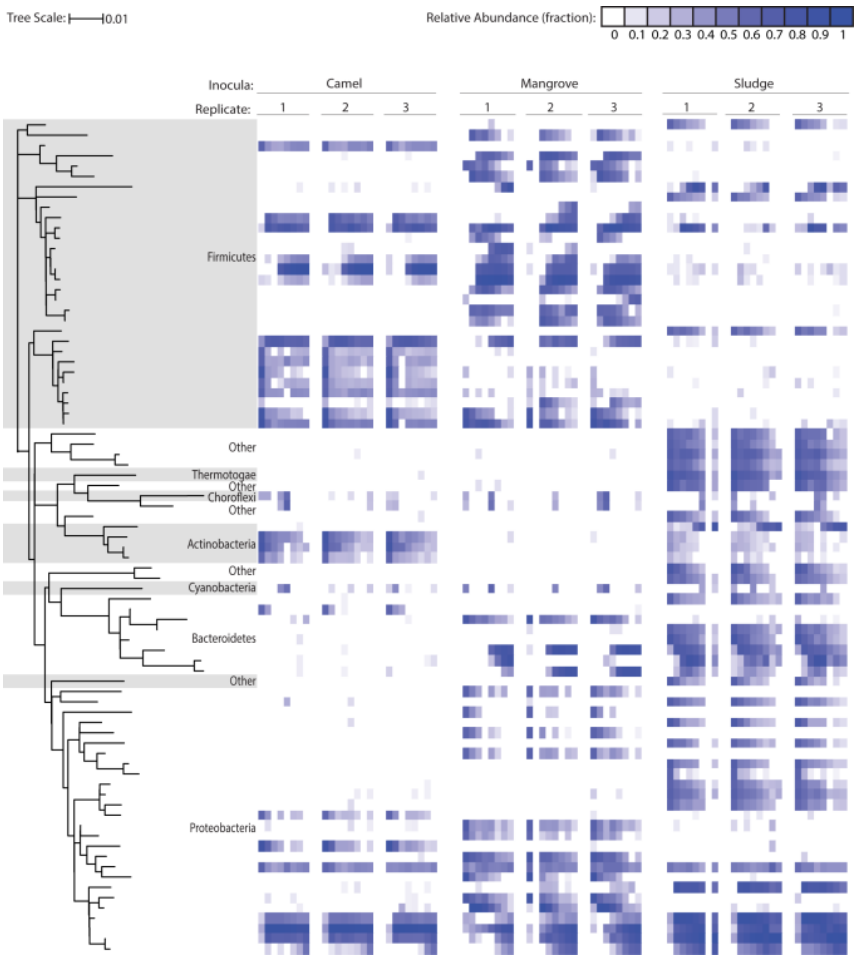


Figure 7 Microbial communities are distinct between inocula but reproducible between replicates (Perrotta et al., 2017)

In sum, the inoculum source matters for experimentation and although determinant phenomena can be observed, microbial structure cannot be expected to approximate. Specific interactions may already have been in place before and at the onset of inoculation. The specificity of inocula must therefore be taken into account for experimentation.

However, to be able to compare an inoculum performance in time-spaced experiments, two possibilities exist. (i) One inoculum can be sampled again in its environment at a different time. But we have seen the importance of initiating process and, furthermore, time differences of a sludge coming from a waste water treatment plan experience different constraints and show different microbial communities structure and efficiency (Valentin-Vargas et al., 2012). (ii) The other possibility of comparing an inoculum performance

is to preserve this inoculum. Whether a preserved inoculum has the same functional and structural properties as a fresh inoculum have been experienced only a few times. Different temperatures, methods (encapsulation, drying and lyophilization) and cryoprotective agents can be used to preserve complex microbial communities for activity recovery. While Kerckhof et al. (2014) have found that activity of fecal communities were recovered after cryopreservation (Kerckhof et al., 2014), Hagen et al (2014) did not manage to recover methanogenic potential of two different inocula for any temperature conservation used (-20°C, 4°C, room temperature) (Hagen et al., 2015). Vogelsang et al. (1999) encapsulated nitrifiers communities into alginate beads and reactivated their activity between 40 to 60% into CSTR reactors after two or three months preservation at -80°C (Vogelsang et al., 1999). In the laboratory, it is admitted to preserve an inoculum at 35°C temperature for a maximum of one month to keep the microorganisms alive and at 4°C or room temperature for longer experiment. The large volumes of digestate are difficult to store at temperature -80°C, even with alginate beads where it would still take a certain number of beads to find the same concentration in the communities. Considering previous results of inocula particularities and preservation contingencies, carrying out tests at the same time is still preferable rather preservation or time-spaced experiments.

Other than inoculum itself, different biotic parameters can be applied on anaerobic digestion, as for example as bioaugmentation. The objective of bioaugmentation is to improve a process by introducing a pure, co-cultures or mixed cultures of microorganisms. Numerous studies have tested bioaugmentation on anaerobic digestion process. The increase of methane yield after bioaugmentation ranged from 120% to 0 and no evident parameter of succeeding or failing bioaugmentation was revealed (De Vrieze

et al., 2016b). The experiment with 120% increase of methane yield has enriched biomass for propionate degradation. In this way, the authors managed to reduce the solid retention time for organic overload (Tale et al., 2011).

Another example of biotic parameter manipulation is synthetic biology. This expanding field can be described as the design of biological pathways, organisms or devices. Bell et al., (2005) build a synthetic community by adding 1 to 72 species and measure the respiration rate (Bell et al., 2005). Through this construction, they artificially increase the diversity and found a positive and decelerating relationship between bacterial diversity and the studied function. Therefore, synthetic approaches have better controlled of evenness, richness, perturbations effect and ecosystem function for diversity-function experiments (De Roy et al., 2014). However, even if synthetic ecosystems allow us to better understand the underlying mechanisms, testing and validating these systems reaction in vivo would endorse their utilization.

Tilman et al. (1994) performed an experiment to study the effect of plant biodiversity on ecosystem functioning (Tilman and Downing, 1994). They observed that the more diversity in an ecosystem, the greater the stability of the community is. Twenty years later, Bell et al. (2005) built synthetic communities and observed decelerating relationship between diversity and function (Bell et al., 2005). Creating interactions between organisms are influenced by different parameters, as seen before (operational conditions, perturbations and resilience, spatial organization, etc.). In batch reactors, Sierocinski et al., (2017), studied complex community coalescence and found that the more communities were mixed, the higher the performance of the process was by selecting the best performing taxa (Sierocinski et al., 2017).

Community assembly is proving to be an interesting parameter for the study of the functioning diversity relationship.

## 2.6 Community assembly

A community is an assemblage of populations that could be defined by their productivity (metabolites, etc.), their species identity and abundance, the diversity and the trophic interactions, bottom-up (nutrient control) and top-down (predation).

Community assembly is driven by different parameters: dispersal, genetic diversification, selection and ecological drift (Vellend, 2010). Dispersal and diversification are the dynamics that generate or introduce new taxa, whereas selection and drifts imply abundance changes. The hypothetical mechanisms driving the community assembly have been based on the contrasting perspectives of the stochastic neutral models and the deterministic niche paradigm. The neutral hypothesis (Hubbell, 2001) implies random organisms dynamics, whereas the deterministic niche paradigm suggests selection processes via abiotic parameters and species interactions.

Applied to microbial communities, some studies found only one mechanism implied (Sloan et al., 2006; Vanwonterghem et al., 2014a), whereas others have suggested that both stochastic and determinism forces act on the populations (Caruso et al., 2011; Dumbrell et al., 2010; Stegen et al., 2012; Van Der Gast et al., 2008). In these works, perturbations and abiotic parameters would drive niches-selection processes and the patchwork environment would favor stochastic events to occur.

Environmental perturbations affect the microbial community in terms of composition and function. However, microbial communities may be resilient or resistant to these changes (Allison and Martiny, 2008; Shade et al., 2012).

And their diversity (genetic, etc.), dynamics and the different interactions (competition, mutualism, predation, etc.) may insure their stability, i.e. 'the community's response to disturbance'. Various parameters affect microbial community responses after disturbance as shown in Figure 8 (Shade et al., 2012). This figure resumes the numerous factors influencing the community assembly at the individual, population and community levels after pulse (short) and pulse or press (short or long) perturbations. Numerous ecosystem drivers (blues boxes) intervene on the community assembly and contribute to resistance and resilience. Resistance is defined as the strength of the community to resist change in the face of disturbance, whereas resilience is the rate at which the community returns to its state after the disturbance. At the individual and population levels, the persistence after disturbances supports microbial interactions network, local-diversity and turnover rate supporting the community stability (orange arrows). These three mechanisms underlie the effect of diversity on ecosystem function, which will be further explored in the next chapter.

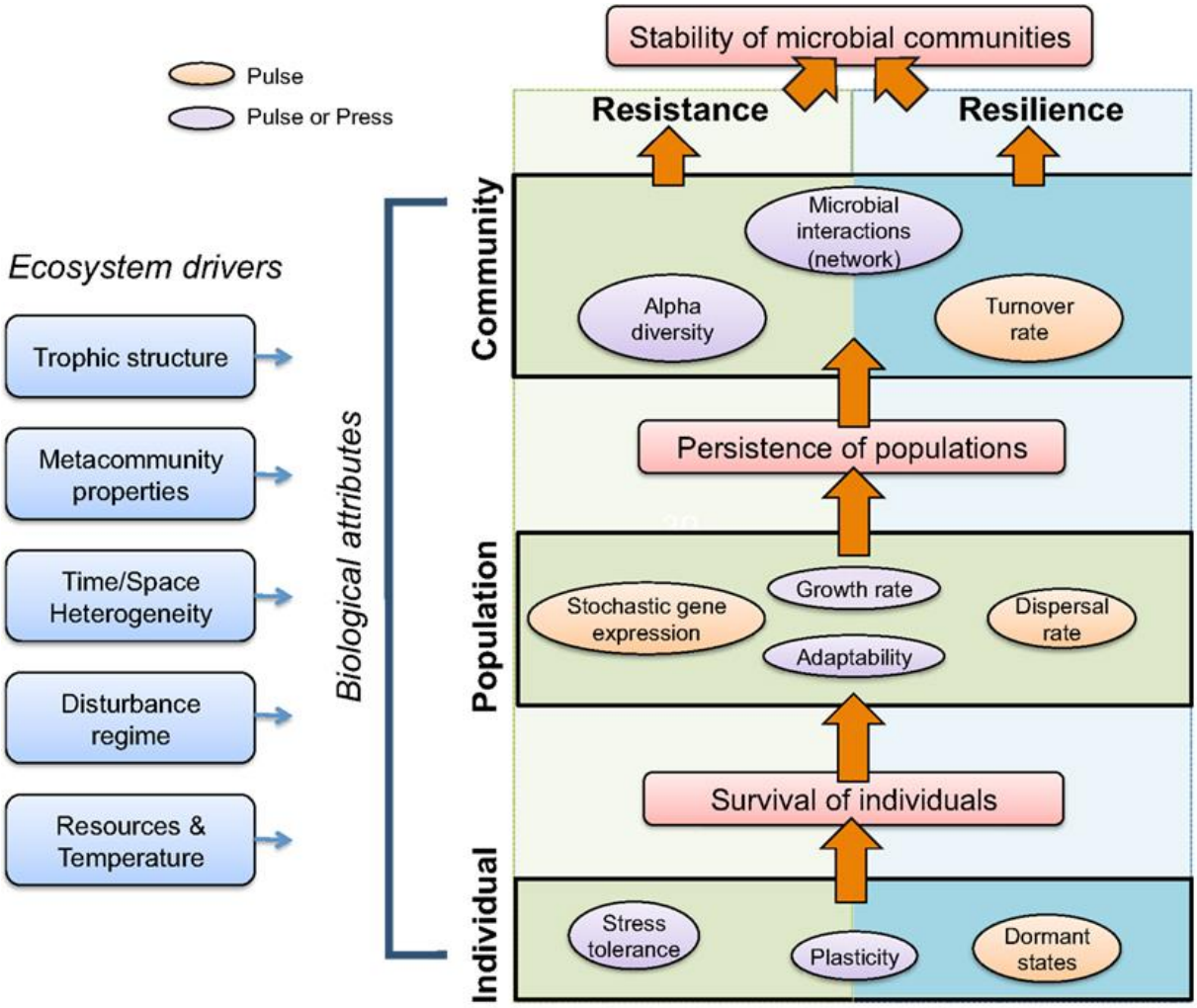


Figure 8 Ecosystem parameters driving resistance and resilience of microbial communities after disturbance (Shade et al., 2012)

Another way of studying community assembly is to mix independent communities and observe their development in either a coalescence or the selection of one community (Rillig et al., 2015). Since microorganisms are ubiquitous, mixing events of communities often occur in various ecosystems, such as leaves or animal excrements falling on the soil, or in an estuary where a river and the sea meet for example. For ecological engineering, studying these mixing events can be strategic at both at the beginning, development and end of the process (Rillig et al., 2016). In anaerobic digestion, mixing events can be important at reactor start-up where the variability is broad;

during the process while communities are selected and drifted; and finally at the end with the digestate valorization for example.

Sierocinski et al., (2017) applied the coalescence concept for studying anaerobic digestion functionality with up to 12 microbial communities mixed in batch reactors. The authors observed that the most methane producing community was dominant after coalescence (Sierocinski et al., 2017). These results may be explained by the co-selection of mutualistic interaction, i.e. the development of better adapted organisms helping each-other. In addition, the more the microbial communities were mixed, the higher was the biogas production. These findings underlined the selection of competitive and best-performing taxa in this process and underline the question of the diversity functioning in ecosystems.

# Chapter 3

# Thesis objectives

# 3 Thesis objectives

Different microbial populations are involved in the anaerobic digestion process, yet their dynamic are not well understood. To study community assembly processes and to understand the dynamics of microbial communities, assembly experiments can be performed at the scale of the whole community by artificially controlling parameters, like dispersal or selection by tuning environmental factors.

Figure 9 presents a diagram with a hypothetical community mixing experiment, where some processes known to shape community assembly are indicated. The effect of dispersal can be studied by mixing several communities in comparison to individual communities, placed in the same conditions. The duration of the experiment is directly linked to the process of drift, which represents stochastic changes in species abundances over time. The process of selection is determined by environmental constraints on the system, as for example temperature or substrate. At the end, the final community structure will result in the combined actions of several processes acting on individual populations that shape the community.

The main challenge of my thesis work is to conduct experiments that allow ranking which process is most important in shaping communities and modulating the performance of the ecosystem, by looking among others at coalescing events.

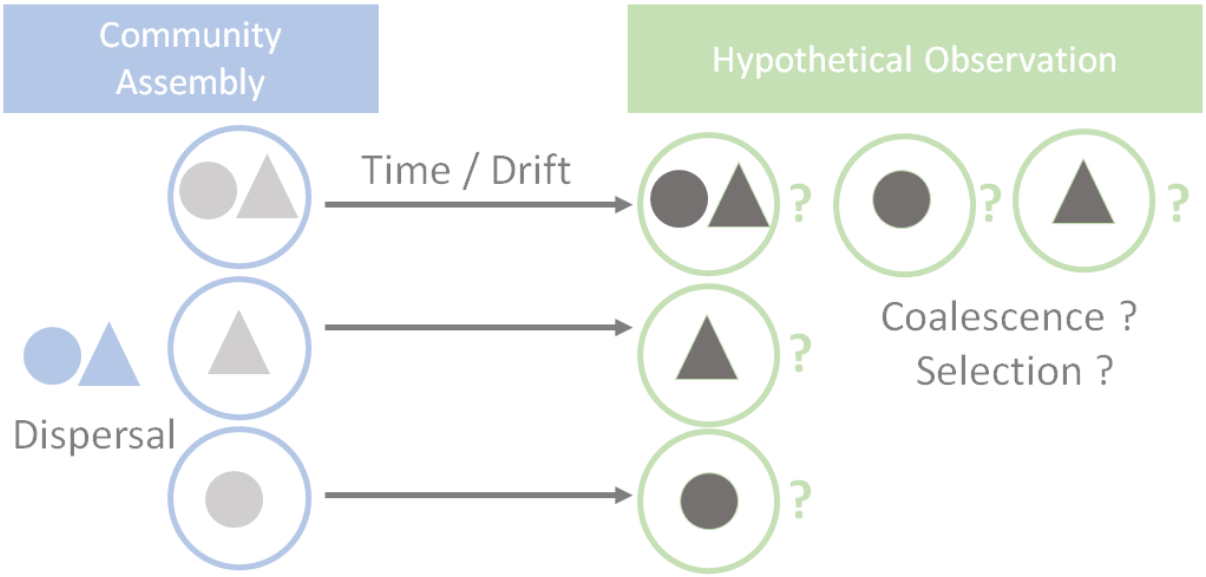


Figure 9 Suggested community assembly experiment of two individual communities and their mix over time

One bottleneck in this kind of experiment is to precisely control the inoculums at origin, and the only solution is to synchronize the start-up of reactor because storage of communities brings too many biases. To address this issue, we propose to operate a series of microbial ecosystems operated on a continuous mode at the same time, but it is not currently available.

My first objective of the PhD thesis is then to develop a system of continuous anaerobic reactors in parallel with enough flexibility in the functioning to be able to answer our questions on community assembly and diversity-functioning relationship.

After having introduced the material and methods used in this thesis work, a chapter is dedicated to the design of a system of several automated anaerobic chemostats. The following chapter describes the study of coalescence of several communities under anaerobic digestion conditions with different feeding regimes. The conclusions of the findings with the perspectives of this work are presented in the last chapter.

# Chapter 4

# Material and methods

# 4 Material and methods

## 4.1 Inoculum and substrate choice

Three inocula were used in the experiment of testing the LAMAC system (chapter 5). These inocula came from pilot-scale, solid-state mesophilic anaerobic digesters that had been inoculated with a sludge coming from an Up-flow Anaerobic Sludge Blanket (UASB) digester in a full-scale sugar factory treatment plant. The three digesters were operated under identical environmental conditions for 1.5 years (Laperrière et al., 2017). One anaerobic digester was fed with readily biodegradable substrates (grass and carrots), the second digester was fed with intermediately biodegradable substrate (grass and manure) and the third digester was fed with slowly biodegradable substrates (manure and dung). The three inocula were named in this manuscript 'INOC A', 'INOC B' and 'INOC C', respectively.

In the experiment of testing the effect of mixed community (chapter 0), five inocula were used: the UASB sludge (UASB from a full-scale sugar factory in Marseille, France), the three sludge sources used in chapter 5 (Laperrière et al., 2017) and another inocula coming from a pilot-scale, solid-state mesophilic anaerobic digester that had been operated under identical environmental conditions for 1 year and fed with cardboard (Capson-Tojo et al., 2017).

In the different experiments, synthetic soluble substrates of different complexity were used. The Table 2 summarizes the substrate use in different experiments.

Table 2 Type of substrate and concentration of the substrate

| | **LAMACs experiment** (chapter 4) | **Mix experiments** (chapter 5) |
|---|---|---|
| **Substrate used and concentration** | switchover between simple (10 gCOD·L-1) and complex (20 gCOD·$L^{-1}$) substrates | Simple, intermediate and complex substrate (10 gCOD·$L^{-1}$) |

On the one hand, the complexity of these substrates was increased by increasing the number of monomer types, and on the other hand by increasing the degree of polymerization. The substrate composition and uses is resumed in Table 3.

Table 3 Substrate composition for the different experiments laid out in chapters 5 and 6

| | Substrate | Formula | monomer | Concentration [g·l$^{-1}$] | | CAS Number | Sigma reference* *exceptions are noted otherwise |
|---|---|---|---|---|---|---|---|
| | | | | Chap. 4 | Chap. 5 | | |
| Simple substrate | Yeast nitrogen base | | | 0.54 | 0.54 | Y0626 | Y0626 |
| | Ethylen glycol | $C_2H_6O_2$ | Ethylen glycol | 1.52 | 1.52 | 107-21-1 | 324558 |
| | Glucose | $C_6H_{12}O_6$ | Glucose | 4.26 | 4.26 | 50-99-7 | G8270 |
| | Fructose | $C_6H_{12}O_6$ | Fructose | 4.21 | 4.21 | 57-48-7 | F0127 |
| Intermediate substrate | Yeast nitrogen base | - | | - | 0.54 | | Y0626 |
| | Polyethylene glycol (MW 8.000) | $H(C_2H_4O)nOH$ | $[Ethylen\ glycol]_n$ | - | 1.47 | 03/12/7554 | W237418 |
| | Dextrin | $C_6H_{12}O_6$ | $[Glucose]_n$ | - | 2.58 | 9004-53-9 | 31405 |
| | Inuline | $C_6nH_{10n}+2O_{5n+1}$ | $[Fructose]_n$ | - | 1.76 | 9005-80-5 | myprotein.com |
| | Lactose | $C_{12}H_{22}O_{11} \cdot H_20$ | [Glucose-Galactose] | - | 2.75 | 5989-81-1 | L3625 |
| Complex substrate | Yeast nitrogen base | NA | - | 1.07 | 0.49 | | Y0626 |
| | Polyethylene glycol (MW 20.000) | $H(C_2H_4O)nOH$ | $[Ethylen\ glycol]_n$ | 1.17 | 0.59 | 25322-68-3 | P2139 |
| | Carboxymethyl cellulose | $[C_{28}H_{30}Na_8O_{27}]n$ | $[Glucose]_n$ | 4.04 | 2.02 | 9004-32-4 | C5678 |
| | Starch | $[C_6H_{10}O_5]n$ | $[Glucose]_n$ | 2.96 | 1.48 | 9005-25-8 | S2004 |
| | Sucrose | $[C_{12}H_{22}O_{11}]n$ | [Glucose-Fructose] | 2.08 | 1.04 | 57-50-1 | 84100 |
| | Inuline | $C_6nH_{10n}+2O_{5n+1}$ | $[Fructose]_n$ | 1.40 | 0.7 | 9005-80-5 | myprotein.com |
| | Malic acid | $C_4H_6O_5$ | | 1.23 | 0.62 | 6915-15-7 | myprotein.com |
| | Lactose | $C_{12}H_{22}O_{11} \cdot H_20$ | [Glucose-Galactose] | 2.20 | 1.1 | 5989-81-1 | L3625 |
| | Trehalose | $C_{12}H_{22}O_{11} \cdot 2H_20$ | $[Glucose]_n$ | 0.67 | 0.33 | 6138-23-4 | T9531 |
| | Raffinose | $C_{18}H_{32}O_{16} \cdot 5H_2O$ | [Glucose-Galactose-[Fructose] | 0.33 | 0.33 | 17629-30-0 | R0250 |
| | Itaconic acid | $C_5H_6O_4$ | | 0.30 | 0.25 | 97-65-4 | I29204 |
| | Glycerol phosphate disodium salt | $C_3H_7Na_2O_6P \cdot xH_2O$ | | 5.46 | 0 | 55073-41-1 | G6501 |
| | α-D gluco-pyranoside | $C_7H_{14}O_6$ | | 0.58 | 0.29 | 97-30-3 | 66940 |
| | Diethyl malate | $C_8H_{14}O_5$ | | 0.40 | 0.2 | 03/12/7554 | W237418 |

Phosphate buffer was prepared to buffer media at pH 7.4 with a final concentration of 0.1 mol·$L^{-1}$. Each substrate was supplemented with nitrogen yeast extract to adjust the carbon-nitrogen ratio to 35.

## 4.2 Design of LAMACs

The design of the multiplexed chemostats is conceived to be as flexible as possible. One module is composed of six chemostats as shown in the Figure 10a. Any number of these modules can be operated in parallel, limited only by the available manpower. The dimensions of one module are 50 cm width × 52 cm length × 100 cm height, and fits well on a standard laboratory bench. Temperature is controlled using a custom-made aluminum heating block (Garaud, Carcassone, France) to fit 250 mL borosilicate graduated laboratory bottles with standard GL45 threading. These bottles were used as reactor vessels. The thermostat integrated in the aluminum block allows setting a temperature range from room temperature to 55 °C. Using Lab Guard II temperature sensors (AES, Chemunex, France), we verified homogeneity of the heat distribution. A standard waterproof magnetic stirring plate was placed underneath the heating block (Variomag Multipoint 6, Thermo Scientific, Waltham, United-States). Three peristaltic pumps with stepping motors (FZ10, A2V, Gazeran, France) were connected to each chemostat for automated substrate loading, biomass wasting and degassing (Figure 10b), thus 18 pumps per module.

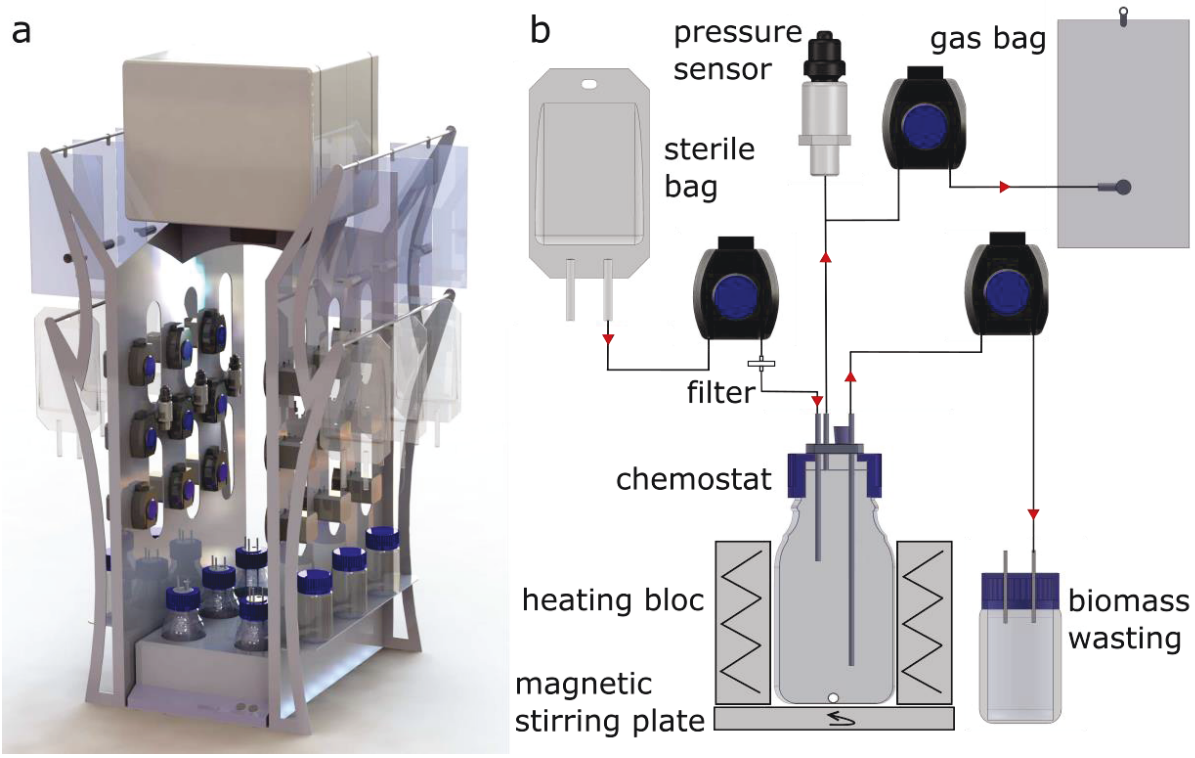


Figure 10 General view of the LAMACs and of components of one chemostat in the LAMACs module. a) 3D view of a LAMACs module, i.e., six chemostats. The electric box containing controlling cards is on the top of the module and above any source of liquids to prevent electric failure in case of leakage. A waterproof stirring plate is underneath the heating block containing the six reactor vessels. Three peristaltic pumps are aligned above each vessel. The upper range of bags is for biogas collection; the lower range serves as substrate reservoirs. The dimensions of one module are 50 cm width × 52 cm length × 100 cm height. b) Schematic view of a chemostat detailing the use of the three-hose connector. One port is used as inlet for feeding, and two ports as outlet for biomass wasting and degassing. One port for manual sampling is sealed with a rubber stopper. A 0.45 µm pore-size filter is placed in the feedline ahead of the reactor to prevent contamination of the medium.

All peristaltic pumps were calibrated before use (Figure 11). Peristaltic pumps were connected to a controller module (TMCM 6110, Trinamic, Hamburg, Germany) and were piloted via a free software (TMCL-IDE, Trinamic, Hamburg, Germany).

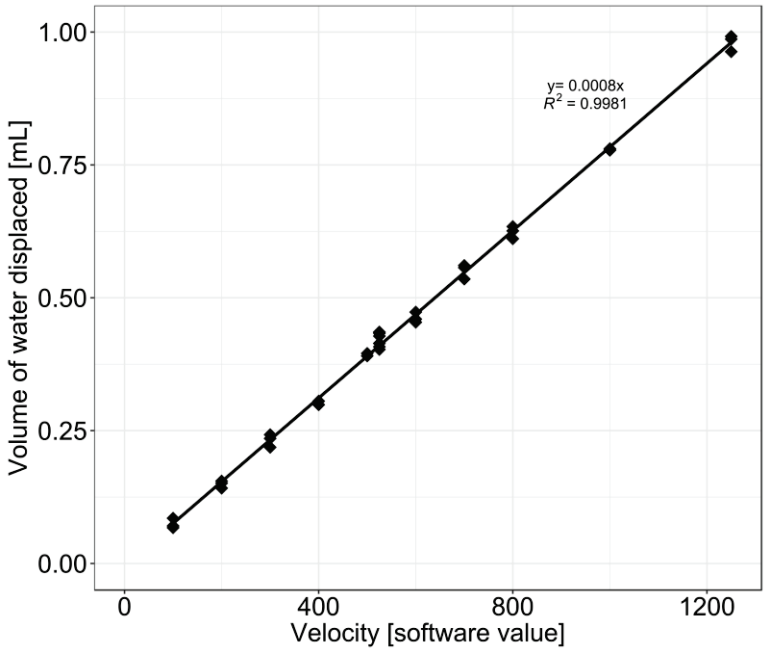


Figure 11 Calibration of one peristaltic pump. The calibration was made with measuring different volumes of water after having rotated the pump one minute at different velocities.

The feed, waste and degassing tubes were connected to the 250 mL reactor vessels through stainless steel three-hose connectors inserted into GL45 caps with aperture. The custom-made cap connector (Garaud, Carcassone, France) is gas tight through a rubber joint between the reactor and the insert under the recommended pressure range of the reactor vessels (maximum of 1.5 bar). The ends of the three-hose connectors are thickened to increase tightness of the tubes. The inner diameter of the connector tubes is 2 mm. In addition to the three-hose connector tubes, we added one 5 mm hole in the cap connector. This hole is sealed with a 10 mm long conical rubber stopper and serves as septum for direct sampling. All parts in contact with the reactor interior can be autoclaved for sterile operating conditions.

We used 100 mL sterile bags (Easyflex+, Macopharma, Mouvaux, France) as nutrient reservoirs. These bags can be easily filled, stored and replaced. Media bags were filled aseptically with enough feed for one week of operation and connected to the reactor. Wasted biomass was temporarily stored in 100 mL graduated laboratory bottles with GL45 threading and

removed for analytical purpose. Biogas was collected in 100 mL Tedlar®Gas Sampling Bags (Sigma-Aldrich, St. Louis, USA). Tygon pump tubing (2 × 4 mm R3603, Saint-Gobain, Courbevoie, France) was used for feeding and wasting. Norprene® tubing (6404 LS14, Saint-Gobain, Courbevoie, France) was used for gas management. Pump tubing was replaced on a weekly basis.

Using pressure sensors, the system is able to detect biological activity directly by measuring the production of biogas. One pressure sensor per chemostat with a sensitivity of 0.0015 bar and an accepted maximum pressure of 3.4 bar (PX2EN1XX050PAAAX, Honeywell, New Jersey, United-States) was placed between the chemostat and the peristaltic pump used for degassing. All pressure sensors were calibrated before use (Figure 12). The pressure sensor tolerates operation in humid environments. Pressure data were constantly monitored by the same controller and software as for the peristaltic pumps. The pressure data collection interval was set to 20 s, i.e., every 20 s, the current pressure in the reactors was recorded using a custom code written in Python 2.7 (https://www.python.org/). For the experiments described here, degassing began when a pressure of 1.2 bar was reached. Gas was pumped out of the system until the pressure fell to 1.05 bar.

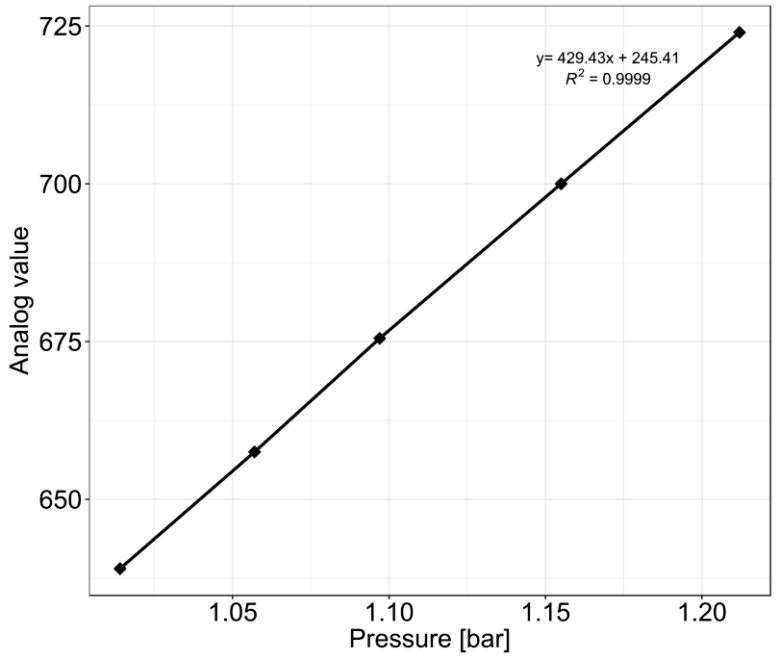


Figure 12 Calibration of one pressure sensor. The calibration was made with a pressure imposed manually, read by the software and checked with a manometer.

All the equipment for the construction of one module (six reactors) cost about 7000€ in 2017 and are detailed in Table 4 below. About half of the budget was used for pumps and sensors with their power supplies. The remainder was used for the chassis, magnetic agitator, heating bloc and three-hose tube connector. Operational costs were estimated to be around 400€ for a one month experiment of six reactors in an anaerobic digestion process, mostly used for consumables, e.g., gas bags (reusable), tubing, sterile bags for substrate and sterile filters. Some of the equipment described in Table 4 as for example the dry bed heating bloc or the magnetic stirring plates can be replaced by less expensive alternatives.

Table 4 Detailed prices of components required for the construction of one LAMACs module containing six reactors.

| | Equipment for one LAMACs module (6reactors) | Supplier reference | Supplier | cost per module (€) |
|---|---|---|---|---|
| **Bottling** | 6 stainless steel three-hose connectors | custom made | Garaud, Carcassonne, France | 222 |
| | 250 mL bottles, cap connectors, magnetic stirrer | laboratory equipment | - | 39 |
| | | | subtotal | 261 |
| **Tempera-ture regulation** | heating bloc | custom made | Garaud, Carcassonne, France | 807 |
| | Temperature regulator | custom made | YESSS Electrique, Francheville, France | 140 |
| | | | subtotal | 947 |
| **Feeding and biomass wasting** | 12 peristaltic pumps with stepping motors (FZ10) | PPELEA02204 | A2V Flowtronique, Gazeran, France | 1320 |
| | Controller module (TMCM 6110) | VARTRI00914 | A2V Flowtronique, Gazeran, France | 615 |
| | motor power supply | - | YESSS Electrique, Francheville, France | 80 |
| | | | subtotal | 2015 |
| **Pressure measure-ment** | 6 peristaltic pumps with stepping motors (FZ10) | PPELEA02204 | A2V Flowtronique, Gazeran, France | 660 |
| | 6 pressure sensor (PX2EN1XX050PAAAX, Honeywell) | 853-6471 | RS components Corby, UK | 380 |
| | pressure sensor power supply | - | YESSS Electrique, Francheville, France | 50 |
| | | | subtotal | 1090 |
| **Unit assembly** | chassis | custom made | Garaud, Carcassonne, France | 707 |
| | electric jacket | - | YESSS Electrique, Francheville, France | 30 |
| | laptop | - | - | 600 |
| | | | subtotal | 1337 |
| **Mixing** | magnetic stirring plate (Variomag Multipoint 6) | 3302060 | Sodipro, Echirolles, France | 1060 |
| | | | total | 6710 |

## 4.3 Pressure data analysis

All pressure data and statistics were analyzed in the R software environment, version 3.3.1 (R Core Team, 2016). Raw pressure data were acquired as

absolute pressure readings in the range of 1.05 to 1.2 bar. Pressure data were converted into biogas volume in three different signal processing steps: first, pressure drops due to automatic degassing after biogas accumulation (1.2 to 1.05 bars) were removed to obtain a curve of cumulated absolute pressure. Secondly, occasional sudden sharp increases or decreases in pressure caused by technical problems (i.e., gas leakage, liquid sampling) were removed so that they did not contribute to the accumulated signal. The limits for this removal were pressure spikes of +/- 10 mbar·min$^{-1}$ for one measurement, e.g., a 20 s time interval. The corrected data were then converted to normalized biogas volume under standard conditions, i.e., 293.15 K and 1.013 bar. Biogas production rates were then estimated from linear regressions.

We tested pressure sensors precision in a dedicated experiment with four reactors under anaerobic digestion conditions over several days in the chapter four.

## 4.4 Analytical methods

### 4.4.1 Biochemical analyses

The pH was measured in the biomass wasted after two days collection (SG23, Mettler Toledo InLab, Greifensee, Switzerland)

Volatile fatty acids (VFA) were sampled directly from the chemostat, filtered (0.45 µm) and injected in a gas chromatograph (CPG Clarus 580, Perkin Elmer, USA) equipped with an auto-sampler, with an Elite-FFAP crossbond®carbowax® 15 m column connected to a flame ionization detector at 280 °C, using nitrogen as carrier gas at a flow rate of 6 mL·min$^{-1}$.

Volatile solids were measured according standard methods of the American Public Health Association (APHA, 2005). The concentration of volatile matter in the manuscript will be designated by the biomass concentration.

After one day collection of wasted biomass (i.e. 12 mL), soluble chemical oxygen demand was measured from 2 mL reactor mixed liquor after centrifugation and filtration (0.45 µm) using prefilled COD tubes (Aqualytic 420721 COD Vario Tube Test MR, 0–1500 $mg \cdot L^{-1}$, Aqualytic, Dortmund, Germany), placed in a HACH COD reactor at 150 °C for 2 h. COD concentrations were determined photometricaly at 620 nm (Photometer MultiDirect, Aqualytic, Dortmund, Germany).

250 µL of biogas were manually sampled directly inside the headspace of the reactors and the biogas composition was measured with a gas chromatograph (Clarus580, Perkin Elmer, Waltham, USA) equipped with a thermal conductivity detector and two columns: RtQBond to split $H_2$, $O_2$, $N_2$, $CH_4$ and RtMolsieve (5Å) to separate $CO_2$. The carrier gas was argon at the initial pressure of 3.5 bar. The temperature was 60 °C in the hoven, 250 °C in the injector and 150 °C for the detector. The gas chromatograph was calibrated using a standard gas mixtures (399152, Linde, Munich, Germany) containing 25 % $CO_2$, 2 % $O_2$, 10 % $N_2$, 5 % $H_2$ and 58 % $CH_4$.

For the chemical oxygen demand balance, all calculations were performed on the COD measures.

### 4.4.2 Biological analyses

Biological analyses were performed for the experiments in the chapter 0.

Twelve mL of biomass wasted were collected and centrifuged 10 min at a G-force set between 3.550 and 7.140g depending on the biomass concentration. After removing 9mL of the supernatant, pellet was resuspended and aliquots

of 500µL were sampled in 2mL sterile Eppendorf tubes and stored at -20°C before use.

FastDNA SPIN kits for soil (MP Biomedicals, Santa Ana, USA) were used for DNA extraction according to the manufacturer's instructions.

For quantitative PCR amplification, two dilutions in triplicate were performed on each sampled for Bacteria and Archaea amplification. For quantitative PCR on Bacteria 16S rRNA gene amplification, the primers W208 and W209 (Yu et al., 2005) were used: W208 F338-354 5'-ACTCC TACGG GAGGC AG-3' at 100nMf; and W209 R805-536 5'-GACTA CCAGG GTATC TAATC C-3' at 250nMf. The probe was Taqman Tamra W210 F516-536 5'-Yakima Yellow-TGCCA GCAGC CGCGG TAATA C-Tamra-3' at 50nMf. For the amplification of bacterial sequences, the PCR mixture contained 6.5 µL Mix Biorad SsoAdvanced Universal Probes Supermix (Bio-rad, Hercules, United States), 0.5 µL of each primers and probe, 2.5 µL water and 2 µL of DNA extracts for a total volume of 12.5 µL. All samples were run at two dilutions in duplicate on a CFX96 (Bio-Rad Hercules, United States) qPCR machine using a program with 2 minutes at 95°C enzyme activation followed by 40 cycles of 7 s at 95°C for dissociation and 25 s at 60°C for hybridization and elongation.

For PCR amplification, the V4- V5 regions of 16S rRNA gene for both archaeal and bacterial genes were amplified with primers 515-532U and 909-928U (Wang and Qian, 2009) with the same method as elsewhere (Venkiteshwaran et al., 2016).

PCR products were sequenced with Illumina MiSeq. Sequencing and library preparation were performed at the GeT PlaGe Sequencing Center of the Genotoul Lifescience Network (Toulouse, France). In order to assemble forward and reverse sequences, mothur version 1.39.0 was used (Schloss et

al., 2009). Alignment and taxonomic affiliations from the 16S sequences were performed with SILVA v128, as provided by mothur. Since sequencing error rate is considered to be 1%, and the sequence length is 400 base pairs, sequences with less than 4 nucleotides different were pre-clustered. Custom R scripts were used to remove sequences appearing less than twice in the whole dataset.

## 4.5 Biomass preparation and inoculation

All inocula used in the different experiments were stored at least one week under anaerobic digestion conditions at 35 °C to wait for a complete degradation of remaining organic matter. Biomass concentration (volatile solids) was adjusted before inoculation. Buffer solution was then added to prevent from acidification due to volatile fatty acids production. The reactors were inoculated at the same volatile matters concentration, 2 or 5 $gVS \cdot L^{-1}$ depending on the experiment (Table 5). Once the biomass is added to the 250 mL reactor vessels, reactors were closed and traces of oxygen removed to maintain anaerobic digestion condition by headspace by nitrogen with the degassing system of the LAMACs.

## 4.6 Statistical analyses

For comparing the biogas production variability between the different inocula, Kruskal-Wallis tests were performed in chapter 0. When the test was significant, a Dunn post-hoc test was performed to account for multiple comparisons of independent samples, using the function '[posthoc.kruskal.dunn.test](posthoc.kruskal.dunn.test)' from the R package 'PMCMR' (version 4.1) (Pohlert, 2016).

Biogas productions between the different mixed and individual communities in chapter 0 were compared with t-tests, while communities fed with

different substrates were compared with pairwise t-test with Bonferroni p-value adjustment.

Variability of methane production ratios between mixed communities and individual communities were compared with Anova tests. Data normality was checked with a 'shapiro.test' and variances with the 'oneway.test', both available in the basic R stats package.

Principal coordinate analysis (PCoA) based on Jaccard distances was used as multivariate analysis to visualize the distances and variations between microbial communities. The distance matrix was calculated with Jaccard similarity from vegan package (Oksanen et al., 2015), using Jaccard as a quantitative index and not qualitative. PCoA was performed with phyloseq package (McMurdie and Holmes, 2013)

Correlations of environmental data to microbial community distance data were performed with the function 'envfit' of the vegan package and the matrix distance was calculated with the quantitative Jaccard index as before. Only data with p-values inferior to 0.07 were displayed on graphics.

The similarity between mixed communities and the corresponding individual communities were determined along the experiment by Jaccard distances. We established an empiric distribution of similarities from 1000 communities with randomly permutated abundance data. Contrasting the observed similarity with the empiric distribution allowed us to assess the significance of the similarity. In a one-sided test a significance level of 0.05 was used.

## 4.7 Summary of experiments performed

Table 5 Summary of parameters used in experiments.

| | **chapter 4** | **chapter 5** |
|---|---|---|
| **Inocula** | Grass-carrots;<br>Grass-manure;<br>Manure | UASB sludge;<br>Grass-carrots;<br>Grass-manure;<br>Manure;<br>Digestate |
| **Substrate** | Alternating substrates every 3 weeks: 1) Complex (20gCOD·$L^{-1}$) 2) Simple (10gCOD·$L^{-1}$) 3) Complex (20gCOD·$L^{-1}$) | Each substrate feeds 10 reactors: Simple (10gCOD·$L^{-1}$) Intermediate (10gCOD·$L^{-1}$) Complex (10gCOD·$L^{-1}$) |
| **Number of continuous reactors** | 12 | 30 |
| **Experiment** | 9 weeks | 12 weeks |
| **Hydraulic retention times** | 15 days | 15 days |
| **Inoculation concentration** | 5gVS·$L^{-1}$ | 2gVS·$L^{-1}$ |
| **Volume** | 180mL | 180mL |
| **Frequency of analytical measurements** | | |
| **pH** | weekly | weekly |
| **sCOD** | weekly | 2 weeks |
| **Gas composition** | weekly | weekly |
| **Volatile Fatty acids concentration** | weekly | 2 weeks |
| **Volatile solids concentration** | weekly | 2 weeks |
| **Biological sampling** | - | weekly |
| **Statistical analyses on pressure data** | Kruskal Wallis test and Dunn post-hoc tests | T-test and pairwise t-test and adjustment of Bonferroni. Data normality verified with Shapiro test. |

# Chapter 5

# Multiplexed chemostat system for quantification of biodiversity and ecosystem functioning in anaerobic digestion

# 5 Multiplexed chemostat system for quantification of biodiversity and ecosystem functioning in anaerobic digestion

Our main goal is to test the development and performance of methanogenic ecosystems during community assembly experiment. To be able to study several mixed communities, we needed a device that allows handling continuous reactors in parallel, since preservation of sludge is problematic. In the previous project (E-NOC, funded by metaprogram INRA MEM), 17 anaerobic digesters were operated in a semi-continuous mode over two months and it appeared it was very time-consuming. It is therefore not conceivable to design a new experiment with 30 anaerobic digesters in parallel. Specific emphasize was therefore given to the automation of many parameters and we developed a system named LAMACs: Lab-scale Automated and Multiplexed Anaerobic Chemostat system. This work benefited from the know-how of several people from the lab with complementary skills: Jérôme Hamelin and Kim Milferstedt conceived the project and ensured its development all along. Guillaume Guizard designed the LAMACs structure, some components of the reactors and set up all electronic equipment of the system. Kim Milferstedt has developed the pressure production analysis scripts. Eric Latrille has developed the automation of analog data acquisition and taught me the fundamentals. I wraped-up all the information and I developed the analog data acquisition and performed tests to ensure the LAMACs results reliability.

The design and the first application of LAMACs are resumed in the chapter below. This manuscript was accepted for publication in the journal PLoS One.

## 5.1 Abstract

Continuous cultures in chemostats have proven their value in microbiology, microbial ecology, systems biology and bioprocess engineering, among others. In these systems, microbial growth and ecosystem performance can be quantified under stable and defined environmental conditions. This is essential when linking microbial diversity to ecosystem function. Here, a new system to test this link in anaerobic, methanogenic microbial communities is introduced. Rigorously replicated experiments or a suitable experimental design typically require operating several chemostats in parallel. However, this is labor intensive, especially when measuring biogas production. Commercial solutions for multiplying reactors performing continuous anaerobic digestion exist but are expensive and use comparably large reactor volumes, requiring the preparation of substantial amounts of media. Here, a flexible system of Lab-scale Automated and Multiplexed Anaerobic Chemostat system (LAMACs) with a working volume of 200 mL is introduced. Sterile feeding, biomass wasting and pressure monitoring are automated. One module containing six reactors fits the typical dimensions of a lab bench. Thanks to automation, time required for reactor operation and maintenance are reduced compared to traditional lab-scale systems. Several modules can be used together, and so far the parallel operation of 30 reactors was demonstrated. The chemostats are autoclavable. Parameters like reactor volume, flow rates and operating temperature can be freely set. The robustness of the system was tested in a two-month long experiment in which three inocula in four replicates, i.e., twelve continuous digesters were monitored. Statistically significant differences in the biogas production between inocula were observed. In anaerobic digestion, biogas production and consequently pressure development in a closed environment is a proxy for ecosystem performance. The precision of the pressure measurement is thus crucial. The measured maximum and minimum rates of gas production

could be determined at the same precision. The LAMACs is a tool that enables us to put in practice the often-demanded need for replication and rigorous testing in microbial ecology as well as bioprocess engineering.

## 5.2 Introduction

Lack of replication (Prosser, 2010) or application of a suitable experimental design (Lennon, 2011) is a recurring problem in experimental work in process engineering and microbial ecology. It is often caused by technical difficulties or the availability of material. The easiest way of replicating experiments in the laboratory from a technical point of view is by multiplying batch experiments (Jessup et al., 2004). An important characteristic of a batch experiment is that nutrients are fed to the system once as an initial pulse. This pulse is consequently degraded by the microbial community that is faced with changing environmental conditions with less and less available nutrients and the potential accumulation of metabolites over time. Batch experiments are widely used and perfectly suited, when for example testing methane and hydrogen production as a function of substrate pretreatment (Eskicioglu et al., 2017), assessing the effect of experimental protocols (Raposo et al., 2011), characterizing key species involved in specific activity (Mosbæk et al., 2016; Sun et al., 2016), or studying transient phenomena like the degradation of crude oil spills (Pereira and Mudge, 2004). In these situations, the batch set-up mimics well the environmental process in question. A continuously operated reactor system like a chemostat is better suited to reproduce the constant exposure of a microbial community to permanently replenished contaminants or nutrients, as for example in soil around a leaking oil tank, in a wastewater treatment plant or an anaerobic digester. Furthermore, the controllable settings in chemostats such as substrate concentration, hydraulic retention time or temperature may make it possible to link molecular data from 'omics' technologies to environment

parameters (Hoskisson and Hobbs, 2005) and allow studying the contributions of microbial diversity, community dynamics, and microbial interactions to process stability (Briones and Raskin, 2003).

Replication or a more complex experimental design can easily require the operation of several reactors in parallel over extended periods. However, operating numerous chemostats over long times is technically challenging. It requires technical expertise and a significant amount of manpower, going along with increasing costs and complexity of the set-up. These factors make experiments in parallel for example in anaerobic digestion difficult where reactor operation may last several months. Several commercial solutions exist for multiplexing chemostat operation. However, the prices of these systems is typically cost-prohibitive for publically funded academic research labs or are missing an important property as for example the suitability to be operated as anaerobic digesters with continuous quantification of ecosystem performance.

The objective was to develop an affordable and versatile system, allowing the operation of a maximum number of chemostats in parallel by a single person. With the Lab-scale Automated and Multiplexed Anaerobic Chemostat system (LAMACs) introduced here, the automated measurement of biological activity in biogas-producing ecosystems is feasible. In this study, the design, application range and limits of the LAMACs are presented, as well as a first application on anaerobic digestion and its suitability for generating biomass samples for molecular ecological purposes.

## 5.3 Results

### 5.3.1 Range of operating conditions of the multiplexed chemostats

Each of the six chemostats in a LAMACs module can be operated independently (Figure 10). While the temperature and mixing regime is fixed

for the entire module, working volume and flow rates can be individually assigned to each reactor. The range of working volume is from 50 to 200 mL. The upper limit is determined by the height of the heating block. The lower limit is determined by the maximum length of the biomass wasting tube that still allows free movement of the magnetic stir bar.

The dilution rate can vary from a zero wastage mode, i.e., fed-batch mode, to a rate compatible with the maximum doubling time of *Escherichia coli* of 20 minutes. The dilution rate can be adjusted by tuning the working volume and the flow rates of the peristaltic pumps. The LAMACs is operated in quasi-continuous mode because of the periodic nutrient addition. Regular pauses are required to prevent overheating of the peristaltic pumps. In our application, the frequency of pulse additions of 24 times a day is high compared to the hydraulic retention time of the system, especially when working in anaerobic digestion with hydraulic retention times of at least 15 days.

As temperature influences biological activity, the temperature homogeneity between reactors positions was considered a priority and investigated in detail. At three distances from the heating probe, temperatures inside reactors were monitored over three days (Figure 13). The average temperature varied from 36.33 °C to 36.48 °C with a standard deviation of 0.13 °C.

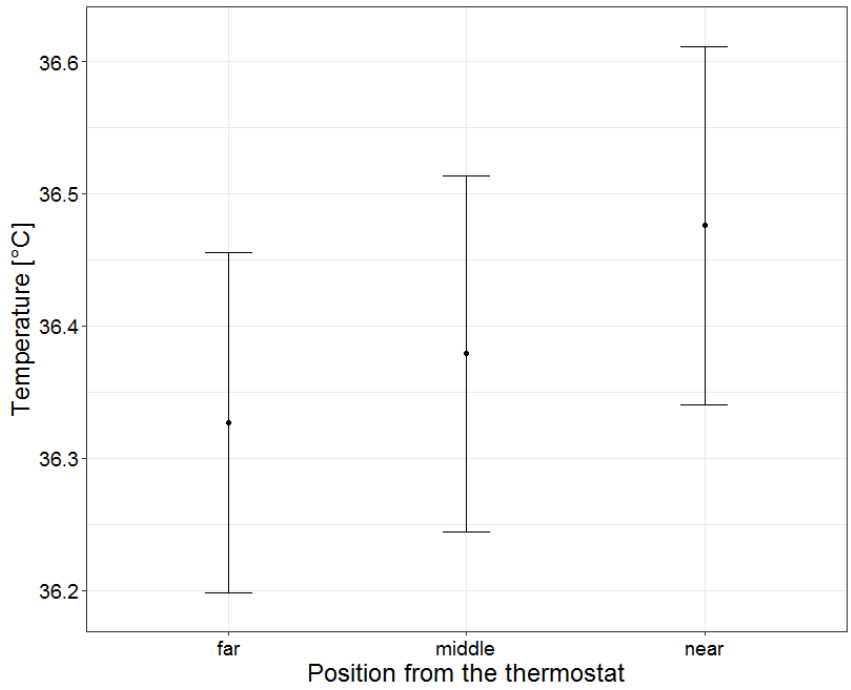


Figure 13 Stable temperature of the LAMACs during operation. Average temperature and standard deviation over three days of operation at three different positions in the heating block. Data points were recorded every 15 minutes, with 280 total data points.

### 5.3.2 Sensitivity of performance measurement

To assess reliability of pressure determination and particularly low production rates, a dedicated experiment was performed with four anaerobic digesters with marked performance differences. In Figure 14a, biogas accumulation over several days of stable reactor operation is shown. Biogas production rates for the same periods are displayed in Figure 14b, ranging from 1.8 $mL \cdot d^{-1}$ (reactor 1) to 63.8 $mL \cdot d^{-1}$ (reactor 4), the latter being the highest observed production rate in the experiment. Reactors 2 and 3 had similar but statistically distinguishable production rates of 7.6 and 7.8 $mL \cdot d^{-1}$, respectively. The lowest detected biogas production rate of 1.8 $mL \cdot d^{-1}$ (Reactor 1) was highly significantly different from signal at ambient pressure without biogas production (Student test, p-value <0.001). Noise introduced by the pressure sensors was therefore negligible. The linearization of pressure increments after degassing events presented small pressure

increases visible in Figure 14, which likely result from gas-liquid transfer of dissolved $CO_2$ and $CH_4$ after the pressure change through degassing. This is not considered a problem for the data analysis. Linear regressions of biogas volume over time allowed the precise determination of biogas production rates

Table 6), with coefficients of determination ($r^2$) above 0.945 and standard error of the slope below 0.01 $mL \cdot d^{-1}$.

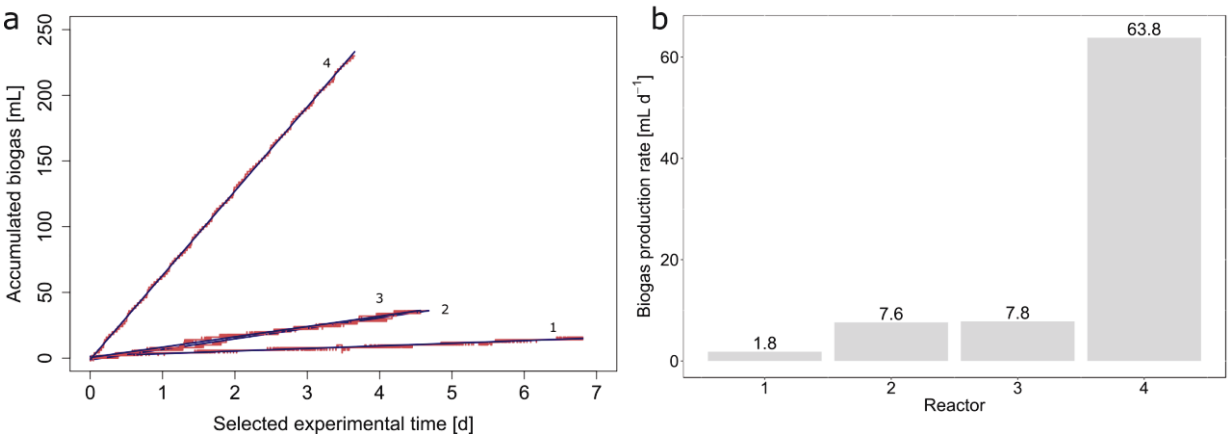


Figure 14 Biogas production of four anaerobic digesters measured by the LAMACs. a) Actual accumulated biogas over time (red curves) and linear regressions (blue lines) b) Biogas production rates derived from linear regressions.

Table 6 Linear regression of biogas accumulation over time.

| Reactor number | Slope[$mL \cdot d^{-1}$] | $R^2$ | Standard error [$mL \cdot d^{-1}$] | Number of data points |
|---|---|---|---|---|
| 1 | 1.8 | 0.930 | 0.003 | 24486 |
| 2 | 7.6 | 0.983 | 0.007 | 22641 |
| 3 | 7.8 | 0.983 | 0.008 | 19182 |
| 4 | 63.8 | 0.999 | 0.009 | 17862 |

### 5.3.3 Long-term operation of the multiplexed chemostats

The LAMACs was designed for a long-term operation of numerous anaerobic digesters at a time. The technical objective of this experiment was to challenge the continuous operation of the LAMACs over a long period, i.e., more than two months. The LAMACs was tested by incubating three inocula in four replicates, therefore twelve anaerobic digesters over nine weeks.

Biogas productions were reported in Figure 15 as a function of inocula origins and time. Results are presented on a weekly basis. Despite having more highly resolved data available, this interval was chosen because soluble COD, hydrogen, methane and biomass production required for establishing a COD balance were measured once a week.

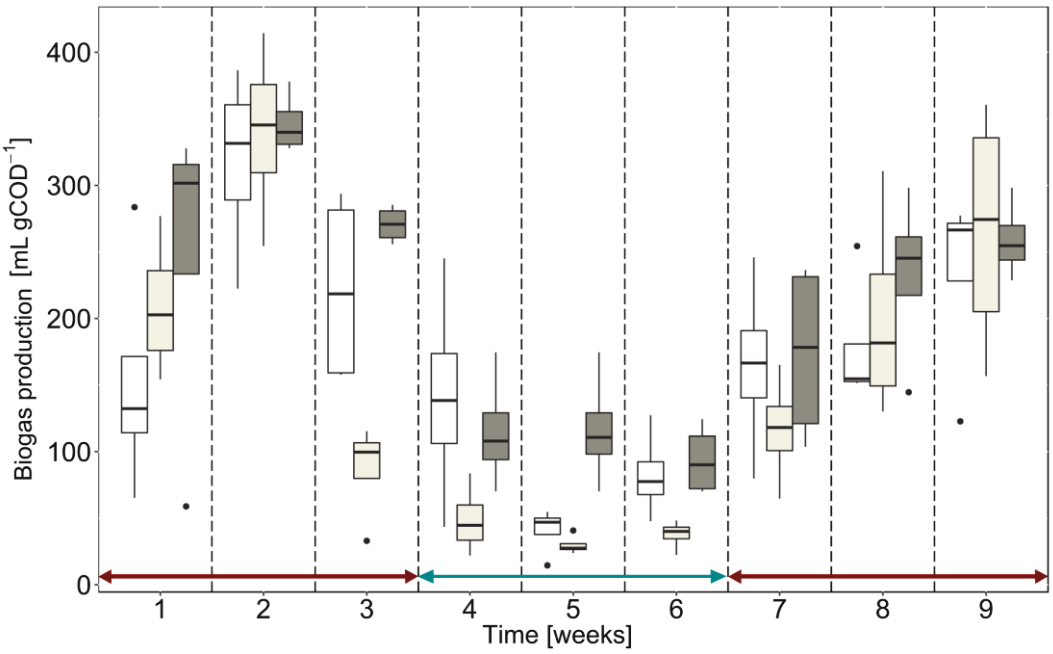


Figure 15 Development of average weekly biogas production over time for anaerobic digesters with three different inocula. Four replicated anaerobic digesters were operated per condition. White boxes stand for the INOC A condition, light gray boxes for INOC B and dark-gray boxes for INOC C. The Kruskal Wallis test was significant in the first eight weeks and the Dunn post hoc test for pairwise multiple comparison displays inocula differences with '*' symbols. In weeks 4 to 6, the simple substrate was used instead of the complex substrate.

Biogas productions ranged from 14.6 to 414.4 mL·gCOD$^{-1}$ (Figure 15 and Table 7). The biological variability of the four replicated reactors is displayed by the size of the boxes in box plots in Figure 15. This intrinsic biological variability was smaller than the variation of performances over time due to operating conditions, and less than the differences between inocula sources. Performances were normalized by the added substrate to be able to compare periods with different substrate loads. The performance decreased when the substrate load was decreased between week 4 and week 6. For example, the inoculum originally fed with slowly biodegradable substrates (INOC C, dark-gray color in Figure 15) had an average production rate of 282 mL·gCOD$^{-1}$ over the first three weeks and then dropped to 108 mL·gCOD$^{-1}$ between week 4 and week 6 when the load was reduced. The performance rose again to 222 mL·gCOD$^{-1}$ between week 7 and week 9 after increasing again the load. Although similar trends were observed, the three inocula sources performed differently most of the time (Kruskal Wallis test, p-value<0.05).

While differences in the biogas production rates were shown according to the origin of the inocula (Figure 15), similar biomass concentrations expressed as volatile solids (Figure 16) and total volatile fatty acid concentrations (Figure 17) were observed in all twelve reactors. Significant differences in biogas production rates (Table 7) were thus related to the specific activity of microbial communities since total biomass did not differ between the different inocula sources (Figure 16). These changes in biogas production rates may be explained by the shift of substrate concentration and composition every three retention times. These shifts can affect the ability of active microorganisms to degrade the actual substrate.

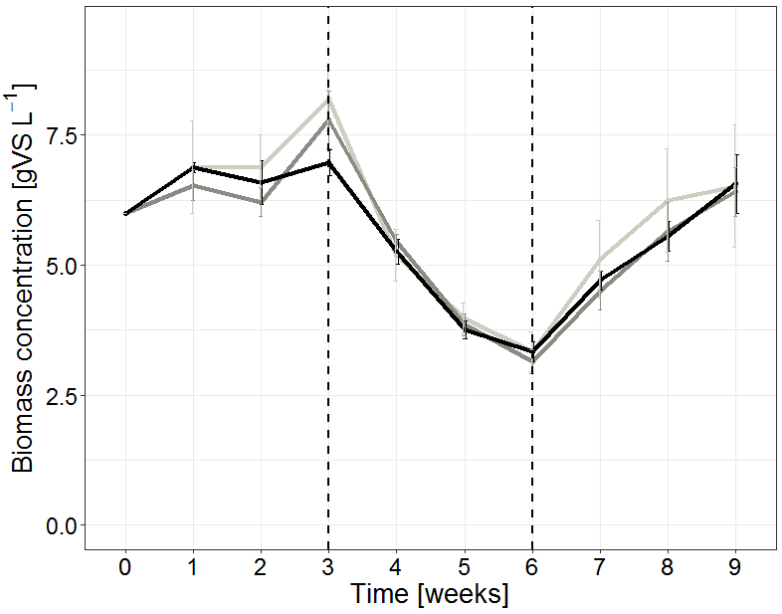


Figure 16 Dynamics of biomass concentration for twelve anaerobic digesters over nine weeks. Biomass concentration is expressed as volatile solids. A color code was applied by inoculum origin; Light gray stands for INOC A replicates, dark-gray stands for INOC B replicates and black stands for INOC C replicates. During weeks 4 to 6, a simple substrate with halved loading rate in terms of COD was applied to the reactors.

## 5.4 Discussion

Operating multiple anaerobic digesters in continuous mode is labor intensive. Braun et al. (2015) worked with 12 manually operated continuous reactors with a working volume of 400 mL and a hydraulic retention time of 20 days over 100 days for testing the fate of polycyclic aromatic hydrocarbons with three different microbial communities (Braun et al., 2015). Operation of these reactors required full-time attention. The apparent need for a multiplexed solution, reducing maintenance and operation requirements is obvious, bearing in mind that the required number of reactors to be operated in parallel may exceed 12 to address many scientific challenges. There are highly multiplexed commercial solutions available for example the AMPTS system by Bioprocess Control (Badshah et al., 2012) that enables the operation of 15 batch reactors in parallel with automated real-time methane flow monitoring. This multiplexed solution allows the use of complex experimental designs. For example, Sierocinski et al. (2017)

presented an application of the AMPTS by testing the effect of community coalescence on ecosystem performance in a gradient of 1 to 12 combined methanogenic communities (Sierocinski et al., 2017). However, AMPTS allows only batch operations where active and dead cells stay in the system. In continuous mode, only actively multiplying cells can maintain their presence when facing washout. Full-scale wastewater treatment plants and also anaerobic digesters are operated in continuous mode. When studying these processes at the laboratory scale from an ecological and bioprocess engineering point of view, mimicking continuous operation is thus essential.

Starting experiments sequentially is one way around the use of a multiplexed continuous reactor system. When using pure cultures, it is possible to repeat experiments or to test alternative experimental conditions even of a complex experimental design one at a time, as the starting point of the experiment is presumably reproducible. When working with complex microbial communities, the assumption of a reproducible starting point is not supported as it is known (Hagen et al., 2015; Kerckhof et al., 2014) that the microbial community structure and ecosystem performance of an inoculum cannot be easily conserved (Perrotta et al., 2017; Rafrafi et al., 2013; Raposo et al., 2011). It is therefore necessary to conduct all experiments belonging to an experimental design at the same time. Knowing the limits of batch operation, it may be desirable to conduct a follow-up experiment to the study of Sierocinski et al. (2017) (Sierocinski et al., 2017), testing the effect of substrate composition on the performance of coalesced microbial communities in continuous operation. Already for a relatively simple design of this experiment, 30 reactors operated in parallel are required when three different substrates and five different inocula with their respective mixtures were considered. This number of continuous reactors is achievable only if most of the operation is automated and if the reactor volume is not too large

to minimize the time-consuming preparation of complex mixtures of organic substrates.

Without even addressing the problem of cost, the commercial system from Anaero Technology (www.anaero.co.uk/) appears most closely related to the LAMACs but has a more than four times larger reactor volume requiring the preparation and storage of larger amounts of feed. While this comparably large reactor volume may be advantageous for various applications, in many other situations (e.g., working with sterile feed), large reactor volumes are unmanageable. In contrast, miniaturization and multiplexing of experimental systems has long been done using flow cells with a volume of as little as 5 mL, for example in biofilm studies (Wolfaardt et al., 1994). More recently, multiplexed chemostats with small volumes on the order of 10 mL (Gebhardt et al., 2011; Kusterer et al., 2008; Schmideder et al., 2015), or even truly microfluidic devices with working volumes of around 2 µL have become available (Steinhaus et al., 2007). However, we consider for our purposes the lower limit of acceptable reactor volume to be approximately 50 mL. With this volume, in combination with a sufficiently short hydraulic retention time, enough microbial biomass is generated for off-line measurements of, for example, metabolites, biomass concentration and or for microbial community analyses (Vuono et al., 2014). With the settings that were used with the LAMACs in this study, 12 $mL \cdot d^{-1}$ of effluent is available for off-line measurements. Currently, the effluent is stored at ambient temperature with the potential exposure to oxygen. These conditions may possibly induce changes in effluent quality, e.g., COD consumption through heterotrophs, growth of biomass and volatilization of organic acids. Refrigeration of the effluent can be envisioned as future improvements to the LAMACs.

A recurring need from experimentalists (Cadotte and Fukami, 2005; De Roy et al., 2013; Venail et al., 2008) as well as modelers in microbial ecology

(Sloan et al., 2006; Wang et al., 2015) is the ability to interconnect ecosystems for testing effects on ecosystem performance by migration, washout or connectedness to a larger metacommunity. Connected chemostats in series may also mimic digestive tract topology (de Wiele et al., 2015; Macfarlane et al., 1998; McDonald et al., 2013; Zihler Berner et al., 2013). This required flexibility is built into the design of the LAMACs where each chemostat can be either operated individually or in series by connecting it to other reactors. The only constrain is a fixed temperature between room temperature and 60 °C and a fixed mixing regime for all reactors within a module (Figure 10).

In the current configuration as anaerobic digester, ecosystem performance is immediately accessible through the rate of biogas production. This parameter of crucial importance for biodiversity and ecosystem functioning experiments is measured directly as pressure development in the LAMACs (Figure 10). The reliability of the LAMACs has been demonstrated in data acquired over the period of nine weeks by showing the biological activity in replicated reactors and three types of inocula (Figure 15). Ritter counters (Dr.-Ing. Ritter Apparatebau GmbH & Co. KG, Bochum, Germany), operating by volume displacement, are frequently used for anaerobic digestion, but they require a minimum flow rate of 24 $mL \cdot d^{-1}$ (https://www.ritter.de/en/products/milligascounters/). Ritter counters were not adapted to the LAMACs because much smaller gas production rates can be expected, and were observed with 1.8 $mL \cdot d^{-1}$. The experimentally determined observed maximum biogas production rate of 415 $mL \cdot gCOD^{-1}$ (Figure 15 and

Table 6) should not be considered as the system's maximum as much more frequent degassing could be accomplished than the two or three degassing events per day that were observed here. Thus, the LAMACs can be used with

a variety of substrates ranging from slowly to highly degradable. Equally, it is possible to use the LAMACs with a more active biomass, i.e., for biohydrogen or ethanol fermentations.

A manageable system of continuous lab-scale chemostats was created, tested and validated for microbial ecology and bioprocess engineering applications. In a recent experiment, one person was able to operate five LAMACs modules simultaneously (Figure 18), i.e., 30 anaerobic digesters, over a twelve-week period. This operation is a significant improvement compared to previous studies (Braun et al., 2015) and was made possible by automation of degassing, feeding and biomass wasting, as well as miniaturization. Samples collected during the experiment were suitable for molecular analyses as we presented in Figure 19, with the quantification of *Bacteria* by quantitative PCR. The full experiment will not be detailed here but serves as proof of concept of the LAMACs. One LAMACs module with six reactors can be built for less than 7000 € (Table 4), thus less than 1200 € per reactor. One LAMACs reactor is four times cheaper than the most comparable commercial solution, e.g., as advertised from Anaero Technology (www.anaero.co.uk/).

The application of the LAMACs may be of particular interest to researchers from various disciplines, not limited to bioprocess engineering and microbial ecology. Screening microbial communities for desired ecosystems functions tasks (Kerckhof et al., 2014; Steinberg and Regan, 2011; Venkiteshwaran et al., 2017) or linking microbial processes to microbial community structure (Rafrafi et al., 2013). The system allows tackling common pitfalls with respect to the statistical evaluation (Bier et al., 2015) or experimental design (Prosser, 2010), and at the same time provides a high-resolution automated sensing approach to monitor ecosystems functioning (Shade et al., 2009). In this framework, LAMACs brings us one step further toward the

understanding of the dynamic and function of complex microbial communities (Widder et al., 2016).

Acknowledgements

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## 5.5 Additional data

Table 7 Comparison of biogas production. Four replicated reactors were used. Mean biogas production rates are expressed over the organic loading rate (ml·gCOD$^{-1}$). Kruskal Wallis tests (Chi$^2$) were performed. When the test was significant (p-value < 0.05), the Dunn post-hoc test was applied to account for multiple comparisons of independent samples.

| Week | INOC A | INOC B | INOC C | Chi$^2$ | p-value | pairwise comparison |
|---|---|---|---|---|---|---|
| 1 | 110.1 ± 39.5 | 177.9 ± 107.5 | 249.2 ± 163.0 | 33.0 | 6.9e-08 | A ≠ B and C |
| 2 | 265.9 ± 144.9 | 331.0 ± 177.3 | 327.0 ± 107.8 | 18.5 | 9.7e-05 | A ≠ B and C |
| 3 | 217.9 ± 106.8 | 85.8 ± 42.8 | 270.9 ± 34.4 | 199.6 | 2.2e-16 | All different |
| 4 | 141.2 ± 109.1 | 48.3 ± 47.0 | 116.9 ± 42.5 | 108.0 | 2.2e-16 | B ≠ A and C |
| 5 | 40.8 ± 16.8 | 29.9 ± 7.5 | 114.8 ± 34.9 | 237.4 | 2.2e-16 | All different |
| 6 | 82.8 ± 39.5 | 37.7 ± 10.8 | 92.4 ± 22.3 | 179.5 | 2.2e-16 | All different |
| 7 | 163.3 ± 72.5 | 113.7 ± 61.7 | 174.5 ± 66.5 | 48.9 | 2.5e-11 | All different |
| 8 | 175.2 ± 78.3 | 200.7 ± 75.9 | 233.3 ± 57.9 | 37.8 | 6.3e-09 | C ≠ A and B |
| 9 | 243.9 ± 69 | 268.2 ± 123.4 | 257.9 ± 38.6 | 3.3 | 0.1886 | - |

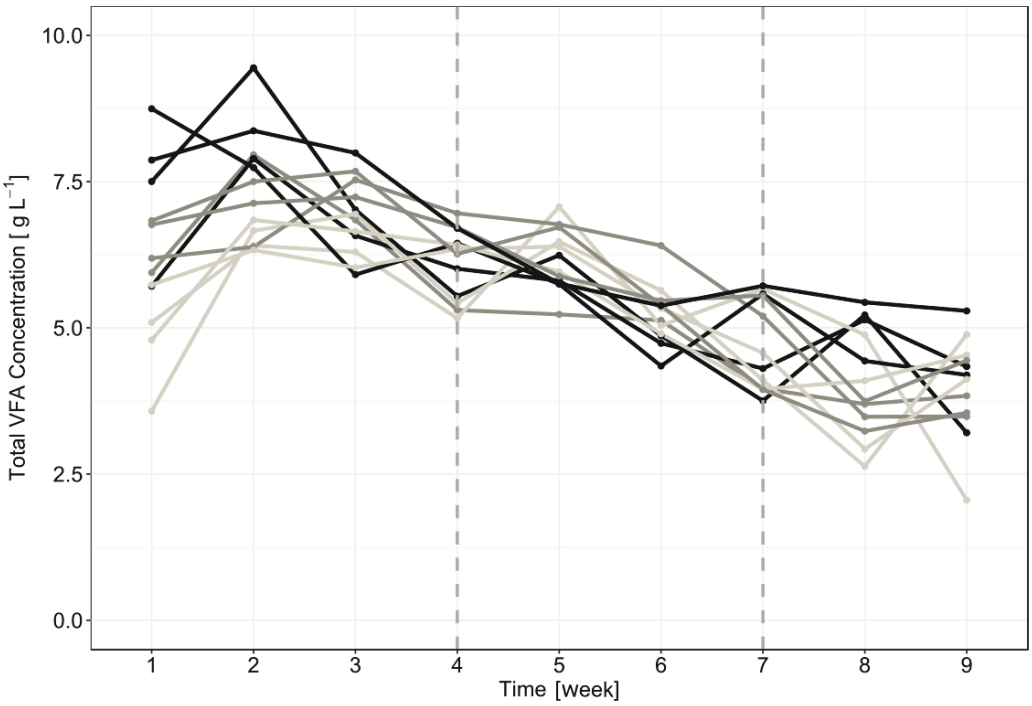


Figure 17 Volatile Fatty Acid concentrations over time in the twelve reactors. Lines in light gray stand for INOC A, dark-gray stand for INOC B, and black stand for INOC C.

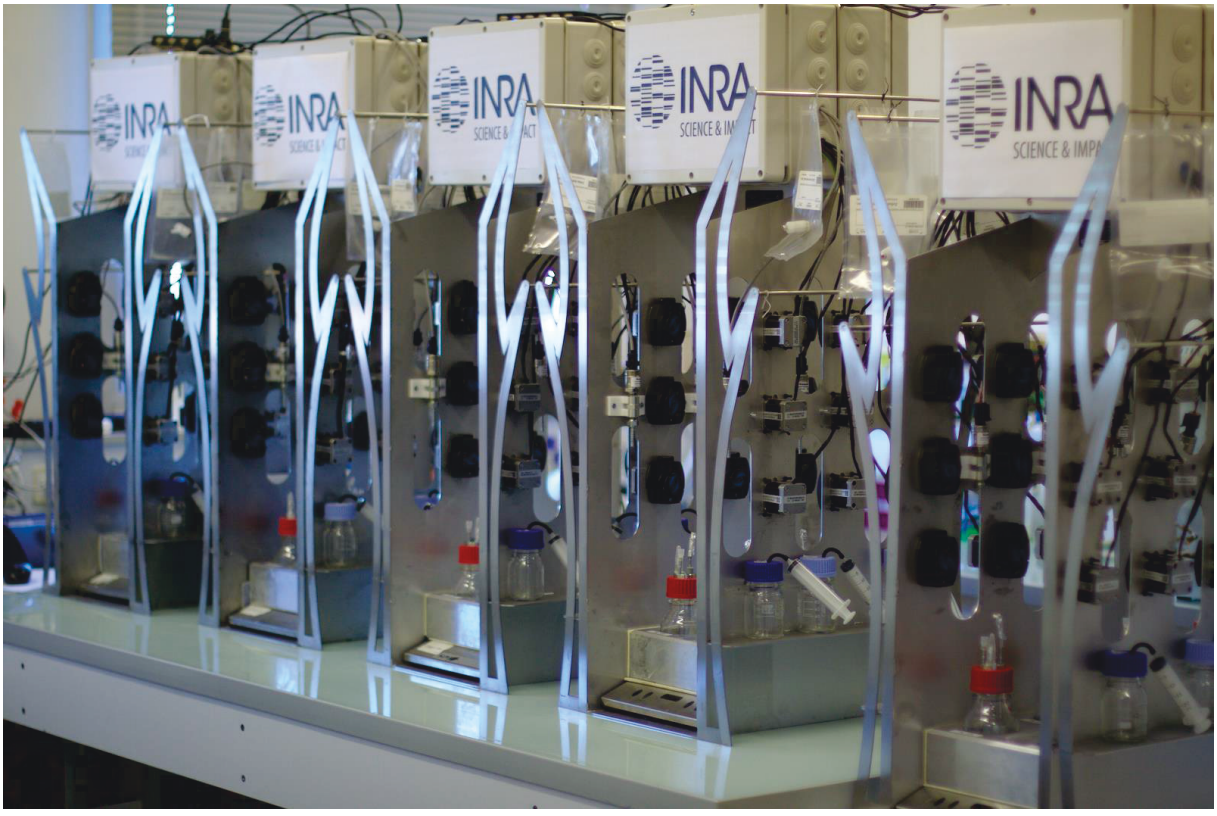


Figure 18 Picture of 5 LAMACs modules.

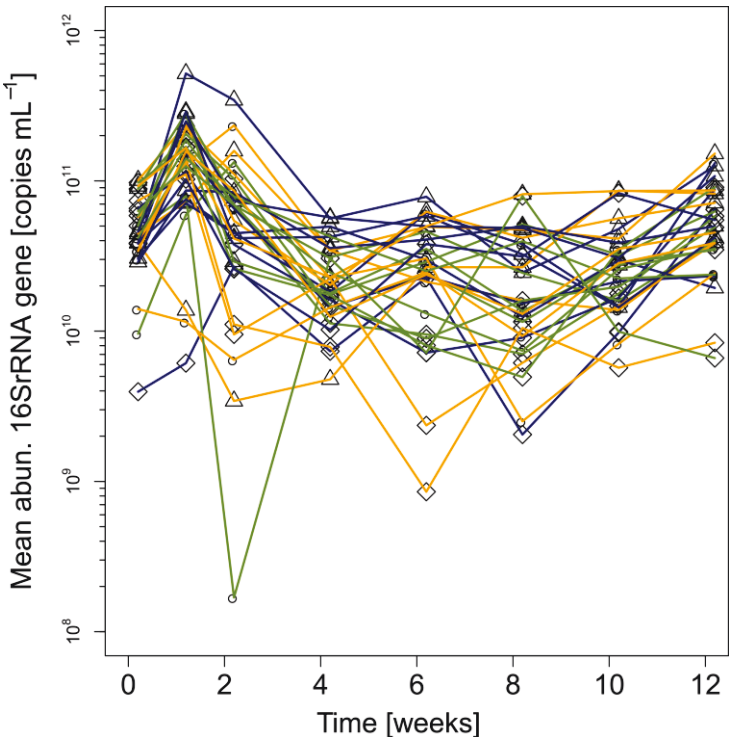


Figure 19 Bacterial abundances in 30 continuous reactors over a period of twelve weeks. Experimental details are given in chapter 2 and data come from chapter 5). Orange lines stand for data from reactors fed with complex substrate, green for simple substrate and blue for intermediate substrate.

# Chapter 6

# Substrate and inoculum choice affect assembly of functionally redundant anaerobic communities

# 6 Substrate and inoculum choice affect assembly of functionally redundant anaerobic communities

The experiment presented in this chapter was developed in collaboration with Dr. Pawel Sierocinski and Professor Angus Buckling from the University of Exeter. Previous work on community mixing at LBE and at Exeter (Sierocinski et al., 2017) were based on batch cultures, whereas the work presented here describes a follow-up experiment based now on continuous cultures. The main driver of this experiment was to test whether the observed effect of mixing on ecosystem performance was only a transitory effect, as observed in the case of hybrid vigor for the F1 generation in plant breeding, or if the effect remained over time.

## 6.1 Introduction

Predicting microbial community dynamics at the community level has been largely studied in various ecosystems, yet researchers struggle to find clear patterns of community assembly. Experiments manipulating microbial community assembly and their functioning have been explored in different ways, either by dilutions (Roger et al., 2016), with synthetic mixture (Bell et al., 2005), or by coalescence studies (Rillig et al., 2015). Coalescence studies have been manipulating community assembly and postulated that the fate of coalescence lead to the dominance of one community (Tikhonov, 2016). Sierocinski et al. (2017) have observed this phenomenon in a batch experiment performing anaerobic digestion. The dominant community was the most efficient for biogas production and this dominance was explained by the selection of competitive and best-performing taxa. In addition, this

experiment showed that the more communities were combined, the higher the biogas production was.

As underlined by Rillig et al., (2015) community coalescence and the functional consequences can be largely influenced by numerous parameters as the variation of abiotic parameters or as the ratios of mixed communities (Rillig et al., 2015). Hence, we propose to study a community coalescence experiment in which we study community coalescence in continuous reactors. After three hydraulic retention times, a continuous reactor often reaches a steady-state because its conditions such as biomass concentration and microbial growth rate become constant.

To carry out this type of experiment we used five inocula from anaerobic digesters from different sources and the five possible mixtures of four inocula. These five mixed communities are compared to the communities derived from the five inocula, here called individual communities. In addition to community assembly, the effect of substrate complexity was tested. Substrate complexity has in fact a large influence on the microbial community structure (Lu et al., 2013; Sundberg et al., 2013). Numerous publications have generally alleged that the use of a simple substrate leads to the development of low diversity microbial communities, sensitive to changes in environmental conditions. More complex substrate may allow the development of a greater diversity of niches and a possible complementarity effect (Evans et al., 2017; Langenheder et al., 2010). Three substrates of increased complexity were therefore tested with all mixed and individual communities.

The multiple points of this chapter will be analyzed both in terms of community assembly and substrate complexity. The objectives aim to: (i) compare the performance of mixed and individual community ecosystems and (ii) evaluate the effect of substrate complexity on performance of

ecosystem functioning. Finally (iii), indicators for community coalescence will assessed.

## 6.2 Results

### 6.2.1 Reactor operation

Thirty reactors were operated under continuous conditions over a period of 12 weeks. As expected, the initially present biomass from the inoculum was partially washed out after about six weeks. Steady reactor operation was obtained in terms of biogas production (Figure 20a) and biomass concentration (Figure 20b). The time of six weeks corresponds to three hydraulic retention times. Biogas production by substrate did not significantly differ after this time. Biogas production during steady operation falls in the range of 60 mL·d-1. This production can be expected assuming an organic loading rate of 0.12 gCOD·d-1 and a buffered biomass (pH>6) fed with an idealized carbon source composed of sugars yielding about 500 mL·gCOD-1. In the first four weeks, the observed biogas production tended to exceed the expected range, likely because of carry-over of organic matter, notably straw and cardboard added the inoculum. However, before use, the inocula had been stirred anaerobically for one month so that carry-over was minimized. Overall, meeting the expected range confirms that the system was tight and operating normally.

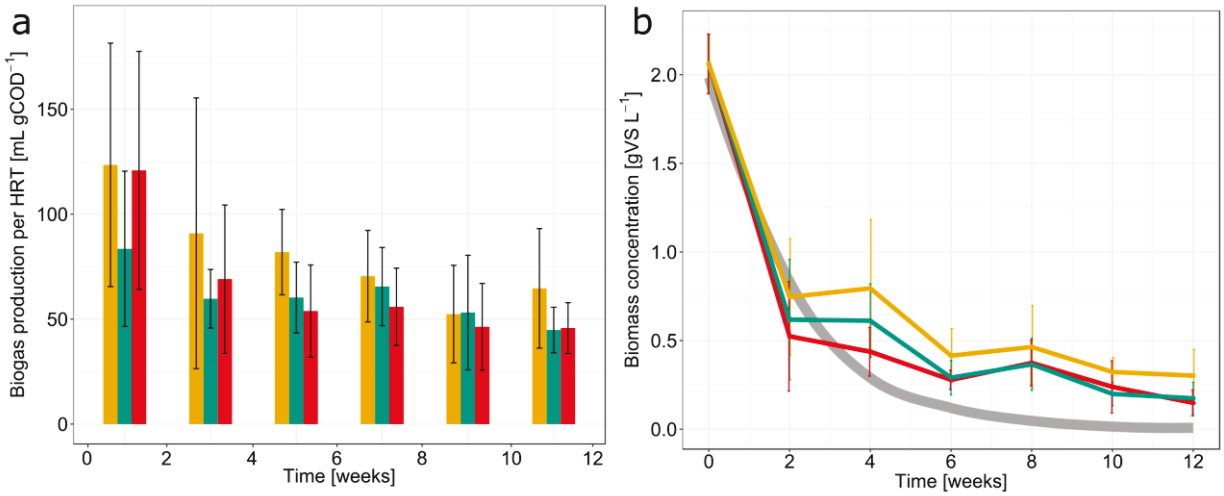


Figure 20 Temporal developments of (a) mean daily biogas production over two week intervals and (b) mean biomass concentration at specific time points by substrate. Yellow, green and red bars and lines represent data from reactors fed with "simple", "intermediate" and "complex" substrates, respectively. Error bars represent standard deviations of measurements in 10 reactors (1 measurement per reactor). The grey line in (b) represents the theoretical washout curve of the initially present biomass in the absence of microbial growth.

The biomass concentration expectedly decreases sharply over the first weeks of reactor operation and then stabilizes after about six weeks. During steady-state, biomass concentrations in the reactors exceed the expected biomass concentration when simulating abiotic washout from a continuously stirred reactor under continuous conditions. The excess of biomass is directly linked to the growth of microorganisms from the substrate load.

In two-week intervals, balances of oxidation-reduction equivalents expressed as chemical oxygen demand (COD) were established (Additional data, Figure 30 – Figure 36). On average, the balances close at 80% ±6, indicating that all major COD fluxes were considered during reactor

operation. For the last three hydraulic retention times, the term of steady-state will be used to refer to the stable conditions in the reactors.

### 6.2.2 Influence of substrate and community assembly on ecosystem functioning

Globally, no significant differences in biogas production were neither detected between the different substrates nor between their community assembly (mixed communities vs. unique communities, Figure 21). Some exceptions were detected, notably, in the third interval covering weeks 5 and 6 of reactor operation in which bioreactors fed with simple substrate produced significantly more biogas than reactors fed with complex substrates (pairwise t-test, p-value<0.05). Likewise, in weeks 7 – 8, individual communities produced significantly more biogas than mixed communities reactors (t-test, p<0.05). Choice of substrate or the community assembly therefore does not have a clear impact on ecosystem performance in our study.

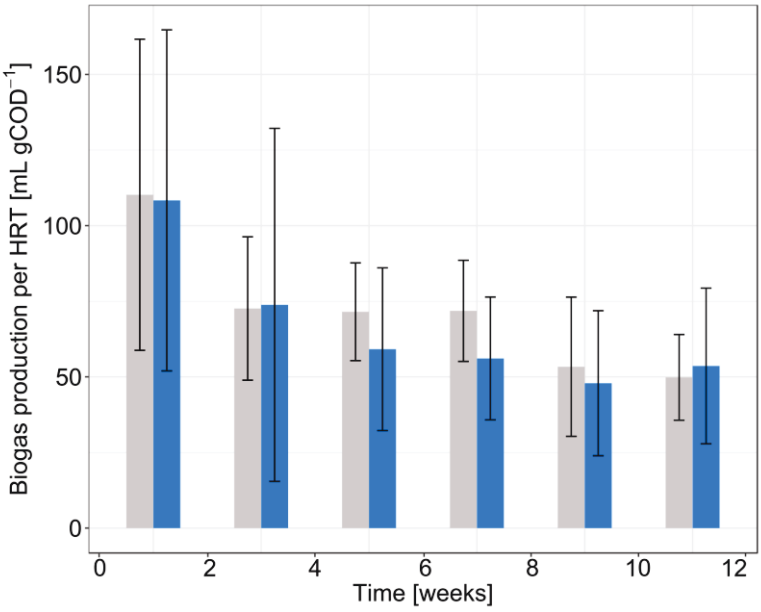


Figure 21 Temporal development of mean daily biogas production over two week intervals by community assembly (gray = individual communities, blue = mixed communities). Each bar represents the mean biogas production of fifteen reactors and its standard deviation.

One important indicator of process performance in anaerobic digestion is the concentration of volatile fatty acids (VFA) (Su et al., 2015). Their high concentrations and particularly that of propionate are known to inhibit anaerobic digestion (Nielsen et al., 2007; Tale et al., 2011). In our experiment, total VFA accumulated and reached a peak of about 5 g·L$^{-1}$ over the first five weeks (Additional data, Figure 28a). Acetate represented on average 71% ±6 of the total VFAs. After the peak, the concentration decreased. While acetate and propionate accumulation was transient for all substrates, butyrate built up to concentrations of around 1 g·L$^{-1}$ notably in reactors fed with substrate of low complexity ("simple") (Additional data, Figure 29). Related to the VFA concentration, a pH decrease was observed (Additional data, Figure 28b). A pH lower than 6.5 may inhibit the anaerobic digestion process. The comparably low COD balance for the first weeks of the experiment (Additional data, Figure 30) is in part explained by VFA accumulation.

As we show later in the text, the methane content in the biogas as measured by off-line gas chromatography explained more variability of the community

structure than the automatically measured biogas production. For that reason, we use for the following analyses methane instead of biogas production.

No differences could be detected for mean biogas production by substrate or community assembly. However, significant differences in the behavior of individual reactors could be detected, notably when contrasting the performance of mixed communities with the individual communities that constituted the mixture, or called here after: reference. In Figure 22, we presented the ratio of methane production by mixed communities at a given time and the mean methane production of the individual communities at the same time. We mixed four out of five inocula in various combinations at the beginning of the experiment, leaving out one inoculum. The mixed communities in Figure 22 are labelled by the disregarded inoculum, e.g., mix without inoculum 1 ("w/o inoc 1"). In this representation, it is impossible to distinguish the putative influence of the missing inoculum on the mixture from the potential effect of bringing together the four communities present in the mixture. Therefore, both possibilities need to be considered. Likewise, it is impossible to see in Figure 22 if an observed behavior is caused by a change in the performance of the mixed community or by the mean of the individuals. As this information is obviously available, it is added in the interpretation.

Generally, two different behavior patterns of the mixed communities with respect to the individual communities can be identified. (1) Statistically different performances by substrate were observed for some mixtures, i.e., "mix w/o inoc1" that produced significantly more methane than the respective individual communities using the simple substrate (anova, p-value <0.005). Similarly, "mix w/o inco2" produced significantly less (anova, p-value <0.01) methane than its individual communities using the complex

substrate. In each case, the performance of the individual communities was stable, whereas the performance of the mixed community caused the change in ratio. For these two inocula, the other two respective substrates yielded performances not distinguishable from the individual communities. Also, the mixed community “mix w/o inoc4” systematically performed worse than its references (anova, p-value <0.05), notably with simple and complex substrates, even though the magnitude was smaller compared to the first two examples with this behavior. (2) A second pattern is displayed by the inocula “mix w/o inoc3” and “mix w/o inoc5”. Their performances over the last six weeks of reactor operation were statistically not different from the individual communities, i.e., mixing did not have an effect on process performance expressed as methane production, even though the reason for the behavior was different. While the individual communities of “mix w/o inoc3” tended to decrease performance over time, leading to an overall high variability, the “mix w/o inoc5” ratio was variable, caused by a high performance variability of mixed community. Despite the observed similarity of mean performance by substrate and by community assembly (Figure 20 and Figure 21), inoculum choice may still have a notable effect on performance of ecosystem function.

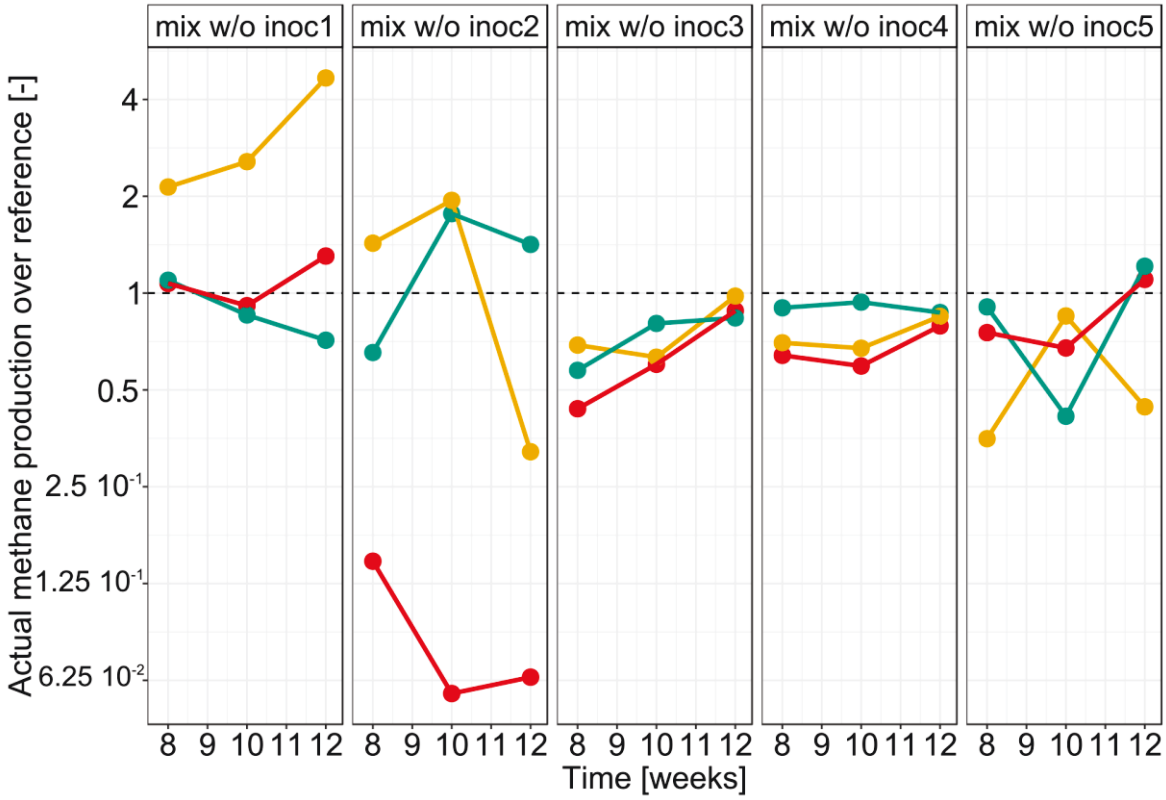


Figure 22 Ratio of methane production of the mixed communities over reference communities, i.e., the mean methane production of the corresponding individual communities by each substrate. The different colors represent the substrate: yellow for the simple substrate, green for the intermediate substrate, red for the complex substrate. Note that the y-axis is log2 transformed to visually treat ratios above and below 1 equally.

A difference between mixed communities is first of all visible, e.g., mixed community w/o inoc1 has ratios up to 4 times higher than the average of reference, while mixed community without inoculum 4 does not exceed the reference value. A closer look at the mixed community without inoculum 1 reveals that in other mixed communities, high ratios are not found; suggesting that inoculum 1 has a negative effect in these communities. In addition, mixed communities without the inoculum 3 did not have a better production than the reference. This result may indicate the (hypothetical) importance of the individual community 3 for the methane production in the experiment.

A substrate effect is also remarkable, as with communities without inocula 1, 2 and 4 have significantly different results depending on their substrate. The mixed community without inoculum 2 clearly has low methane production with complex substrate, suggesting that for mixing the inocula fed with complex substrate, the inoculum 2 may be of importance. This inoculum may also have no effect and the mixed community “mix w/o inoc2” has not developed the interactions or the microbial network to degrade the complex substrate into methane.

Finally, despite the fact that the system is in steady-state, a temporal effect plays on these production ratios: either it doesn't change or only slightly as with the mixed without inoculum 4, either time has an effect such as for example with mix without inoculum 3, which has methane ratios increasing over time.

Community mixture is therefore interesting for the anaerobic digestion functioning here, especially without inoculum 1 and certainly with inoculum 2 and 3, demonstrating here once again the importance of the inoculum. Relating microbial diversity to ecosystem functioning

### 6.2.3 Relating microbial diversity to ecosystem functioning

Mixing microbial communities had immediate effects on the diversity and structure of the resulting communities. In the following, we differentiate the responses by domains of Bacteria and Archaea.

The temporal development of bacterial and archaeal diversities in mixed and individual communities is presented in Figure 23 with the accompanying statistical analysis in Table 8. Generally, as expected, the diversity of Bacteria is significantly higher than that of Archaea.

Globally, the differences in bacterial diversity between mixed and individual communities at any time point are not significant and mixing consequently did not have a lasting effect on the bacterial diversity. In contrast to this, all diversities measured in the reactors at any time were significantly lower than diversity of the individual inocula and the mixed inocula. The initial diversity of the mixes was calculated from the sequence inventories of the inocula and not measured in a biomass sample. The initial diversities of the mixes were greater than the mean diversity of the inocula, indicating that the sequence inventories of the individual inocula were at least in part complementary. The initial bacterial diversity of the mixes was equally significantly different from the diversity measured during reactor operation. Mixing the communities lead to a transient, significant increase in bacterial diversity that was, however, quickly lost in the system, even already after 0.1 weeks of reactor operation. This early loss is likely related to the substantial washout at the beginning of the continuous operation of the reactors.

When calculating the initial diversity in the mixes for the archaeal community, a significant, transient, initial increase of archaeal diversity was observed. Therefore, mixing did not have a lasting effect on the overall archaeal diversity. Conversely, the differences in archaeal diversity between the inocula and individual communities, are not significant at any time point. A notable difference between mixed and individual archaeal communities was observed when considering loss of diversity over time.

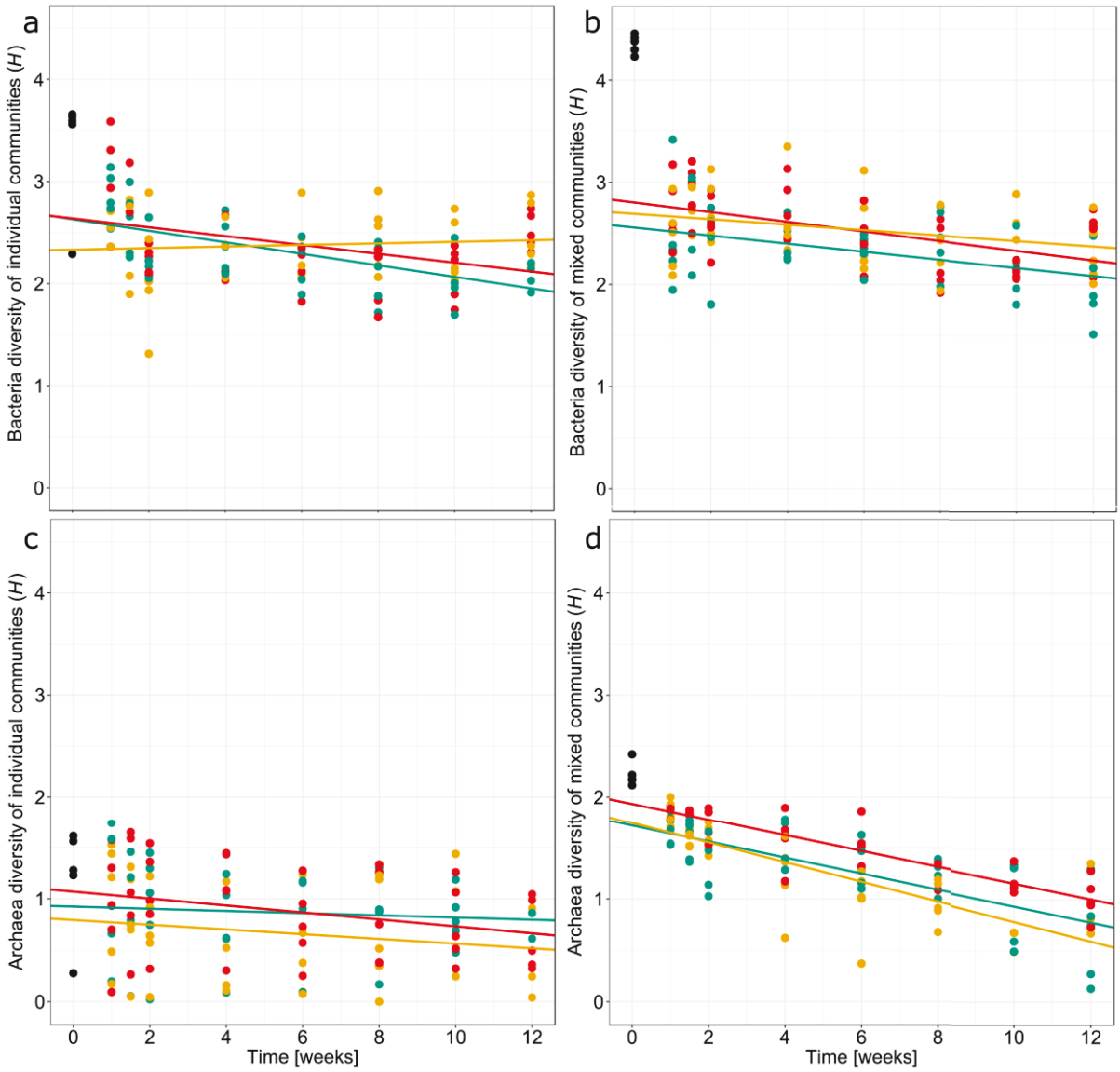


Figure 23 Linear regressions of Bacteria and Archaea diversity calculated with Shannon diversity index along time and separated as a function of community assembly: in panel a-c) individual communities and panel b-d) mixed communities. At time 0, the diversity of the five inocula have been calculated and represented in dark points. For mixed communities, diversity is the average of the 4 inocula of the mixture. Along the experiment, each point is calculated from ten reactors fed with different substrate: yellow for simple substrate, green for intermediate substrate and red for complex substrate

Table 8 Bacterial and archaeal diversities calculated as Shannon diversity were compared using t-test. Diversity of inocula at 0 weeks was compared to the mixed and individual communities.

| Domain | Time [weeks] Comparison | 0.1 | 1 | 2 | 4 | 6 | 8 | 10 | 12 |
|---|---|---|---|---|---|---|---|---|---|
| Bacteria | t0 vs individual | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | t0 vs mixed | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | Mixed vs indiv | NS | NS | <0.05 | <0.01 | NS | NS | NS | NS |
| Archaea | t0 vs individual | NS | NS | NS | NS | NS | NS | NS | NS |
| | t0 vs mixed | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | Mixed vs indiv | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.05 | NS | NS |

We estimated the rate of loss as the slopes of linear regressions (Table 9). We observed the highest loss for all substrates in mixed communities considering the Archaea, converging to the diversity found in the individual communities. Loss of archaeal diversity in individual communities was not different from 0. For Bacteria, the loss was significantly lower than for Archaea or not different from 0.

Table 9 Linear regressions slope of Archaea and Bacteria diversity over time as a function of community assembly and substrate. Loss rates derived from linear regressions fitted to archaeal and bacterial diversity over time (Figure 23).

| | | **Individual communities** | | | **Mixed communities** | | |
|---|---|---|---|---|---|---|---|
| **Domain** | Substrate | Simple | Interm. | Complex | Simple | Interm. | Complex |
| **Bacteria** | Slope [$\Delta H \cdot \text{weeks}^{-1}$] | -0.04 | -0.07 | -0.09 | 0.01 | -0.11 | -0.08 |
| | p-value | NS | 0.01 | <0.001 | NS | <0.001 | <0.001 |
| **Archaea** | Slope [$\Delta H \cdot \text{weeks}^{-1}$] | -0.04 | -0.03 | -0.06 | -0.17 | -0.14 | -0.14 |
| | p-value | NS | NS | NS | <0.001 | <0.001 | <0.001 |

Diversity of Archaea has been proven to be higher in mixed communities than in individual communities in the first weeks of the experiment. In the

following, we related the microbial diversity to ecosystem functioning using methane production during steady reactor operation, i.e., the last six weeks of operation.

The bacterial diversity was not significantly correlated to methane production, whereas different behaviors are observed for Archaea. The diversity of mixed archaeal communities linearly correlated with methane production (slope of 12.34 $mL \cdot gCOD^{-1} \cdot \Delta H^{-1}$, $p<0.05$), whereas no correlation is observed for individual communities (Figure 24 and Table 10). The two regressions intersect in the range of the highest observed archaeal diversities. This means that at any observed level of archaeal diversity, on average, individual communities showed stronger or at least equal performance of ecosystem function as mixed communities. These results suggest that the effect of diversity on ecosystem functioning may depend on the history of the diversity, i.e., whether diversity is derived from coalescence, simply mixing or through a shared developmental history of the community. In our situation, mixing and putative coalescence of communities did not lead to changes in observed ecosystem function, even though the community was notably altered.

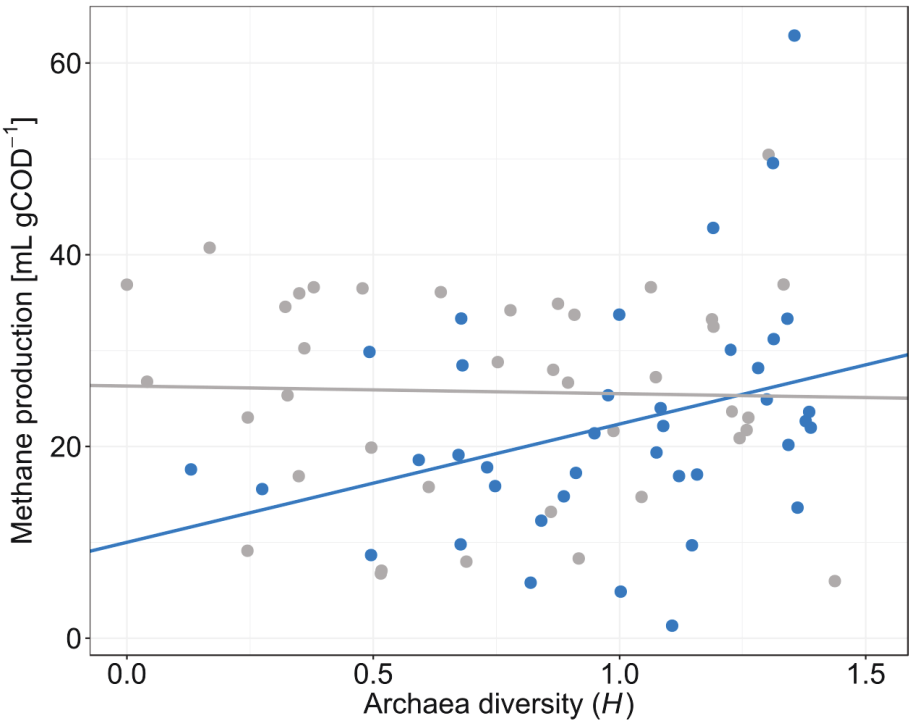


Figure 24 Methane production during steady state (weeks 8 to 12) as a function of archaeal diversity expressed as Shannon index (*H*). Grey symbols and lines represent the data points and the regression for individual communities; blue symbols and lines represent mixed communities.

Table 10 Linear regressions of archaeal diversity measured as Shannon index (*H*) and steady-state methane production (weeks 8 to 12).

| | **Individual and mixed communities** | **Individual communities** | **Mixed communities** | | | |
|---|---|---|---|---|---|---|
| **Substrate** | All | All | All | Simple | Intermediate | Complex |
| **slope [mL·gCOD$^{-1}$ Δ$H^{-1}$]** | 2.56 | -0.8 | 12.34 | 28.6 | 14.74 | 13.08 |
| **p-value** | NS | NS | 0.036 | NS | 0.02 | NS |

When resolving the diversity – performance data by substrate, only methane production from the substrate of intermediate complexity ("intermediate") was significantly correlated to archaeal diversity (Table 10).

While ecosystem functioning has not been linked to the diversity, it will be interesting to assess the relevance of biotic and abiotic parameters to the structure of microbial communities.

### 6.2.4 Relating microbial community structures to ecosystem functioning

In the following, we relate the structure of microbial communities to performance under steady state conditions. The aim is to determine how measured biotic and abiotic parameters correlate with functioning and the microbial community structure. Figure 25 highlights the dissimilarities of structures between microbial populations calculated with the quantitative Jaccard index, i.e. the differences in OTU abundance between populations. The microbial community composition represent 19.1% over the first two principal coordinate analysis axes for Bacteria and 45.2% for Archaea.

Bacterial communities under steady state are correlated to substrate ($R^2$ = 0.40, linear correlation of community ordination with substrate over all time points) and under steady state ($R^2$ = 0.49 linear correlation of community ordination with substrate), as shown in Figure 25a and Table 11. Even though performances are not significantly correlated to the substrates used for feeding the reactors, the choice of substrate is leaves traces in the bacterial community.

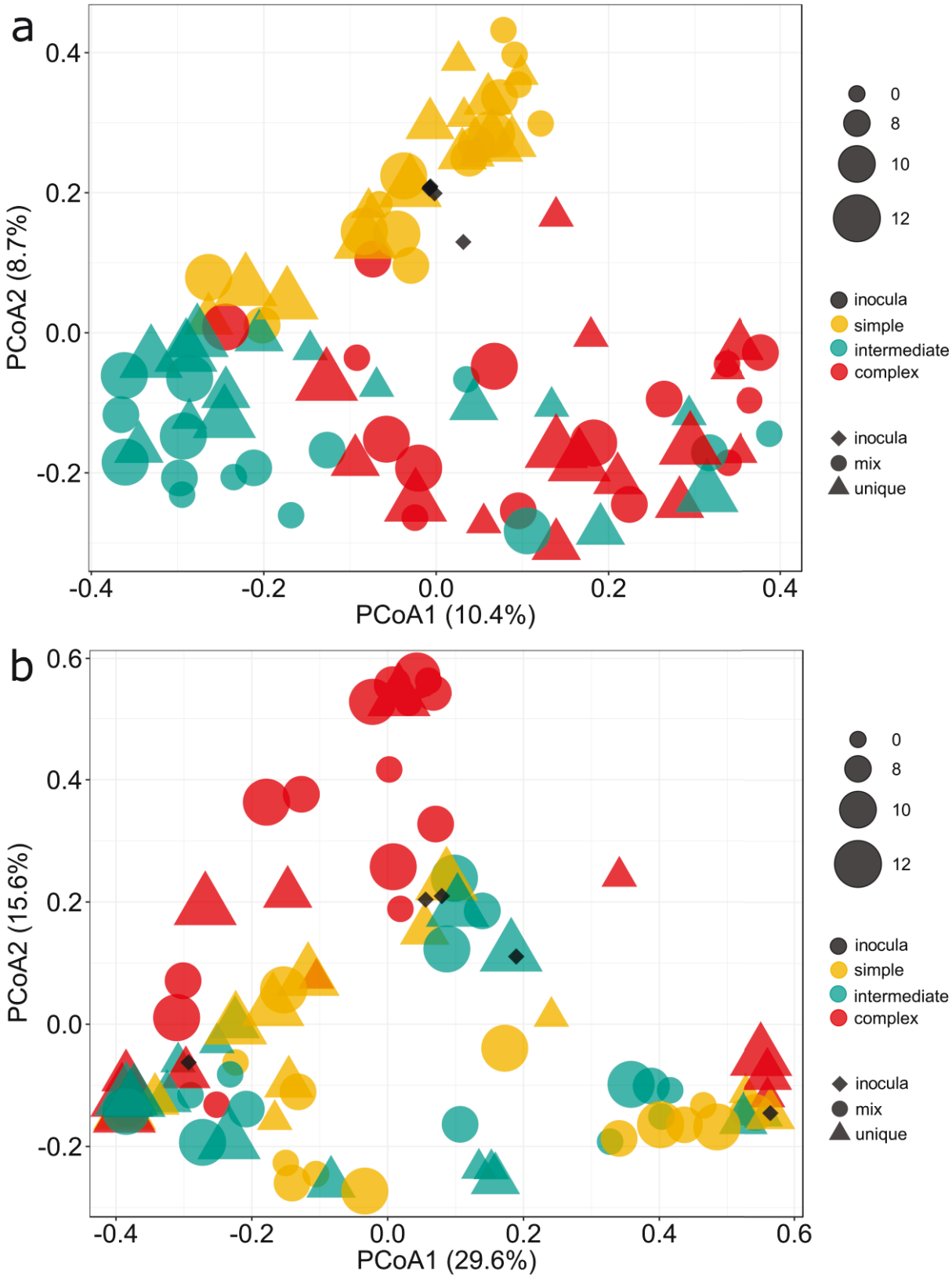


Figure 25 Ordinations of microbial communities with principal coordinate analysis (PCoA) from a distance matrix calculated with Jaccard distance: a) Bacteria b) Archaea.

There is equally a strong correlation between experimental time and bacterial community structures when separated by substrate. Especially with the intermediate substrate where the correlation is $R^2$=0.47 compared to 0.24 and 0.36 with the simple and complex substrates, respectively. The correlation with experimental time indicates that the bacterial communities evolve over time, even when the performance of the reactors reached a steady state.

Also pH and VFA concentrations correlate detectably when data are separated by substrate, with a strongest correlation between data obtained with the intermediate substrate ($R^2$ = 0.60 and 0.63 respectively for pH and VFA concentrations). The observed anti-correlation between pH and VFA concentrations is expected as there is an immediate effect of volatile fatty acid concentration on the pH of the system (Figure 28). In contrast, the chemical oxygen demand (COD) tends to have a low or no correlation with the bacterial structure. Substrates are apparently differently degraded, leading to the transient accumulation of metabolites as VFAs, despite the fact that the overall performance of ecosystem function, i.e., methane production, is not significantly different by substrates.

When substrates are considered separately, bacterial communities correlate strongly with their inocula, whether it was an individual inoculum or a mixture, i.e. the inocula have a lasting effect on the bacterial communities without a convergence towards a common community structure. The $R^2$ for the correlations are 0.59 for the simple substrate and 0.69 and 0.53 for the intermediate and complex substrates respectively. The fact that the correlation between community structure and inocula was considerably lower when all substrates were treated together ($R^2$ = 0.9) indicates that substrate effects were stronger drivers for community structure than the inocula.

The directly measured performance indicator measured in the LAMACs, i.e., biogas production, correlates weakly with the bacterial community structure ($R^2$ =0.06, over total experiment, $R^2$ = 0.08, over steady state phase). In these last weeks in particular, when the data are split by substrate, the bacterial communities fed with the intermediate substrate correlate to the biogas concentration ($R^2$ = 0.30). Once again, only the correlation of performance data with the community structure of the reactors fed the intermediate substrate is notable and significant (see also 0.03 and 0.12). In the last weeks of the experiment, hydrogen concentrations increased in reactors fed with simple substrate. However the correlation is not quite high ($R^2$ = 0.21) since only 4 out of 10 reactors produce hydrogen. Strikingly, none of the reactors fed with complex substrate produced significant amounts of hydrogen, as a result as the anti-correlation is quite high 0.46 (<0.001), as shown in the ordination represented in Figure 37. In addition, those reactors were quite correlated to methane production 0.53 (<0.001).

Table 11 Correlations of environmental data to microbial community distance data. Correlations were performed with the function 'envfit' of the vegan package and the matrix distance was calculated with the Jaccard index. These correlations were tested on all data from week 2 to 12, then on the six last weeks of the experiment. The parameter of the community assembly (mixed or individual communities) has been tested but is never significant.

| | Substrate | Inoculum | Time [h] | Biomass concentration [gVS·L$^{-1}$] | pH | VFA [g·L$^{-1}$] | COD [gCOD· L$^{-1}$] | Biogas [mL·d$^{-1}$· gCOD$^{-1}$] | Methane [mL·d$^{-1}$· gCOD$^{-1}$] | Hydrogen [mL·d$^{-1}$· gCOD$^{-1}$] |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacteria weeks 2 to 12 (number of data points = 180) | | | | | | | | | | |
| $R^2$ | 0.407 | 0.089 | 0.067 | 0.074 | 0.002 | 0.177 | 0.012 | 0.059 | 0.017 | 0.12 |
| p | 0.001 | 0.029 | 0.001 | 0.001 | NS | 0.001 | NS | 0.005 | 0.216 | 0.001 |
| Bacteria weeks 8 10 12 (number of data points = 90) | | | | | | | | | | |
| $R^2$ | 0.494 | 0.091 | 0.102 | 0.117 | 0.560 | 0.444 | 0.267 | 0.083 | 0.187 | 0.145 |
| p | 0.001 | NS | 0.011 | 0.002 | 0.001 | 0.001 | 0.001 | 0.026 | 0.002 | 0.001 |
| Bacteria weeks 8 10 12 simple substrate (number of data points = 30) | | | | | | | | | | |
| $R^2$ | | 0.593 | 0.244 | 0.066 | 0.422 | 0.477 | 0.269 | 0.032 | 0.515 | 0.209 |
| p | | 0.002 | 0.032 | NS | 0.002 | 0.001 | 0.014 | NS | 0.001 | 0.043 |
| Bacteria weeks 8 10 12 intermediate substrate (number of data points = 30) | | | | | | | | | | |
| $R^2$ | | 0.690 | 0.468 | 0.117 | 0.601 | 0.631 | 0.013 | 0.301 | 0.395 | 0.203 |
| p | | 0.001 | 0.001 | NS | 0.001 | 0.001 | NS | 0.009 | 0.001 | 0.03 |
| Bacteria weeks 8 10 12 complex substrate (number of data points = 30) | | | | | | | | | | |
| $R^2$ | | 0.534 | 0.361 | 0.147 | 0.319 | 0.223 | 0.057 | 0.12 | 0.5324 | 0.458 |
| p | | 0.013 | 0.003 | NS | 0.010 | 0.039 | NS | NS | 0.001 | 0.001 |
| Archaea weeks 2 to 12 (number of data points = 180 – 12 (<30 sequences) =168) | | | | | | | | | | |
| $R^2$ | 0.028 | 0.491 | 0.245 | 0.095 | 0.014 | 0.291 | 0.440 | 0.193 | 0.063 | 0.057 |
| p | NS | 0.001 | 0.001 | 0.001 | NS | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 |

| Archaea weeks 8 to 12 (number of data points = 90 – 12 (<30 sequences) =78) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $R^2$ | 0.148 | 0.415 | 0.054 | 0.083 | 0.016 | 0.130 | 0.088 | 0.079 | 0.071 | 0.076 |
| p | 0.003 | 0.001 | NS | 0.041 | NS | 0.008 | 0.036 | 0.052 | NS | NS |

To highlight these conclusions for the six weeks of the experiment, the ordination is represented in Figure 37 in the Additional data. The different substrates represented by different colors appear to separate, although some points of the reactors fed with the intermediate substrate mix with those fed with the complex substrate. Clearly, the VFA concentration is linked with the microbial structure of reactors fed with simple substrate, as are COD, volatile or biomass concentration. Expectedly, correlations of pH and VFA concentration are positive and hydrogen is anti-correlated to the reactors fed with the complex substrate.

In comparison to the bacterial communities, archaeal communities do not appear to be structured by the substrate as shown in Figure 25b and Table 11 with an $R^2 = 0.15$. Another marked difference between the bacterial and archaeal communities is the relative difference of the inocula. For Archaea, the inocula are relatively more different from each other than for the Bacteria (black points in Figure 25a and b). It appears that the archaeal communities were structured according to these initial inocula as seen in the comparably strong correlations ($R^2$ =0.49) and in steady-state ($R^2$ =0.42). These environmental correlations are resumed in the Additional data Figure 38. These results underline once again the structural difference between communities of Archaea and Bacteria. It is important to note that in a process implemented for anaerobic digestion, the structure of Archaea communities is not correlated with methane production.

In addition to a comprehensive analysis of the entire microbial communities, also highly abundant OTUs with their taxonomic affiliation were characterized. The most abundant phyla in the bacterial communities are the *Firmicutes* and the *Proteobacteria* Table 12. The *Bacteroidetes* phylum is significantly less represented in the reactors fed with simple substrates than in reactors fed with intermediate or complex substrates. Within the

*Firmicutes,* the *Clostridia* dominate the bacterial community independent of the substrate. *Gammaproteobacteria* and *Betaproteobacteria* are the most represented class in *Proteobacteria*.

Table 12 Bacteria absolute abundances in steady state reactors represented for phylum total abundances superior to $10^{10}$ and class abundances superior to $10^{7}$ expressed in percentage of total abundance

| | simple | intermediate | complex |
|---|---|---|---|
| ***Bacteroidetes*** | **0.3%** | **13.1%** | **7.9%** |
| *Bacteroidia* | 0.26% | 12.8% | 7.5% |
| *Cytophagia* | 0% | 0% | 0.01% |
| *Flavobacteriia* | 0.01% | 0.03% | 0.2% |
| *Sphingobacteriia* | 0.02% | 0.24% | 0.2% |
| ***Firmicutes*** | **20%** | **23.7%** | **13.3%** |
| *Bacilli* | 1.6% | 0.46% | 0.4% |
| *Clostridia* | 17.4% | 21.68% | 10.7% |
| *Negativicutes* | 0.93% | 1.51% | 2.2% |
| ***Proteobacteria*** | **7.5%** | **9.4%** | **5%** |
| *Alphaproteobacteria* | 0.07% | 0.62% | 0.2% |
| *Betaproteobacteria* | 2.66% | 6.62% | 2.7% |
| *Deltaproteobacteria* | 0.01% | 0.04% | 0.01% |
| *Gammaproteobacteria* | 4.75% | 2.12% | 2.1% |

It is interesting to separate the Archaea community in the different family by the inoculum since it is the biotic parameter that correlates the most with the community, as previously shown in Table 11. In relative abundances, the main archaeal order is *Methanobacteriales,* hydrogenotrophic methanogens. Abundances of the order *Methanosarcinales*, acetoclastic methanogens, are low (Additional data, Figure 39). In reactors fed with complex substrate, the

order *Thermoplasmatales* is quite present particularly in mixed communities and not in reactors fed with the other substrate.

We have therefore seen that the Bacteria communities are mainly driven by the substrate, and the Archaeal communities are driven by inocula. Interestingly, the community assembling parameter is not correlated with microbial structures but inocula are. The effects of inocula have therefore independent effects that act on microbial structures and the structure of microbial communities show some differences with respect to substrate and assembly. The coalescence of these inocula in mixed communities is then studied in a new chapter.

### 6.2.5 Coalescence of mixed communities

We were able to show that, on average, mixing communities did not lead to significantly improved performance of ecosystem function, irrespective of the substrates added (Figure 20a and Figure 21), albeit marked differences in the behavior of some of the mixtures (Figure 22) and a notable distinction of microbial communities by substrate (Figure 25a) and by inoculum (Table 11). This leads to the question of how the choice of inocula and the substrate used for feeding affect the assembly of the mixed communities.

On the broad range of possible assemblies, the extreme positions may be (1) coalesce of all initial communities. This can be imagined as a complete reshuffling of the interactions between individual community members to find their place in a new, synthetic microbial community with putative new interactions, (2) the prevalence of one of the inocula communities that consequently displaces as one unit the other communities, or (3) the de-novo assembly of a novel community from low-abundant community members, leading to a community in which the inocula are unrecognizable.

To test what type of assembly likely shaped the mixed community, we expressed the trace of each inoculum in the developing mixed community by the Jaccard similarity at every given time point between the developing inocula in the individual communities and the respective mixture (Figure 26 and Figure 27). The observed similarity is then compared to a reference distribution of similarities. This distribution was derived from 1000 permutations of communities in which the abundances of each OTU were randomly sampled from the respective 245 abundances available for the OTUs from the samples considered in this study. It must be noted that the Jaccard similarity as used here deviates from the traditional definition in that it takes sequence abundances (and not only presence-absence information) into consideration (Oksanen et al., 2015).

Globally, we were able to find significant similarities for most of the mixed communities with at least one if not all inocula, even after twelve weeks of reactor operation. In cases where significant similarities with several individual communities are detected, it is parsimonious to interpret the results as a coalescence. At steady state performance (weeks 8 to 12), for the bacterial communities in reactors fed with the complex substrate (columns 11 – 15 in Figure 26), the systematic presence of elevated, significant similarity with all four individual communities is found. This corresponds to the first extreme position laid out above.

In several instances three out of four communities (columns 4 and 9) or two out of four (column 10) had significant similarities with the mixed communities. These observations can be approximated by the number of red circles over the last three time points in the figure (39 out of 60 possibilities). For the other substrates, typically fewer inocula were significantly similar to the mixed communities (23 and 17 out of 60 for simple and intermediate substrates, respectively). We were therefore able to observe complete

coalescence of the inocula when reactors were fed with complex substrate and partial coalescence for simple and intermediate substrates.

In one situation (column 7), a behavior is observed that corresponds to the second scenario developed above, in which the mixed community shares significant similarity with only one of the individual communities. This may be a situation in which one initial community acting as a unit has outcompeted the other inocula.

In two reactors, none of the inocula is significantly similar to the respective mixed community (third scenario above). This suggests the development of a unique bacterial community, likely developed from bacteria that were present in low abundance in the inocula. One of these communities was fed the complex substrate (column 12) and is the only exception to otherwise completely coalesced inocula for this substrate. For this mixed community, no significant similarity was observed during the steady state interval. Similarly, one community fed with the simple substrate (column 1) shares few significant similarities with some inocula, all of which at comparably low absolute values. Also in this reactor, it seems that a novel microbial community developed from community members with an initially low abundance.

Figure 26 was used above to interpret coalescence as a function the individual reactors and of substrate. The figure equally allows an interpretation by inoculum. Following this logic, it appears that inoculum 2 was particularly successful in leaving a lasting mark in the mixed communities. In every mixed community where this inoculum was used, significant similarities were observed during steady state operation. Inoculum 5 was least successful as its traces were rarely found in coalesced communities with the exception of reactors receiving the complex substrate. However, this inoculum was the only one that managed to gain as a

community the competition against the other three inocula (column 7). The rare coalescence with other communities when fed with simple and intermediate substrate, as well as the outcompetition of the other inocula in one case may indicate that inoculum 5 has a stable network of interactions that is not easily opened to new interactions, i.e., the integration into a new ecological context.

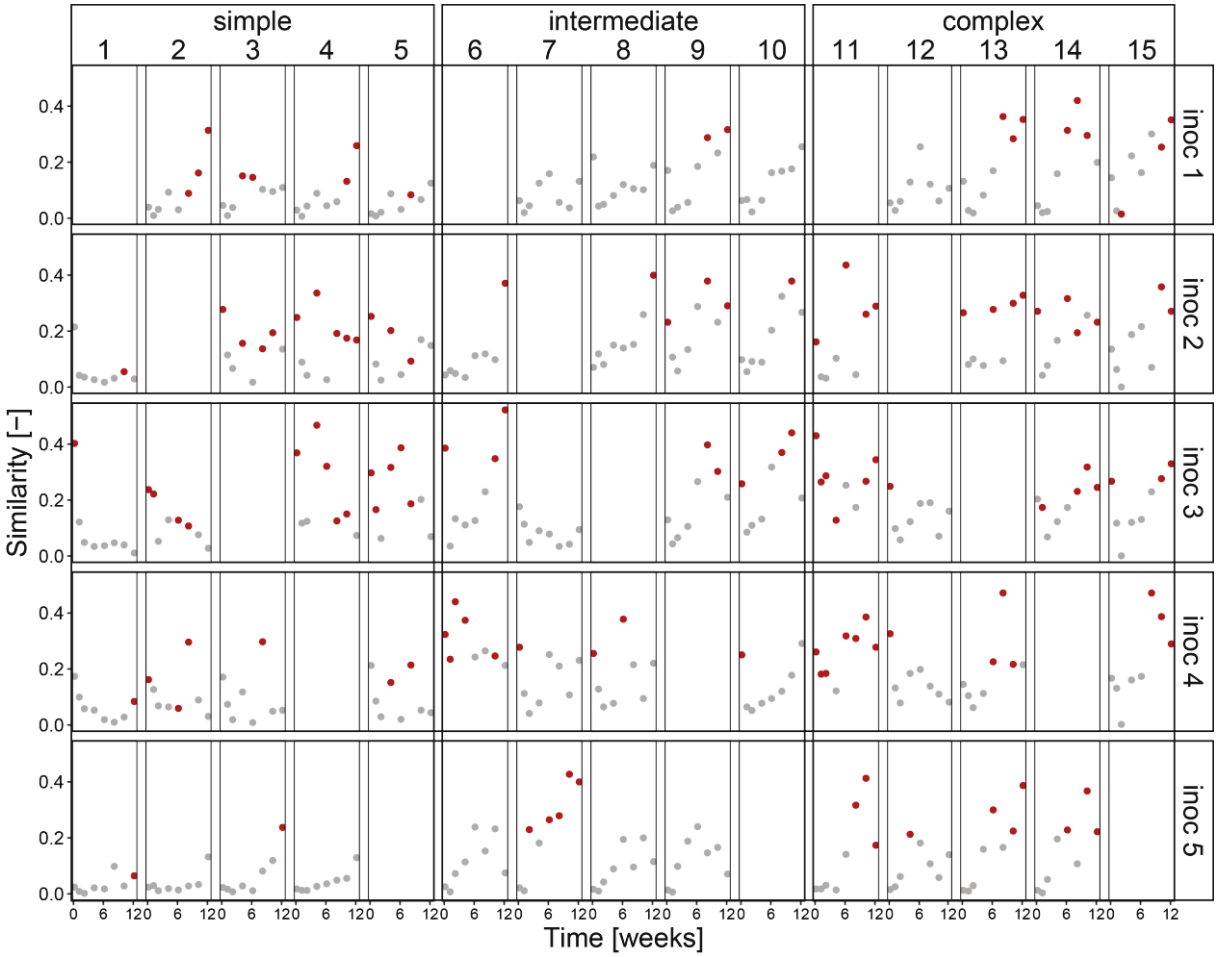


Figure 26 Similarity between mixed bacterial communities (columns) and the respective individual communities derived from the inocula (rows). The similarities are calculated based on the Jaccard distances (1-dissimilarity) and significant results are displayed in red.

For archaeal communities few significant similarities are observed between inocula and mixed communities (Figure 27). Significant similarities during steady state operation are slightly more abundant with complex substrate

(13 times) than with the simple (10 times) and intermediate (8 times) substrate. It appears that also for the Archaea, traces of multiple inocula are most likely found for the complex substrate and least likely for the intermediate substrate. As for the bacterial data, also here, the mixed archaeal community in column 7 is most similar to inoculum 5, while similarities to all other inocula are not significant. Globally, the Archaea of inoculum 5 are the least successful community as a significant similarity to this community under steady state performance is only observed once. Communities of Bacteria and Archaea in inoculum 5 appear to behave similarly, i.e., are unable to coalesce with other communities, but able to outcompete the others under certain conditions. Curiously, also the mixed community in column 12 of the bacterial community stands out in the archaeal data. For both datasets, no significant similarity to any of the individual communities was observed under steady state. As before for the Bacteria, also in the archaeal data, inoculum 2 is most successful when considering the times a significant similarity under steady state is detected. Globally, community assembly of Bacteria and Archaea appears to similar in our experiment. Major differences in the behavior of the individual communities was observed, e.g., between inoculum 2 and 5.

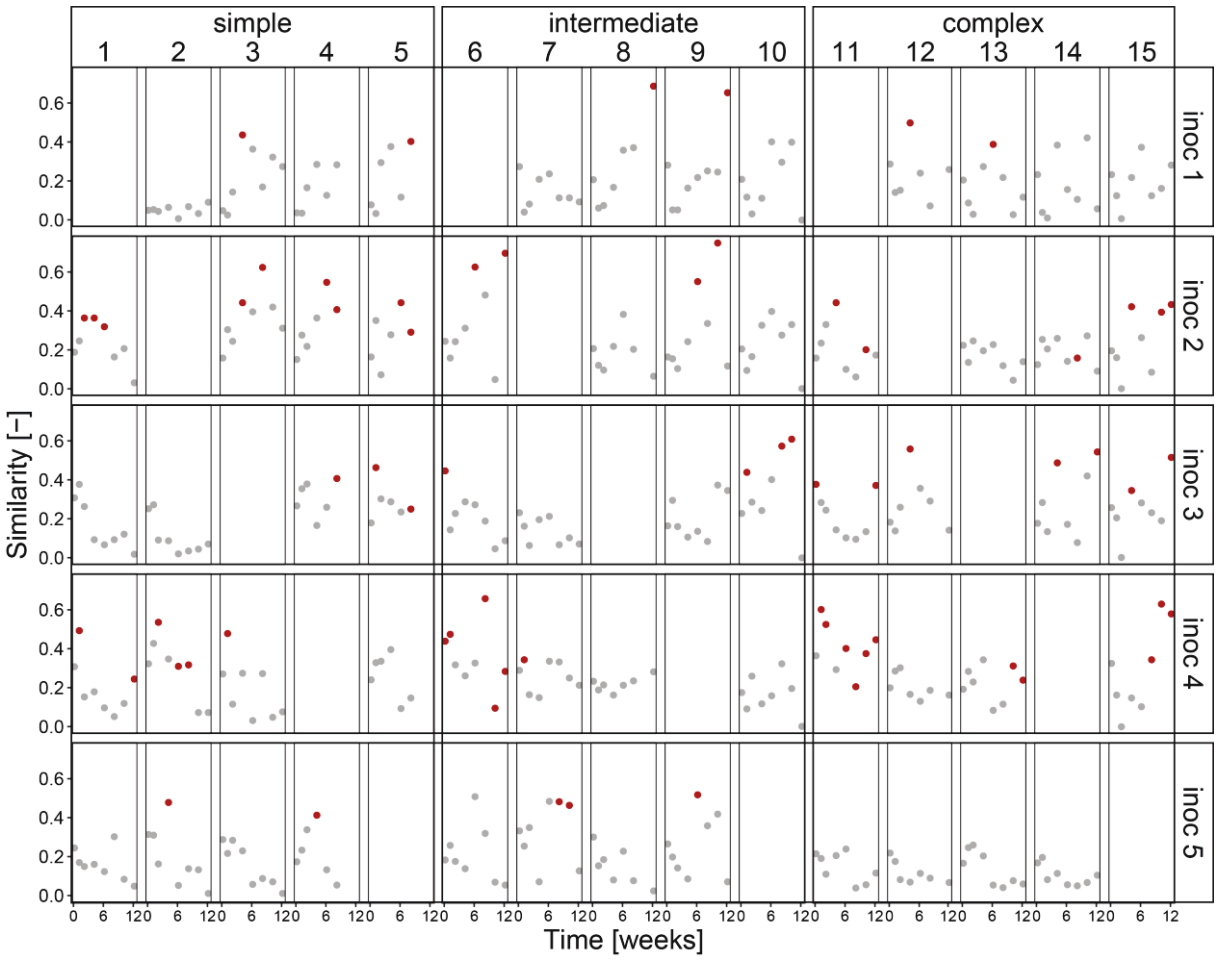


Figure 27 Similarity of archaeal communities (columns) with each used inocula in the different mixed communities (rows) distinguished per feeding substrate. The similarities are calculated based on the Jaccard distances (1-dissimlarity) and significant results are displayed in red.

In sum, mixed communities of Bacteria are more often close to individual communities than mixed communities of Archaea. This result may be linked to the fact that diversity of bacterial communities decrease after inoculation in individual and mixed communities, whereas diversity of Archaea communities increased after inoculation at the contrary to individual communities Figure 23. This increase diversity may have allowed Archaea to develop mixed communities different from those of individuals.

Also, mixed communities are more often similar to individual communities when fed with complex substrate. We hypothesized that a more complex substrate would promote greater diversity. However, we do not confirm this

hypothesis with the previous results and here we can suggest that, on the contrary, the degradation network of the complex substrate is typically borrowed by the different individual communities and this network is found in the mixed communities. Conversely, a simpler substrate can possibly make numerous microorganisms compete each other, as a result as a different development of communities. Unfortunately, this hypothesis cannot be confirmed because the intermediate substrate is not the average of simple and complex substrates.

Finally, some inocula have a higher similarity occurrence in mixed communities as inoculum 2 and in contrast inocula 1 and 5 are less represented in both bacterial and archaeal communities.

## 6.3 Discussion

The objectives of the chapter were to study the performance of anaerobic digestion ecosystems by highlighting the effects of a substrate complexity gradient and community assembly. These parameters were tested by implementing individual (1 single inoculum) and mixed (4 inocula) communities and feeding each condition with three different substrates continuously for 12 weeks. In steady-state, the expected biogas production was reached and the biomass concentration stable; the ecosystems functioning is therefore stable and the conditions comparable.

Biogas production has not been improved with the substrate complexity (Figure 20a). These results differ from those of a previous study, where methane yield was favored with the most complex substrate (Lu et al., 2013). However, in this study, the complex substrate was food waste and led to a higher diversity of archaeal and bacterial communities. Unlike this study the bacterial diversity in the experiment was unchanged with the substrate complexity and the increase in archaeal diversity is transient (Figure 23).

No distinction of biogas production differences between individual and mixed communities was established at first (Figure 21). Singularities of methane yields have emerged with certain mixed communities (Figure 22). Two interpretations were likely to explain the increase of methane yield: either an increase in performance of the mixed community, or a decrease performance of the individual communities (reference). For the mixed community “mix w/o inoc1” a clear improvement in methane production was observed. At contrary, with “mix w/o inoc2” a small ratio was observed and was due to a low production of this mixed community concomitantly with a high reference value. In both cases, the performance of the mixed community was substantial. The choice of inocula is therefore important, as has already been demonstrated for the resistance to the stress (De Vrieze et al., 2014) or for the performance (Koch et al., 2017; Raposo et al., 2011). The property of ecosystems to interact, to form effective interactions with assembled ecosystems have been thus suggested.

By studying the assembly of these communities, individual communities composing mixed communities are leaving their trace in bacterial and archaeal communities (Figure 26 and Figure 27). The coalescence was increased with complex substrate. The use of this substrate may have enabled the network development for substrate degradation and favor the coalescence. These results are in contradiction to the selection of a particular inoculum as it has already been found elsewhere (Chapleur et al., 2014; Sierocinski et al., 2017). The mixed bacterial communities were not selected but resilient and persistent in the mixtures. Moreover, these communities have evolved in the same way over time, reinforcing the idea of solidifying interactions over time. This proposal is emphasized by observations of the historical importance of assemblages on ecosystem functioning in other environments (Fukami, 2015; Rummens et al., 2018). Yet, the method used

to statistically test whether the similarity of a community is significant to a mixed community could not be tested extensively and deserves further analysis. The experiment has nonetheless allowed us to observe the coalescence of a mixed community and additional testing of these interactions should be expanded.

In mixed archaeal communities, increase diversity was observed after inoculation. However, at same level of diversity, individual communities showed stronger or equal performance of ecosystem function as mixed communities. These results differ from previous study where a strong correlation between the diversity and the functioning of communities was observed (Carballa et al., 2011; Venkiteshwaran et al., 2017; Werner et al., 2011). One explanation might be explained with the use of functionally redundant inocula in the experiment. The five inocula had seen relatively little different substrates and 4 out of 5 inocula come from mesophilic solid-state reactors. The functional redundancy of these ecosystems was maybe important and may explain why no complementarity effect was observed in mixed bacterial communities.

Similarly to other studies, the bacterial community structures have been particularly linked to the substrate (Table 11) (Regueiro et al., 2012; Sundberg et al., 2013). *Bacteroidetes* are found mainly in reactors fed with intermediate and complex substrate and only few in reactors fed with simple substrate (Table 12). This Bacteria phylum has been shown to degrade complex sugars by fermentation (Wexler, 2007), yet there are no complex sugars in the simple substrate unlike the other two substrates where sucrose or dextrin are contained for example. Within the *Firmicutes*, the *Clostridia* dominate the bacterial community independent of the substrate. This is not further surprising as *Clostridia* are capable of degrading simple and complex carbohydrates and have been recognized for their metabolic importance into

biofuels and biotechnologies applications (Tracy et al., 2012). In anaerobic digestion studies, members of *Clostridia* class have been shown to be key phylotype and often most abundant (Hao et al., 2016; Nelson et al., 2014; Sundberg et al., 2013; Treu et al., 2016). *Chloroflexi* phylum is a one of the foremost populations in anaerobic digesters (Nelson et al., 2014; Rivière et al., 2009; Sundberg et al., 2013), yet this phylum was not found in abundance in our study (<0.01%). Unlike bacterial communities linked mostly to the substrate, archaeal communities were principally linked according to the initial inoculum (Table 11). The majority order of the Archaea is *Methanobacteriales*, corresponding to hydrogenotrophic methanogens, the predominant metabolic pathway for methanogenesis (Hao et al., 2016; Sundberg et al., 2013). Interestingly, most of the reactors fed with complex substrate have their composition marked by the presence of the order *Thermoplasmatales*, particularly in mixed communities (Additional data Figure 39). Archaea belonging to this order appear in extreme and diverse ecosystems (Adam et al., 2017) as well as in anaerobic digesters in the presence of hydrogenotrophic methanogens (Chouari et al., 2015).

## 6.4 Conclusion

Individual communities used for mixtures are found in the mixed communities after 2 months of continuous cultivation. There was coalescence and incorporation of elements from several original communities to form a new assembly, contrary to the idea of selection that was first proposed. Further work would be interesting to test this coalescence effect with diverse ecosystems.

## 6.5 Additional data

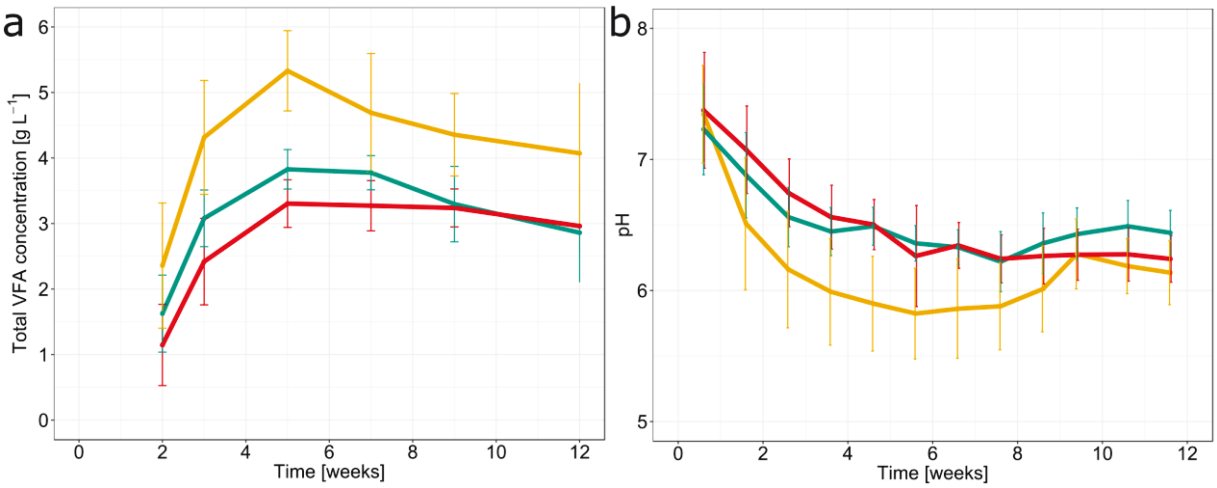


Figure 28 Monitoring along the experiment A) Volatile fatty acids concentrations B) pH. Each point is calculated from ten values. Colors stand for substrate used: yellow line for simple substrate, green line for intermediate substrate, red line for complex substrate

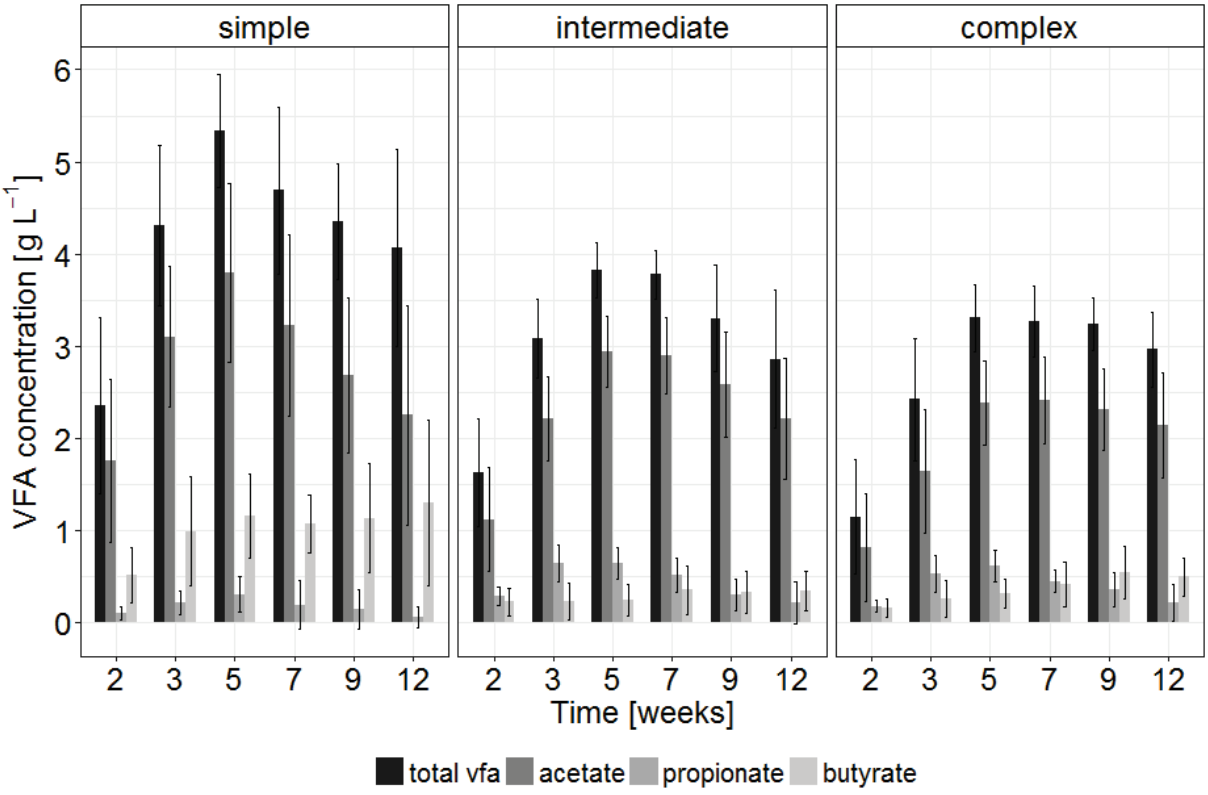


Figure 29 Volatile fatty acids concentrations monitoring along the experiment as a function of substrate. Each mean is calculated from ten values

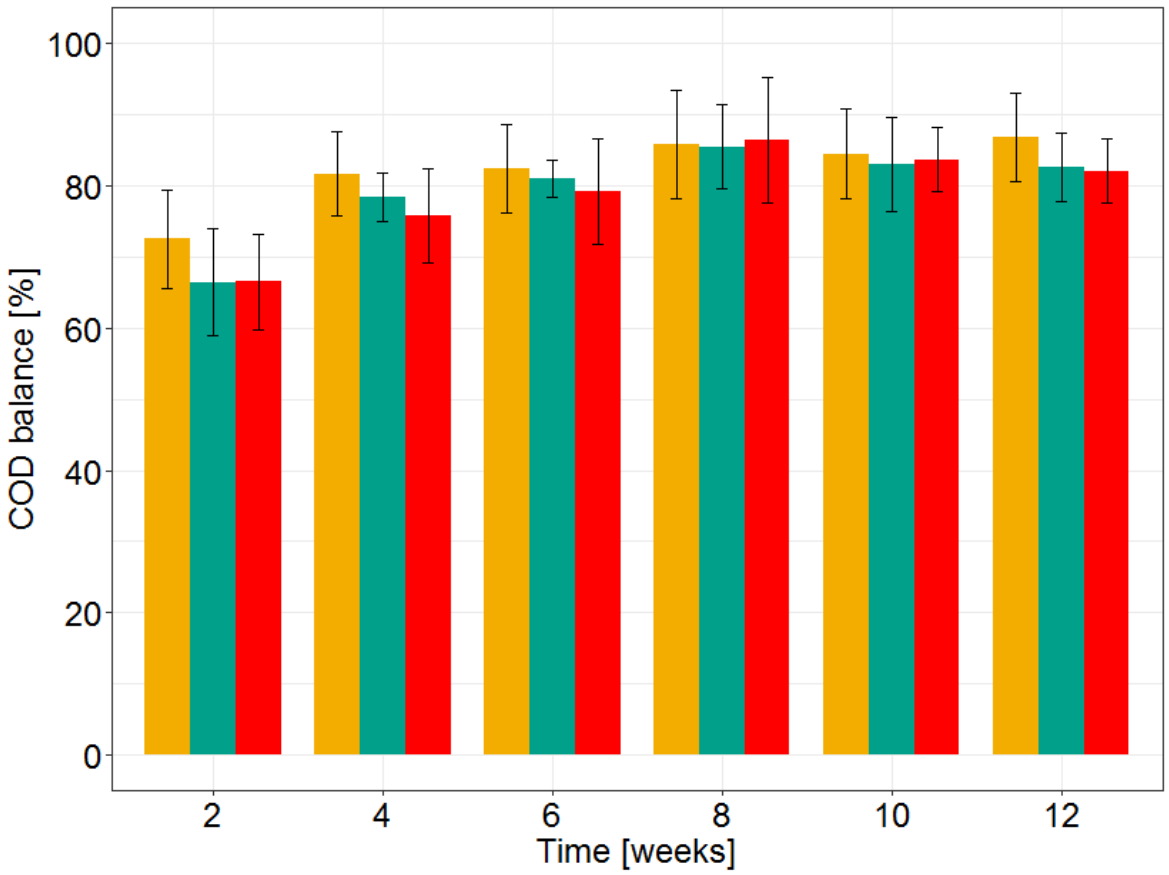


Figure 30 Percentage of COD balance calculated after two weeks. Each bar represent ten reactors fed with different substrates : yellow for simple substrate, green for intermediate substrate and red fo compex substrate

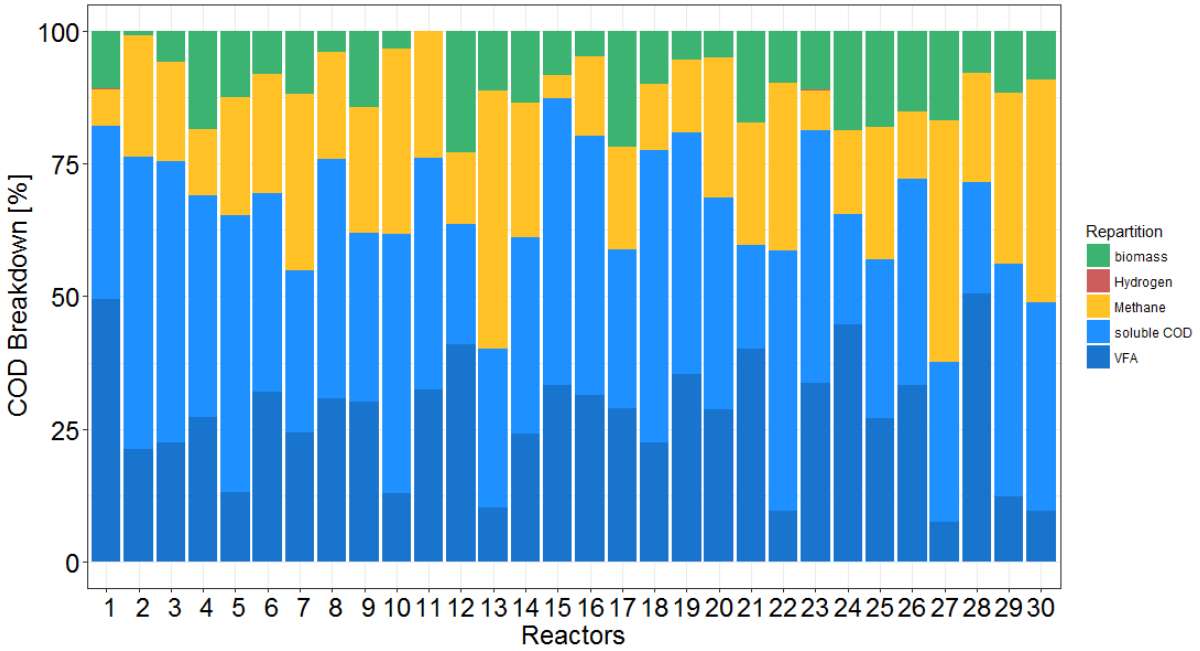


Figure 31 Percentage of COD breakdown in hydraulic retention time 1. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA

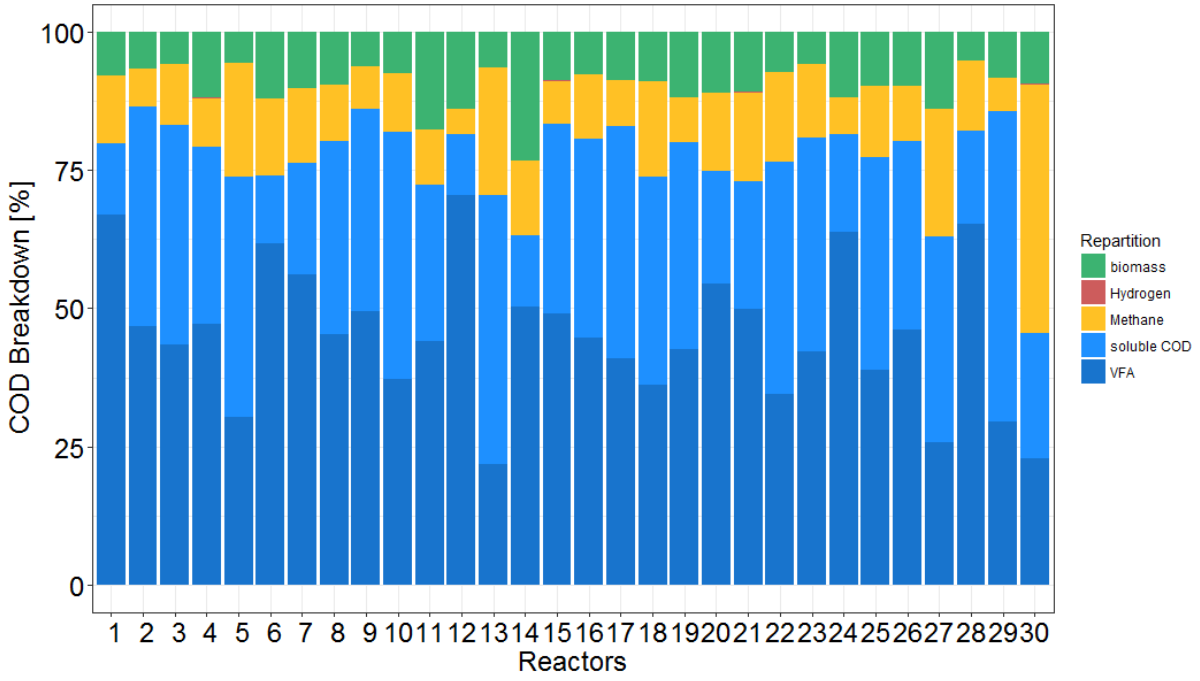


Figure 32 Percentage of COD breakdown in hydraulic retention time 2. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA

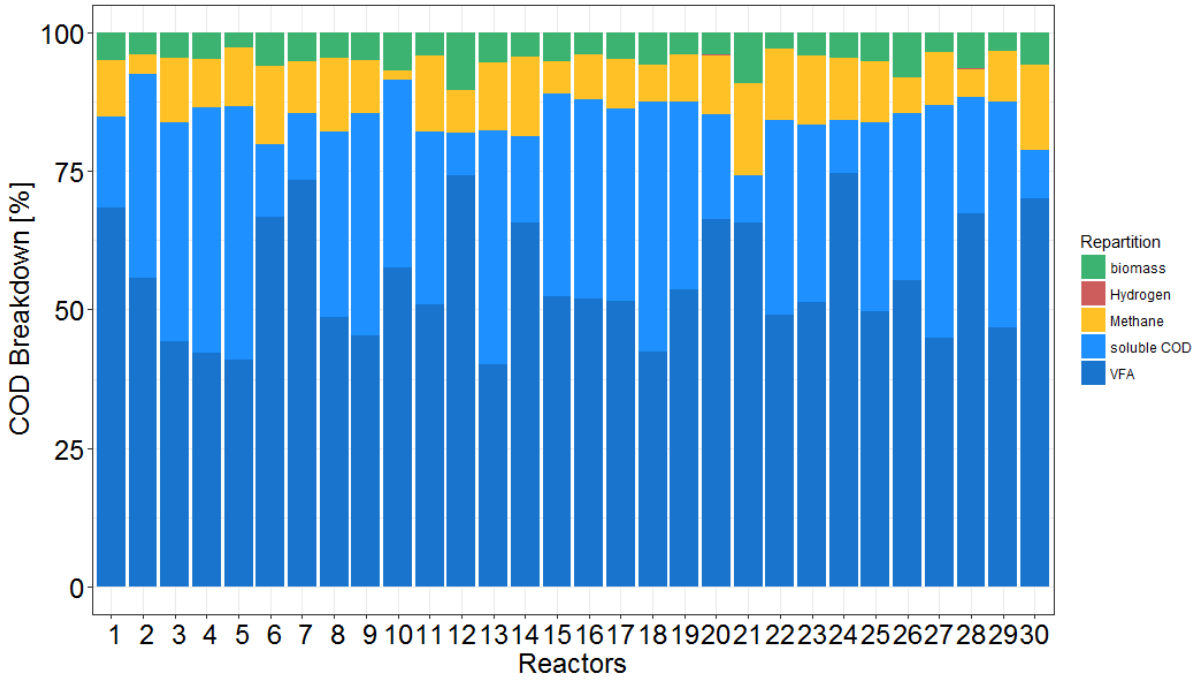


Figure 33 Percentage of COD breakdown in hydraulic retention time 3. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA

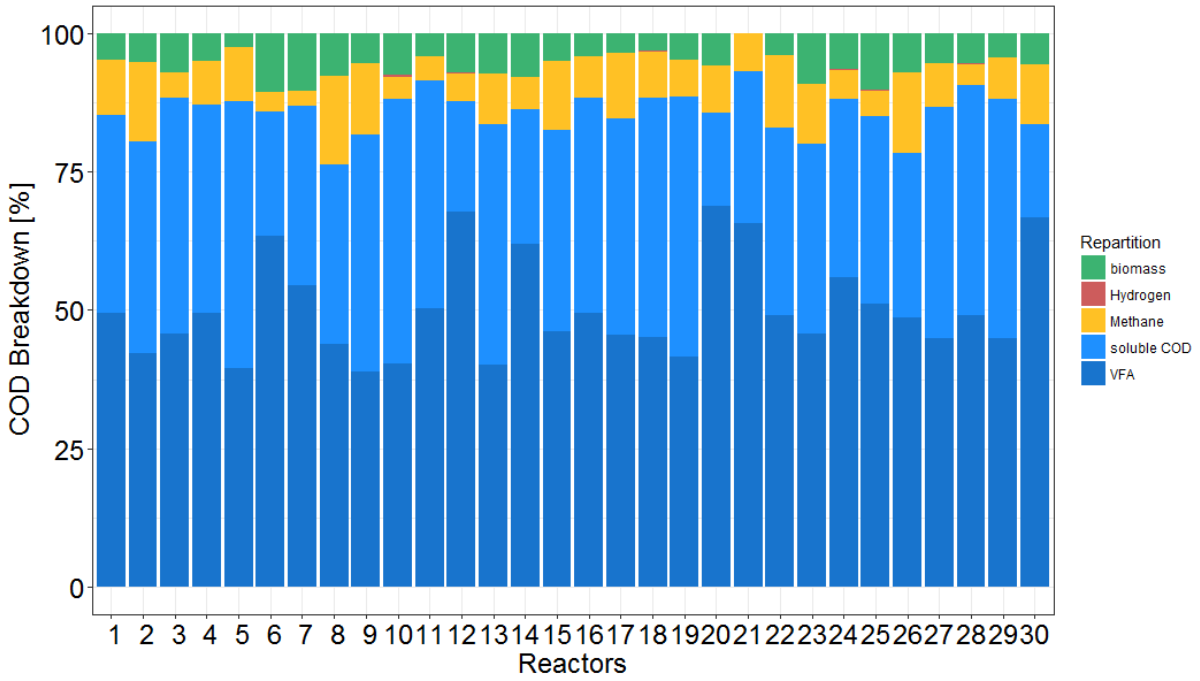


Figure 34 Percentage of COD breakdown in hydraulic retention time 4. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA

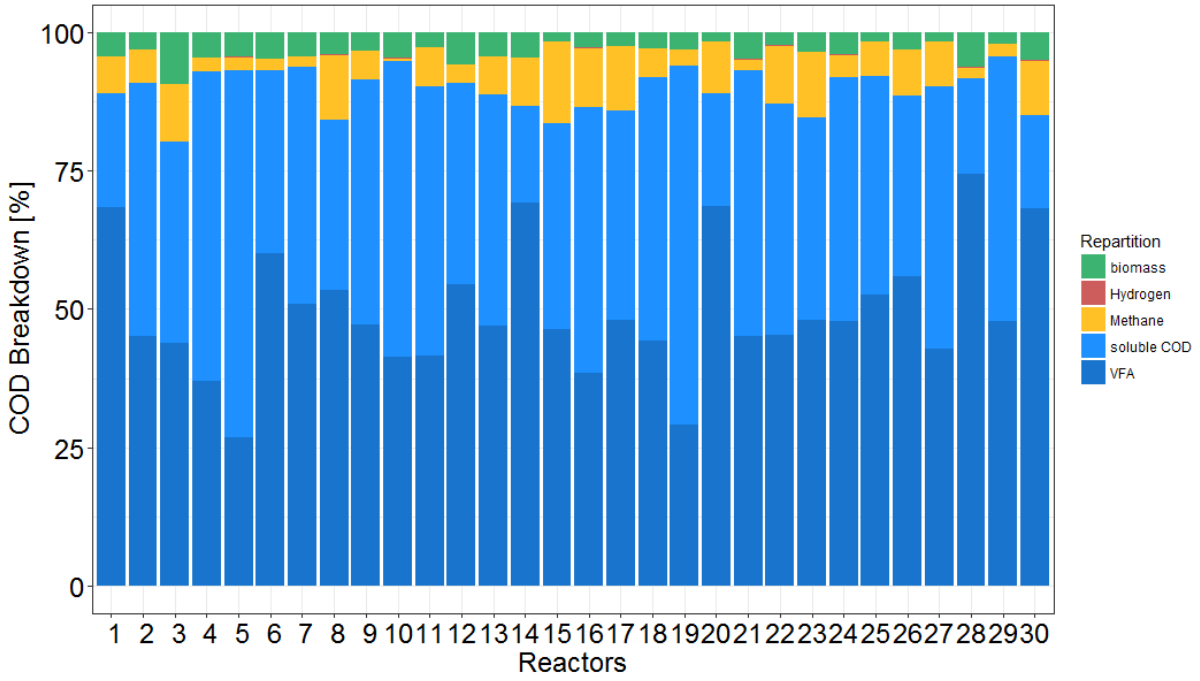


Figure 35 Percentage of COD breakdown in hydraulic retention time 5. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA

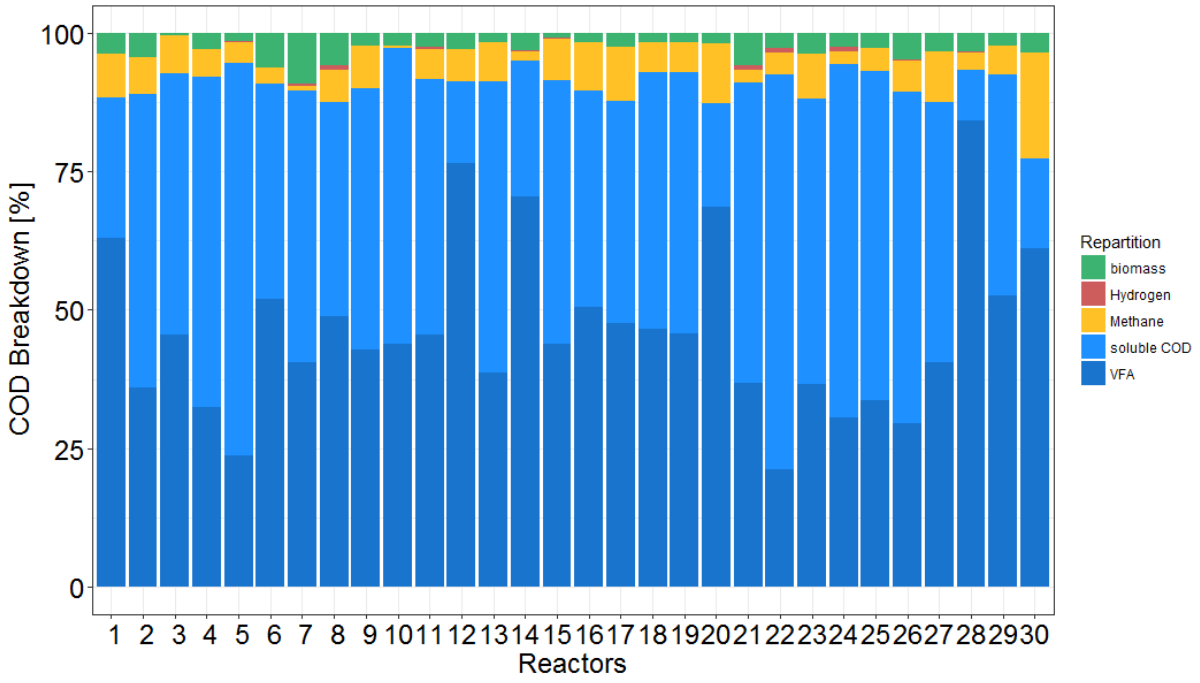


Figure 36 Percentage of COD breakdown in hydraulic retention time 6. Biomass is represented in green, hydrogen in red, methane in yellow, soluble COD in blue with darker blue for VFA

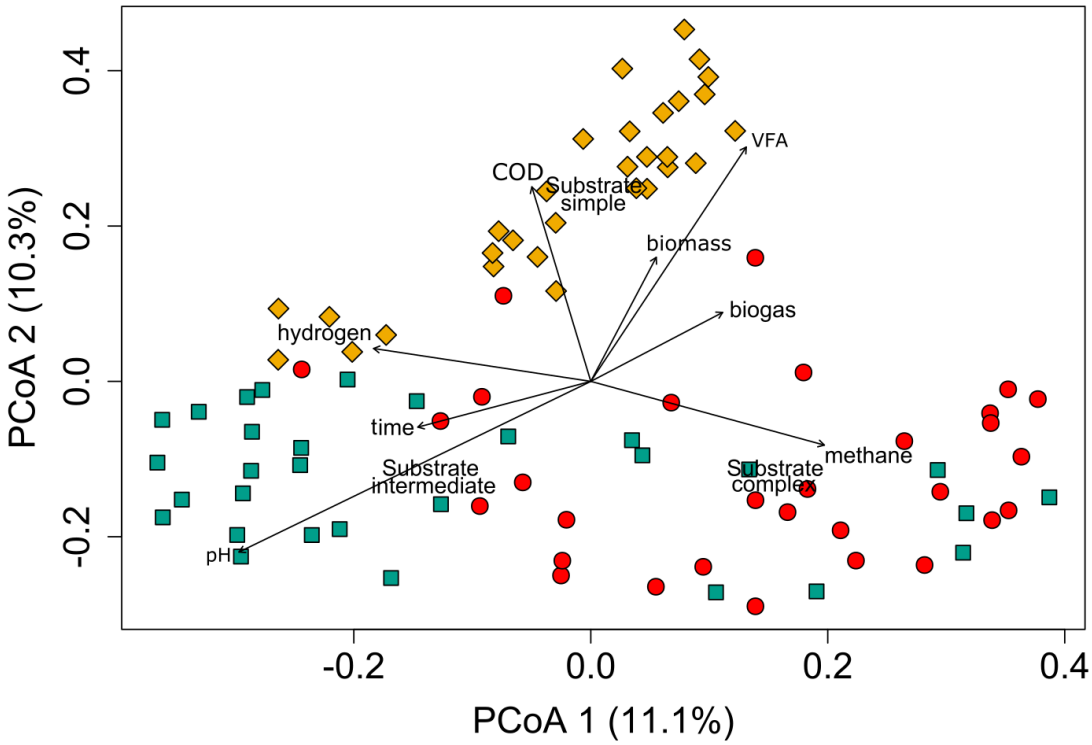


Figure 37 Environmental variables represented on bacterial data ordinations made with Jaccard distance index for the last six weeks of the experiment. The different substrate use is highlighted by the different forms and colors: in yellow diamonds the simple substrate, in green squares the intermediate substrate and in red circles the complex substrate

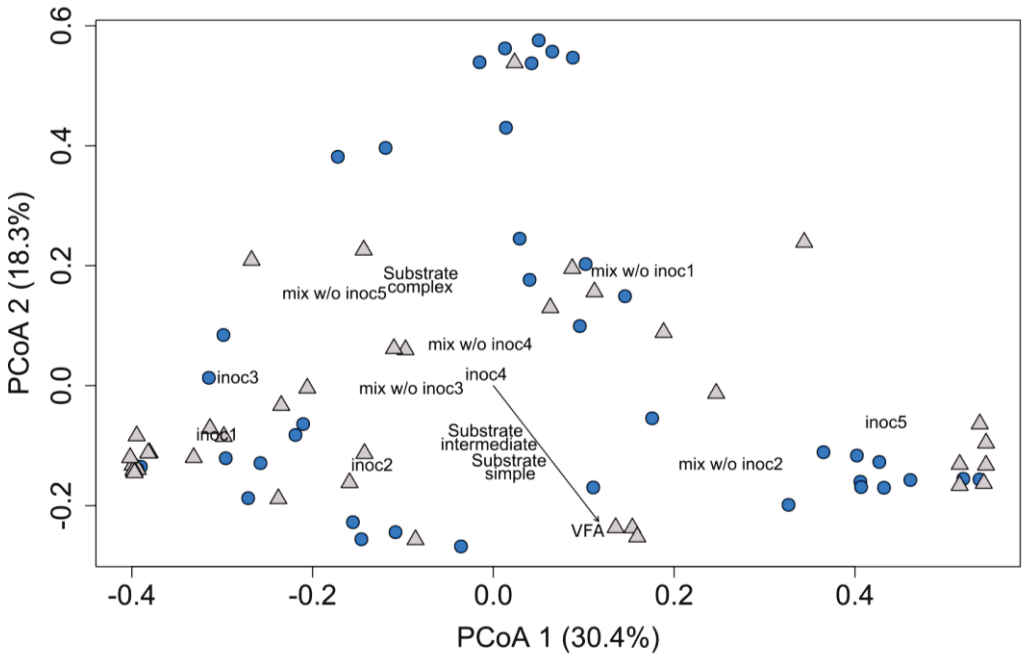


Figure 38 Environmental variables (p-value <0.01) represented on Archaea data ordinations made with Jaccard distance index for the last six weeks of the experiment. The shape and color of the plot represent the community assembly: grey triangle for the individual communities and blue circle for the mixed communities

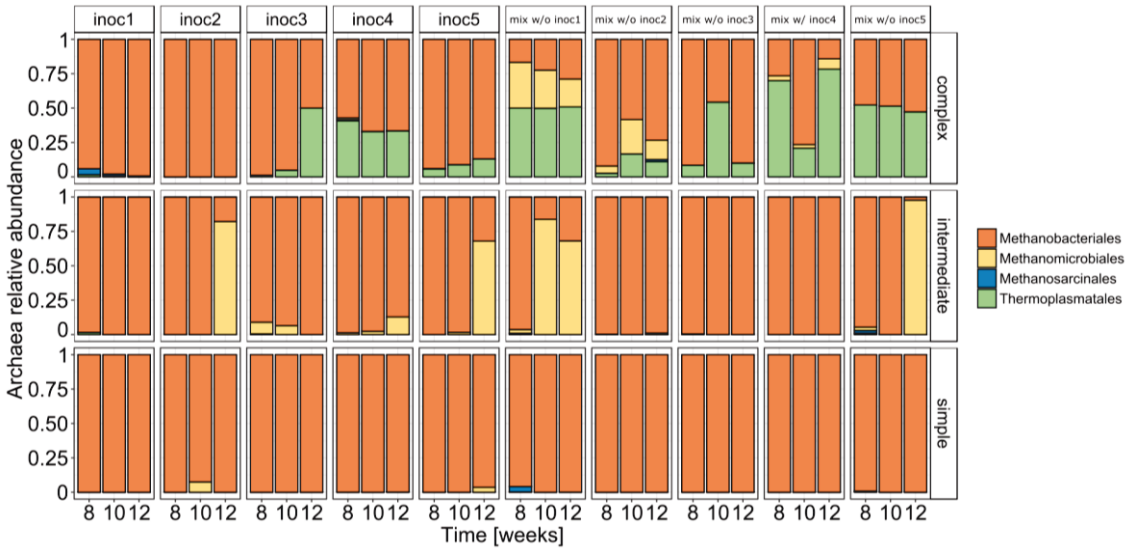


Figure 39 Different Archaea relative abundances Order for each inoculum per substrate in steady state conditions (weeks 8 to 12)

# Chapter 7

# General conclusion

# 7 General conclusion

Coalescence of microbial communities, for example by mixing various inocula at the startup of a reactor, or by augmenting an operating reactor with a microbial community may be one way of engineering a target community with desirable properties, e.g., performance of ecosystem function. We are far from formulating generalized laws of microbial community assembly. However, first important steps are being made in this thesis and in the literature (Rummens et al., 2018; Sierocinski et al., 2017).

One central need towards the goal was the development of a continuously operating reactor system that allows testing of various conditions at the same time. The development of the LAMACs and the ability to multiplex ecological experiments is a milestone as it enabled us to overcome a serious technical bottleneck, i.e., the conservation and revitalization of inocula to ensure experimental reproducibility which has been shown to be problematic in anaerobic digestion studies (Hagen et al., 2015; Kerckhof et al., 2014). Since the end of the experimental work of this thesis, the LAMACs has been already been requested for various experiments in microbial ecology in our laboratory. The flexibility of the system has proven a valuable feature as it is currently being used for the aerobic screening and enrichment of inocula for the degradation of lignocellulosic compounds. For this experiment, our suggestion of connecting reactors in series was implemented. In another experiment in preparation, the use of the LAMACs as photobioreactor will be tested. A LED lighting device in between the heating block and the mixing plate has already been fitted to the system. The experimental system so far is a success and fulfills our ambitious expectations.

As methane is the performance proxy for the ecosystem functioning in anaerobic digestion, the direct quantification would have been a significant improvement for the LAMACs. A simple method for removing carbon dioxide from biogas has been tested, involving a silica capsule absorbing $CO_2$ connected in series with the pressure sensor. However, positioning the capsule above the reactor increases the partial pressure, which raises the gases solubility in the medium and alters the gas balance. This solution was therefore not adapted and removed to the system. Another major limitation of the LAMACs is the impossibility of digesting solid wastes due to the small peristaltic pumps and thin tubes.

In our application of the system as anaerobic digesters, we were able to describe coalescence of microbial communities as a function of substrate complexity. We demonstrated that substrate complexity favored coalescence, even though no detectable difference in performance of ecosystem function, here methane production, was observed. For Bacteria and Archaea, the effect of mixing was different when considering the resulting microbial diversity and correlations to environmental or operational parameters. Bacterial and archaeal communities appear to behave differently possibly linked to the differing overall community sizes. However, community assembly for the two domains, i.e., the observed degree of coalescence, seems similar. This may be a consequence of the tight syntrophic links between the two domains in the anaerobic foodweb, in which Archaea and Bacteria act as one interacting unit.

# Chapter 8

# Perspectives

# 8 Perspectives

Technically, we are currently aware of two major limitations of the LAMACs. We were forced to use soluble substrates in our experiments because of the difficulty of pumping and homogeneously sampling particulate substrates. The use of particulate substrates is desirable for applications in anaerobic digestion. Also in the above mentioned context of inocula screening for lignolytic activity, the possibility of using a particulate substrate is obviously interesting. Extending the LAMACs operation to particulate substrate would be a highly desirable development and should be considered for the future but remains a challenge. Much less a challenge is overcoming the limitation of using biogas as performance proxy even though we demonstrated that methane production is more powerful. Measuring methane online using for example a multiplexed micro-GC, already existing in the laboratory may be feasible.

We were able to demonstrate that reactors produced methane at similar rates irrespective of substrate addition or community assembly. Functional complementarity was not observed in *Bacteria* communities in terms of performance, i.e., improved performance after coalescence. This leads to the pertinent question in what measurable aspect the coalesced communities differ from the individual counterparts. One often hypothesized feature could be a different behavior of the reactors when facing environmental stress, e.g., changes in loading conditions, temperature or pH shocks. We have currently only limited knowledge of the stress responses of the reactors. Some preliminary experiments were presented in Figure 15. Stress responses of newly assembled communities certainly deserve thorough testing. One possible experiment can be easily carried out with the LAMACs as already indicated in chapter 4 where substrate type and organic loading

rates are periodically changed, as shown in Figure 15. In fact, a significant number of studies have reported that digesters overcoming minor stresses were better able to adapt to larger disturbances later on (De Vrieze et al., 2013; Laperrière et al., 2017).

The application of a combinatorial model may help us assessing beneficial and unfavorable communities in a mixture in terms of a defined functional parameters (Jaillard et al., 2014). The individual beneficial properties of the communities in mixtures are currently under test with this model.

Another approaches based on species are used to describe the active players in the ecosystems; several studies have established a functional core, i.e. active taxa found in several communities correlated to the ecosystem functioning (Nelson et al., 2011; Solli et al., 2014; Venkiteshwaran et al., 2017). One particular study described a direct relationship between the most abundant species retrieved from anaerobic digesters and the digester performance with a quantitative relationship (Venkiteshwaran et al., 2017). These methods focusing on the microbial core aim to improve digester functioning prediction as statistic models of anaerobic digestion control as ADM1 do not take microbial communities into account (Batstone et al., 2002). The interpretation of the core is though limited by the methods of determining the number of shared OTUs: the sequence identity degree and the taxonomic-level (Shade and Handelsman, 2012).

The effects of bioaugmentation are sought to provide desired functionality, such as probiotics for their health benefit or in the long-term investment as the addition of propionate-degrading consortium to prevent from propionate accumulation in anaerobic digester (Tale et al., 2011). However, bioaugmentation experiments are not always conclusive and some communities do not provide the desired functionality (Bouchez et al., 2000). In the community assembly experiment, we have shown that communities

have the capacity to establish themselves. The inoculation of more different ecosystems or ecosystems with specialized functions could possibly imprint the community and increase the ecosystem functionality as a bioaugmentation event. Explaining and testing why some communities lead functionality or not can therefore be tested with LAMACs and methanogenic microbial ecosystems providing a measure of performance.

In the community assembly experiment, the coalescence of communities did not increase the functionality and could be explained by the fact that they come from too similar conditions: mesophilic solid-state digesters and operated for more than a year. We can imagine that these communities are functionally stable and might have developed strong interactions. The strength of these interactions and whether one 'young' community can imprint a 'mature' community can be tested with the LAMACs. The addition of an individual community in a mixture where it is not initially present can be studied at several stages of development of this mixed community. Such research question is a current topic particularly with complex communities, which has been studied in environments as freshwater bacterioplankton community (Rummens et al., 2018). Here also the introduction of a new community seems to colonize the structure. However, the mechanism of the progressive community construction and imprint has not been understood. A similar research question can be assessed in continuous anaerobic digesters with the LAMACs. The advantage of such a system is that the continuous configuration makes it possible to perform a natural washout of the non-growing microorganism in the community. For example, 6 communities and their mixtures of 5 communities can be tested in five replicates to which is added the individual community not presents in the mixture of different degrees of development, as simplified in Figure 40. In this experience, adding an external community to different levels of

development of a mature community would test the strength of interactions within that community. This experiment can used a bacterioplankton community in LAMACs, as the addition of LEDs can be added as already did in the laboratory. Yet, with methanogenic communities, a performance measure can be assessed and would help to understand the functioning of the ecosystems.

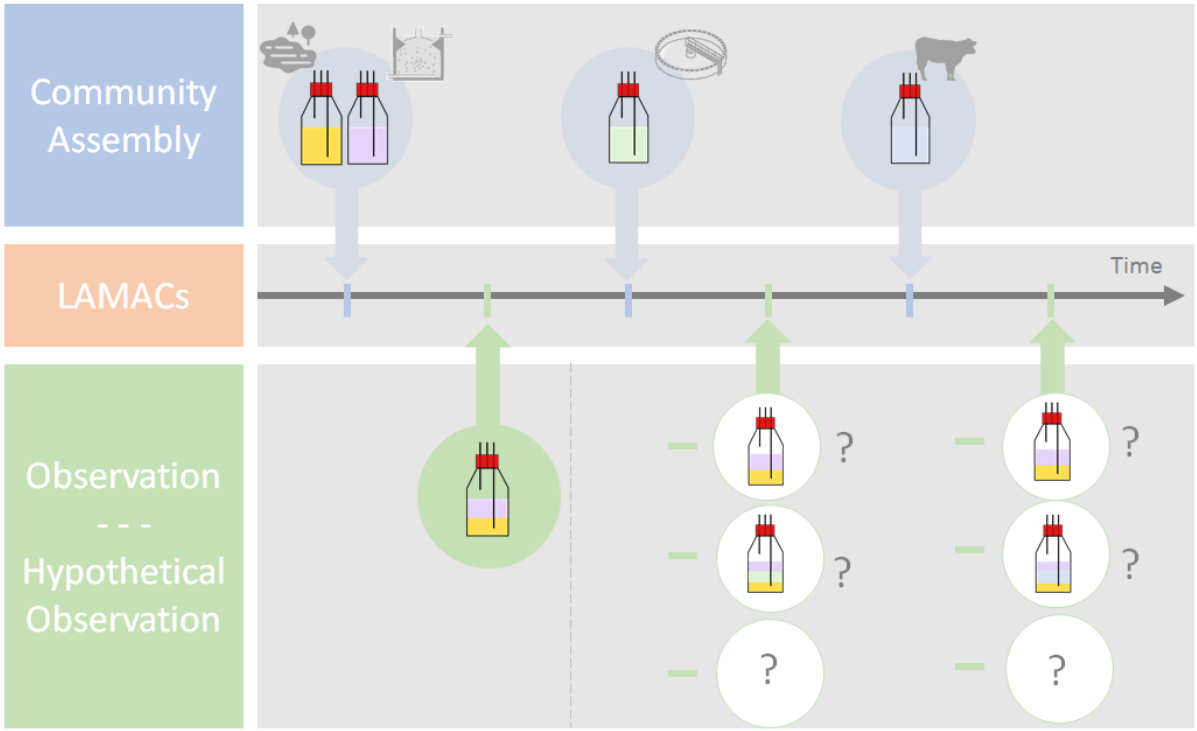


Figure 40 Experiment testing the strength of different community assembly with different communities coming from different sources and the addition of community at different degrees of development.

# Chapter 9

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