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Anaerobic digestion of biodegradable plastics : performances and microbial diversity

Guillaume Cazaudehore

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THÈSE

UNIVERSITE DE PAU ET DES PAYS DE L'ADOUR
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(ED 211 SEA)

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par **Guillaume CAZAUDEHORE**

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**Spécialités : Génie des procédés, Physiologie et Biologie des
organismes et des populations-interactions**

Méthanisation des plastiques biodégradables : performances et diversité microbienne

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A ma grand-mère, Odette

Abstract

Growing concern regarding non-biodegradable plastics and the impact of these materials on the environment has promoted interest in biodegradable plastics. Biodegradable plastics offer additional waste management options (e.g., anaerobic digestion or composting) over conventional plastics. However, the treatment of biodegradable plastics under anaerobic digestion is only in its infancy. Therefore, the aim of this thesis was to investigate the fate of biodegradable plastics in anaerobic digestion systems and the microorganisms involved in the plastic conversion to methane.

For this purpose, batch anaerobic digestion experiments were performed on the main biodegradable polymers and on three commercial blends of biodegradable polymer, under both mesophilic and thermophilic conditions. Only Poly(3-hydroxybutyrate) (PHB) and Thermoplastic starch (TPS) exhibited rapid (25-50 days) and important (57-80.3% and 80.2-82.6%, respectively) conversion to methane under both mesophilic and thermophilic condition. Methane production rates from poly(lactic acid) (PLA) was very low under mesophilic condition, to such an extent that 500 days were required to reach the ultimate methane production, corresponding to a PLA conversion to methane of 74.7-80.3%. Methane production rate from PLA was greatly enhanced under thermophilic condition since only 60 to 100 days were required to reach the same ultimate methane production. Lactate-utilizing bacteria such as *Tepidimicrobium*, *Moorella* and *Tepidanaerobacter* were revealed to be important during the thermophilic digestion of PLA. Similarly, starch-degrading bacteria (from *Clostridium* genus) were highlighted during TPS digestion at 38 °C and 58°C. Previously known PHB degraders (i.e., *Enterobacter*, *Ilyobacter delafieldii* and *Cupriavidus*) were observed during mesophilic and thermophilic AD of PHB.

The low biodegradation rate of most of the biodegradable plastics in mesophilic anaerobic digesters is a major hindrance to their introduction at industrial scale. Thermal (at 120 or 150 °C) and thermo-alkaline (at 70°C or 90 °C with calcium hydroxide addition) pretreatments were successfully implemented on PLA. These strategies were tested on PLA, which is one of the main biodegradable polymer, accounting for 25% of the biodegradable plastic production. PLA pretreated with these treatments, achieved biodegradation yield of 73% after 15-20 days; a similar biodegradation yield was obtained after 500 days for untreated PLA.

PHB and PLA are among the most studied polymer to replace conventional plastics. Finally, the stability and performances of the co-digestion of these plastics (with and without PLA pretreatment) with food wastes fed semi-continuously under mesophilic conditions was investigated. The addition of biodegradable plastics resulted in a more stable process (in comparison with stand-alone biowastes reactor) and no negative effects could be detected. PHB was estimated to be fully biodegraded in the reactors. By contrast, PLA was accumulating in the reactor, and an average biodegradation of 47.6% was estimated during the third hydraulic retention time. Thermo-alkaline pretreatment of PLA improved the biodegradation yield of PLA to 77.5%. The identification of specific microorganisms implicated in the biodegradable plastic degradation was complicated; the majority of the microorganisms correlated with the methane production from reactors co-digesting PLA and PHB were implicated in the anaerobic digestion of the biowaste, which can be explained by the low proportion of biodegradable plastics introduced.

Résumé

L'impact environnemental des plastiques conventionnels conduit à un développement et à un déploiement de matériaux alternatifs comme les plastiques biodégradables. Ces plastiques biodégradables ont pour avantage, par rapport aux plastiques conventionnels, de pouvoir être traités en filière de recyclage organique (méthanisation ou compostage). Cependant, l'étude de la fin de vie des plastiques biodégradables en méthanisation en est encore à ses débuts. Par conséquent, l'objectif de cette thèse est d'étudier le devenir de ces matériaux en digestion anaérobie (DA) mésophile et thermophile, leurs performances de biodégradation et les microorganismes qui sont impliqués dans leur biodégradation.

Des expérimentations de DA en mode batch ont été réalisées sur les principaux polymères biodégradables (PHB, PLA, PCL, PBAT, TPS, PBS) et sur trois mélanges commerciaux, en conditions mésophiles et thermophiles. Seul le poly(3-hydroxybutyrate) (PHB) et l'amidon thermoplastique (TPS) ont présenté une conversion en méthane rapide (25-50 jours) et importante (57-80,3% et 80,2-82,6%, respectivement). Des bactéries précédemment identifiées comme des dégradeurs de PHB (i.e., *Enterobacter*, *Ilyobacter delafieldii* et *Cupriavidus*) ont été observées pendant la dégradation mésophile et thermophile du PHB. De la même manière, des bactéries dégradant l'amidon (du genre *Clostridium*) ont été retrouvées lors de la dégradation thermophile et mésophile du TPS. La cinétique de biodégradation du PLA était très lente en conditions mésophiles (500 jours pour une biodégradation du PLA de 74,7 à 80,3%). La condition thermophile était beaucoup plus favorable (60 à 100 jours pour la même biodégradation). Les bactéries consommatrices de lactate, comme *Tepidimicrobium*, *Moorella* et *Tepidanaerobacter* ont été mises en évidence durant la dégradation thermophile du PLA. La faible cinétique de biodégradation de la plupart des plastiques biodégradables dans les digesteurs anaérobies mésophiles est un obstacle majeur à leur introduction à l'échelle industrielle. Des prétraitements thermiques (120 ou 150 °C) et thermo-alcalins (70 °C ou 90 °C avec ajout d'hydroxyde de calcium) ont été mis en œuvre avec succès sur le PLA qui représente 25% de la production de plastique biodégradable. Ces traitements permettaient d'atteindre un rendement de biodégradation de 73% après 15-20 jours.

La stabilité et les performances de la co-digestion du PHB et du PLA (avec et sans prétraitement) avec des biodéchets en conditions mésophiles ont ensuite été validés à l'échelle pilote semi-continu afin d'être plus représentatif de la réalité industrielle. L'ajout de plastiques biodégradables a donné lieu à un processus plus stable par rapport à la condition biodéchets seul et aucun effet négatif n'a pu être détecté. Une biodégradation complète du PHB a été mesurée alors que le PLA s'est accumulé dans le réacteur, et une biodégradation moyenne de 47,6 % a été estimée pendant le troisième temps de rétention hydraulique. Le prétraitement thermo-alcalin du PLA a amélioré le rendement de biodégradation à 77,5%.

Enfin, une zone d'ombre autour de la qualité et de l'innocuité des digestats ayant traités des plastiques biodégradables subsiste, celle-ci devra être impérativement levée dans un avenir proche.

Résumé étendu

L'utilisation excessive des plastiques conventionnels, la mauvaise gestion de leur fin de vie ainsi que leur faible biodégradabilité a engendré une forte accumulation de ceux-ci dans l'environnement. Ces plastiques s'accumulant dans l'environnement sont néfastes à différents égards. Ils peuvent être consommés tel quels par les organismes causant des occlusions intestinales ou s'emmêler autour des organismes et ainsi provoquer leur mort. Ils peuvent également se fragmenter et se transformer en microplastiques pouvant affecter les écosystèmes terrestres et marins et s'accumuler dans la chaîne alimentaire. Les plastiques biodégradables ont été développés comme une alternative aux plastiques conventionnels qui serait plus respectueuse de l'environnement. Les polymères communément qualifiés de biodégradables le sont à minima en condition de compostage industriel, mais ne le sont pas forcément dans d'autres milieux. L'essor de la méthanisation au niveau Européen ainsi que la mise en place de la collecte sélective des biodéchets à l'horizon 2024 ouvre de nouvelles perspectives pour les flux de biodéchets et plastiques biodégradables. Si la biodégradabilité des plastiques biodégradables en compostage industrielle est bien renseignée, le traitement des plastiques biodégradables par voie de la digestion anaérobie et la production de méthane en est encore à ses balbutiements. Par conséquent, l'objectif de cette thèse a été d'étudier les performances de dégradation des plastiques biodégradables en méthanisation et les microorganismes qui sont impliqués. Dans un premier temps, une étude bibliographique sur la fin de vie des plastiques biodégradables en méthanisation mettant en avant l'état de l'art et les verrous a été réalisée.

Ensuite une première étude scientifique a visé à déterminer l'influence du ratio Inoculum / Substrat utilisé pour évaluer la biodégradabilité des plastiques biodégradables en test BMP (Biochemical Methane Potential). Cette étude a été réalisée sur deux polymères, l'acide poly(lactique) (PLA) et le poly(3-hydroxybutyrate) (PHB). Une augmentation du rapport Inoculum/Substrat (IS) était associée à une cinétique de biodégradation améliorée et à une augmentation de la variabilité. Au contraire, des IS faibles étaient associés à un risque d'inhibition de la production de méthane (accumulation d'acides volatiles). Les données obtenues lors de ces tests ont permis de déterminer le ratio idéal (I/S ratio = 2,85 en masse de matière organique) qui

a été appliqué par la suite à l'ensemble des essais en BMP. Cette étude donne aussi des indications importantes en vue de l'amélioration du cadre normatif existant sur la biodégradabilité des plastiques en méthanisation.

Dans un deuxième temps, les principaux polymères biodégradables, à savoir l'acide poly(lactique) (PLA), le poly(3-hydroxybutyrate) (PHB), l'amidon thermoplastique (TPS), Poly(ϵ -caprolactone) (PCL), le poly(butylène adipate/terephthalate) (PBAT) et le poly(butylène succinate) (PBS) ont été en mono-digestion en BMP en condition mésophile (38°C) et thermophile (58°C). De la même manière, la biodégradation en méthanisation de trois mélanges commerciaux de plastiques biodégradables a été suivie en utilisant ce même protocole. Seuls le PHB et le TPS présentaient une conversion en méthane rapide (25-50 jours) et importante (57-80,3% et 80,2-82,6%, respectivement) en conditions mésophiles et thermophiles.

Les cinétiques de biodégradation du PLA et du PCL étaient très lentes en condition mésophile, à tel point que 500 jours ont été nécessaires pour atteindre la production maximale de méthane. Cette dernière correspond à 49,4% de biodégradation pour le PCL et 74,7 à 80,3% pour le PLA mais le temps nécessaire rend leur utilisation incompatible en méthaniseur industriel. La condition thermophile était bien plus favorable à la biodégradation du PLA ; seulement 60 à 100 jours étaient nécessaires pour atteindre le même niveau de biodégradation. L'amélioration de la cinétique à une température plus élevée pourrait être en partie attribuée à la température de transition vitreuse du PLA (environ 60 °C) proche de la température en thermophilie (58°C). Cette température favorise l'hydrolyse chimique et facilite l'accès aux microorganismes et aux enzymes. Au contraire, la digestion anaérobie du PCL était nulle en conditions thermophiles. Cette observation n'était pas en accord avec les données de la littérature. Des investigations supplémentaires sont nécessaires pour comprendre l'origine de cette observation et les mécanismes impliqués. Le PBAT et le PBS qui subissent une biodégradation complète ou presque dans une période relativement courte en condition de compostage industriel ont montré une biodégradation mineure ou nulle en conditions de méthanisation, quelle que soit la température envisagée et même sur une période de 500 jours. Ceci souligne le besoin critique de spécifier l'environnement dans lequel un plastique est biodégradable et de développer à l'avenir un cadre normatif.

Une meilleure connaissance des microorganismes impliqués dans la digestion anaérobie des plastiques biodégradables pourrait ouvrir la voie à l'utilisation de techniques de bioaugmentation ou de biostimulation qui permettraient d'améliorer la biodégradation. Les micro-organismes impliqués dans le processus ont été étudiés en utilisant le séquençage de l'ARNr 16S. Malheureusement, il était compliqué voire impossible d'identifier les micro-organismes spécifiques de la biodégradation d'un polymère lorsque le taux de biodégradation était faible. En revanche, les bactéries utilisant le lactate, comme *Tepidimicrobium sp.*, *Moorella sp.* et *Tepidanaerobacter sp.*, se sont révélées importantes lors de la digestion thermophile du PLA. De même, les bactéries dégradant l'amidon (du genre *Clostridium*) ont été mises en évidence pendant la digestion du TPS en condition de thermophilie et de mésophilie. Des bactéries connues pour dégrader les PHB, (*Enterobacter sp.*, *Ilyobacter delafieldii* et *Cupriavidus sp.*) ont quant à elles été observées pendant la méthanisation mésophile et thermophile du PHB.

Les plastiques du commerce sont souvent formulés à partir de mélange de différents polymères en présence d'additifs afin d'atteindre les caractéristiques techniques visées. La biodégradation de trois mélanges commerciaux de plastique biodégradable a également été étudiée en BMP. Les mélanges, composés de Mater-bi®, Eco-vio® et Vegemat®, ont été achetés sous forme de capsules de café certifiées biodégradables dans des conditions de compostage industriel (selon la norme de spécification EN 13432). Les trois mélanges ont eu un comportement similaire en méthanisation. La digestion mésophile a entraîné une faible conversion en méthane entre 12% et 24% en 100 jours. La digestion thermophile était plus performante, la biodégradation atteignant entre 47 et 69%, en 100 jours. Le manque de connaissance autour de la composition de plastiques commerciaux rend les interprétations des données microbiologiques plus compliquées. Par exemple, on a constaté que *Tepidimicrobium* était dominant dans les digesteurs thermophiles alimentés par les différents mélanges utilisés pendant la phase de forte production de méthane. *Tepidimicrobium* a été identifié précédemment comme une bactérie clé utilisant le lactate dans les réacteurs alimentés en PLA. Cependant, le PLA n'était pas censé être un composant de toutes les capsules de café étudiés.

La cinétique de biodégradation lente de la plupart des plastiques biodégradables dans les digesteurs anaérobies mésophiles est un obstacle majeur à leur introduction à l'échelle industrielle. Différentes stratégies peuvent être mises en œuvre pour améliorer la biodégradabilité

de ces plastiques en digestion anaérobie. Le PLA a été choisi dans cette étude spécifique en raison de son faible taux de biodégradation (500 jours ont été nécessaires pour atteindre la production finale de méthane en mésophile) et il représente l'un des plastiques biodégradables les plus utilisés (25% de la production). Une réduction mécanique de la taille des particules de PLA a augmenté le taux de biodégradation du PLA. Cependant, même avec de très petites particules ($\approx 272\mu\text{m}$), la cinétique de biodégradation n'était pas satisfaisante pour une utilisation industrielle. De plus, un tel prétraitement à l'échelle industrielle n'est techniquement pas réalisable. Au contraire, le prétraitement thermique et thermo-alcalin permet une amélioration significative de la cinétique de biodégradation du PLA. Les prétraitements thermiques n'ont permis une solubilisation importante ($> 60\%$) du PLA qu'à des températures très élevées (120 et 150 °C). À des températures plus basses (70 et 90 °C), une solubilisation négligeable (entre 1 et 6 %) s'est produite après 48 h. En revanche, le couplage du prétraitement thermique et alcalin (ajout de $\text{Ca}(\text{OH})_2$) a augmenté de manière significative la solubilisation à des températures plus basses (70 et 90 °C). Le meilleur prétraitement, qui réduit la consommation de $\text{Ca}(\text{OH})_2$ et la température utilisée, tout en maintenant une cinétique de biodégradation élevée, a été obtenu à 70 °C en utilisant 2,5 % p/v de $\text{Ca}(\text{OH})_2$ pendant 48 h. Ce prétraitement a permis d'obtenir un rendement de biodégradation du PLA de 73 % après 15 à 20 jours. Un rendement de biodégradation similaire a été obtenu après 500 jours pour le PLA non traité. Le prétraitement à cette température est relativement simple à mettre en œuvre à l'échelle industrielle, car les digesteurs anaérobies traitant les biodéchets effectuent généralement une étape de pré-hydrolyse ainsi qu'une étape d'hygiénisation à 70°C visant à inactiver les pathogènes potentiellement présents dans les biodéchets.

L'ensemble des expériences présentées ci-dessus ont été effectuées en mode batch grâce au protocole des TPMs. Ces tests, bien que très utiles et permettant de comparer de nombreuses conditions et substrats, ne donnent pas une image fidèle des performances à l'échelle industrielle, notamment au niveau de la stabilité du procédé. De plus, le développement de la collecte sélective des biodéchets d'ici 2024 en France va générer de nouveaux flux organiques valorisables en méthanisation. Ces flux de biodéchets seront probablement mélangés à l'avenir avec des plastiques biodégradables et, de ce fait, il est important de générer des connaissances sur une base scientifique en ce qui concerne le devenir des plastiques biodégradables lors de la co-digestion avec des biodéchets en méthanisation.

La co-digestion anaérobie dans des réacteurs alimentés en semi-continu avec des biodéchets et des plastiques biodégradables (PHB et PLA prétraités ou non) a été étudiée. L'ajout de plastiques biodégradables a amélioré la stabilité du procédé en comparaison avec le réacteur alimenté uniquement avec des biodéchets. De plus aucun impact négatif n'a pu être détecté. On a estimé que le PHB avait été entièrement biodégradé dans le réacteur. En revanche, le PLA s'est accumulé dans le réacteur, et une biodégradation moyenne de 47,6 % a été estimée pendant le troisième temps de rétention hydraulique. Le prétraitement du PLA, consistant en une hydrolyse thermo-alcaline à 70 °C, avec 2,5 p/v de $\text{Ca}(\text{OH})_2$ pendant 48 h, a amélioré le rendement de biodégradation du PLA à 77,5%. L'identification des microorganismes spécifiques impliqués dans la dégradation des plastiques biodégradables a été compliquée ; la majorité des microorganismes corrélés à la production de méthane dans les réacteurs de co-digestion du PLA et du PHB étaient impliqués dans la digestion anaérobie des biodéchets, ce qui peut s'expliquer par la faible proportion de plastiques biodégradables introduits. Les microorganismes intervenant dans la dégradation des plastiques biodégradables étaient probablement parmi les OTU rares. Un dégradateur de PHB déjà connu, *Ilyobacter delafieldii*, a quand même été observé dans le réacteur co-digérant le PHB mais son abondance était relativement faible (entre 0,1 et 0,3% des séquences). Les digestats obtenus pour les différentes conditions ont montré des paramètres agronomiques et sanitaires en adéquation avec la réglementation Française et Européenne. Cependant, une zone d'ombre autour de la qualité et de l'innocuité des digestats ayant traités des plastiques biodégradables subsiste, celle-ci devra être impérativement levée dans un avenir proche.

METHANISATION DES PLASTIQUES BIODEGRADABLES : PERFORMANCES ET DIVERSITE
MICROBIENNE

Guillaume CAZAUDEHORE - 2022

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Communications

Peer reviewed papers:

- Cazaudehore, G., Monlau, F., Gassie, C., Lallement, A., Guyoneaud, R., 2021. Methane production and active microbial communities during anaerobic digestion of three commercial biodegradable coffee capsules under mesophilic and thermophilic conditions. *Science of The Total Environment* 784, 146972. <https://doi.org/10.1016/j.scitotenv.2021.146972>
- Cazaudehore, G., Schraauwers, B., Peyrelasse, C., Lagnet, C., Monlau, F., 2019. Determination of chemical oxygen demand of agricultural wastes by combining acid hydrolysis and commercial COD kit analysis. *Journal of Environmental Management* 250, 109464. <https://doi.org/10.1016/j.jenvman.2019.109464>
- Cazaudehore, G., Guyoneaud, R., Evon, P., Martin-Closas, L., Pelacho, A.M., Raynaud, C., Monlau, F., 2022. Can anaerobic digestion be a suitable end-of-life scenario for biodegradable plastics? A critical review of the current situation, hurdles, and challenges (**under revision**). *Bioresource Technology*.
- Cazaudehore, G., Guyoneaud, R., Lallement, A., Gassie, C., Monlau, F., 2022. Influence of the inoculum-substrate ratio on the biochemical methane potential and active microbial communities during anaerobic digestion of biodegradable plastics (**under revision**). *Frontiers in Bioengineering*.
- Cazaudehore, G., Guyoneaud, R., Vasmaras, C., Greuet, P., Gastaldi, E., Marchetti, R., Leonardi, F., Turon, R., Monlau, F., 2022. Impact of mechanical and thermo-chemical pretreatments to enhance anaerobic digestion of poly(lactic acid) (**under revision**). *Chemosphere*.
- Cazaudehore, G., Monlau, F., Gassie, C., Lallement, A., Guyoneaud, R., 2022. Active microbial communities during biodegradation of biodegradable plastics through mesophilic and thermophilic anaerobic digestion (**in preparation**).
- Cazaudehore, G., Guyoneaud, R., Cauzi, P., Gassie, C., Lallement, A., Monlau, F., 2022. Simulation of the co-digestion of biowastes and biodegradable plastics in semi-continuous reactors: performances and microbial communities. (**in preparation**).

Oral communications:

- Cazaudehore, G., Guyoneaud, R., Vasmaras, C., Marchetti, R., Monlau, F., 2021. Anaerobic digestion of the main biodegradable polymers and thermo-chemical pretreatment of poly(lactic acid). Oral presentation held at the 8th International conference on sustainable solid waste management, Thessaloniki, Greece.
- Cazaudehore, G., 2021. Les plastiques biodégradables: un outil pour la valorisation des biodéchets par méthanisation. Invited speaker at Bio360 Open, Retiers, Bretagne
- Cazaudehore, G., Monlau, F., Lallement, A., Gassie, C., Guyoneaud, R., 2020. Valorization of biodegradable plastics through anaerobic digestion: performance and microbial communities. Oral presentation held at the 15th SDEWES conference Cologne 2020.
- Cazaudehore, G., Monlau, F., Vasmaras, C., Marchetti, R., Gassie, C., Jacquet, C., Guyoneaud, R., 2019. End-of-life of biodegradable plastic supports through anaerobic digestion: performance and microbial study. Poster and short oral presentation held at the 16th international water association world conference on anaerobic digestion, Delft, Netherlands.

List of abbreviations and symbols

AD	Anaerobic Digestion
BDL	Below Detection Limit
BMP	Biochemical Methane Potential
CSTR	Continuous Stirred-Tank Reactor
DM	Dry Mass
HRT	Hydraulic Retention Time
ISR	Inoculum Substrate Ratio
OFMSW	Organic Fraction of Municipal Solid Waste
OLR	Organic Loading Rate
OTU	Operational Taxonomic Unit
PBAT	Poly(Butylene Adipate-co-Terephthalate)
PBS	Poly(Butylene Succinate)
PCB	Poly(Chlorinated Biphenyl)
PCL	Poly(CaproLactone)
PCR	Polymerase Chain Reaction
PAH	Polycyclic Aromatic Hydrocarbon
PHAs	Poly(HydroxyAlkanoates)
PHB	Poly(HydroxyButyrate)
PHBV	Poly(3-HydroxyButyrate-co-3-hydroxyValerate)
PHO	Poly(hydroxyoctanoate)
PLA	Poly(Lactic Acid)
PVOH	Poly(vinyl alcohol)
SEM	Scanning Electron Micrographie
TPS	ThermoPlastic Starch
TS	Total solids
VFAs	Volatile fatty acids

General introduction

Over the last century, plastic have provided innovative solutions to society's permanent evolving needs and have become an indispensable part of our modern lives. In Europe, the main end-use market for plastics is packaging, representing 39.6% of the plastic demand, followed by building and construction (20.4%), automotive (9.6%), electrical and electronic (6.2%), household, leisure and sports (4.1) and finally agriculture (3.4%) (Plastics Europe, 2020). Plastic can be defined as a material which contains as an essential ingredient a high molecular weight polymer and which, at some stage in its processing into finished products, can be shaped by flow (ISO 472, 2013). Due to the wide variety of polymers used, plastics can exhibit very different properties making them useful in a vast range of applications. Plastics have the benefit of being inexpensive, lightweight, resistant to water, chemicals or light, as tough or flexible as desired (Andrady and Neal, 2009; Dilkes-Hoffman, 2020). In addition, plastics are easily moldable, which makes them easy for use in products manufacturing (Andrady and Neal, 2009; Dilkes-Hoffman, 2020). The widespread use of plastics really started after World War II and, since then, nearly 8 billion tons of plastic have been produced (Geyer et al., 2017). The annual global demand for plastics has been growing ever since, reaching in 2019, 368 millions of tons (Plastics Europe, 2020). Consequently, plastics as a waste are representing a growing part of the municipal solid wastes. For example, in 1960, plastics made up less than 1% (by mass) of municipal solid wastes in the United States; while in 2018, this proportion raised to 12% (US EPA, 2020).

The European waste policy defines a 5-layer strategy to prevent and reduce the negative impacts caused by the generation and management of waste and to improve resource efficiency: 1) Prevention; 2) Preparation for re-use; 3) recycle; 4) recover; 5) disposal. Obviously, the waste having the lower impact is the waste that is not produced or the waste that can be reemployed. This strategy resulted in the ban of several single-use products in European countries (*Directive (EU) 2019/904*, 2019). Plastics Europe, (2020) estimated that only 32.5% of the collected plastic were actually recycled in 2018. Indeed, plastic recycling is not that simple and often requires an efficient identification and sorting of the different polymers (Singh et al., 2017). Additionally, some plastics are not eligible for recycling, especially in the field of packaging, notably due to their multi-

layered composition and the possibility of being contaminated by organic matter (L. S. Dilkes-Hoffman et al., 2019; Singh et al., 2017). Then, 42.6 % of the collected plastic wastes in Europe were processed in energy recovery facilities (*i.e.*, incinerated) and 24.9% were disposed in landfills (Plastics Europe, 2020). Landfills is the worst end-of-life management method resulting in the use and contamination of valuable space (L. S. Dilkes-Hoffman et al., 2019). Landfills from higher income countries are well regulated and maintained, limiting leakages to the environment (L. S. Dilkes-Hoffman et al., 2019). However, in low and middle-income countries, plastics collection and recycling rates are lower and plastics are often disposed in unregulated and unsecured landfills with a high risk of environmental leaking (Jambeck et al., 2015).

Geyer et al., (2017) estimated that almost 60% of all plastics ever produced, corresponding to 5 billion of tons, have been discarded to the environment (natural environments or managed landfills). Due to their persistence, conventional plastics accumulate in the environment (*i.e.*, terrestrial and aquatic ecosystems). Jambeck et al. (2015) estimated that between 4.8 and 12.7 millions of tons of plastics were contaminating the oceans in 2010. Large plastic particles have negative impact on marine life causing notably ingestion, entanglement and finally death (Li et al., 2016). Microplastics from primary (plastics used in a microscopic size) and secondary (resulting from the fragmentation of the macroplastic) sources are also a threat to wildlife and humans (Ajith et al., 2020). These microplastics are accumulating in the food chain and can act as vehicles to carry heavy metals or organic pollutants into the food chain (Ajith et al., 2020; Li et al., 2016).

Biodegradable plastics were developed to have a lower persistence than conventional plastic and therefore, reduce the accumulation of solid plastic waste in the environment (European Bioplastics, 2020). However, the biodegradation of a plastic is strongly tight to the environmental conditions in which it occurs (Narancic et al., 2018). The plastics that are commonly qualified as biodegradable, are at least biodegradable under industrial composting condition according to the EN 13432 specification standard. This means that these plastics meet a number of criteria related to: 1) the content of heavy metals and toxic compounds; 2) their ability to undergo disintegration and 3) biodegradation under industrial composting; and 4) that the disintegration of plastics result in good compost quality without phytotoxic effect. However, biodegradation of these plastics in other environments is not guaranteed, highlighting the importance of specifying the environmental conditions allowing their biodegradation (Narancic et al., 2018). Therefore, the

mention biodegradable should not represent a "right to throw away", and the biodegradation of these plastics should preferably take place in managed environments (anaerobic digestion and/or composting). Biodegradable plastics and bioplastics should not be confused. Bioplastics are defined as "plastics that are bio-based, biodegradable or both" (European Bioplastics, 2020). Therefore, a large part of the bioplastics are not biodegradables, the bio-based and not biodegradable plastics represents 44.5% of the 2020 bioplastic production. These bioplastics are partly or fully produced from renewable carbon sources, thus their production reduce our dependence on fossil fuels.

Biodegradable plastics offer an additional waste management option (organic valorization) over conventional plastics, but they can also be processed through conventional methods (*e.g.*, mechanical or chemical recycling). Among the wastes treatment options, anaerobic digestion was found to be the more environmentally favorable option in comparison with composting (home and industrial) or incineration (only if efficiency of incineration plants do not increase) by Hermann et al. (2011). Moreover, in a context of development of source separation and collection of organic wastes, the use of biodegradable plastic collection bags could be a useful tool for achieving higher collection rates, as consumers perceive biodegradable plastic bags as safe and clean (European Bioplastics, 2016; Kern et al., 2018). The use of products made of biodegradable plastic able of being digested in the same period as other biowaste would be a great advantage for anaerobic digesters allowing to avoid costly deconditioning steps and to recover more biowaste (Kern et al., 2018).

Anaerobic digestion (AD) is a waste management method allowing a simultaneous generation of renewable energy (*i.e.*, through biogas generation) and treatment of organic wastes. This process uses microorganisms in an oxygen-free environment. The main products are biogas (mainly composed of methane and carbon dioxide) and digestate (the non-degraded fraction, which is rich in mineralized nutrients). Biogas can be valorized by different methods: converted in heat and electricity, upgraded into biomethane for introduction in the gas grid or used as biofuel (Miltner et al., 2017; Sahota et al., 2018). Digestate is mostly used as fertilizers or soil amenders (Monlau et al., 2015; Nkoa, 2014; Sheets et al., 2015). Anaerobic digesters are mainly operated at two temperature ranges, namely mesophilic (35-38 °C) or thermophilic (55-58 °C). Additionally, different reactors configuration can be used to treat feedstocks with different total solid (TS):

upflow anaerobic sludge blanket and anaerobic fluidized bed technologies for treating liquid feedstocks (< 3% TS); continuous stirred-tank reactors for feedstocks between 8 and 15% TS; and solid-state reactors designed for feedstocks with TS contents higher than 15%. Due to favorable conditions, the number of AD plants has grown significantly in Europe, with 6 227 biogas plants in 2009 and 17 783 biogas plants at the end of 2017 (Scarlat et al., 2018; Wu et al., 2021).

The selective collection of bio-wastes by 2024 on the National Territory will generate new organic flows for anaerobic digestion. These biowaste flows will certainly be mixed in the future with biodegradable plastic supports and for this purpose, it is important to generate scientific knowledge on the fate of these biodegradable plastic supports in the anaerobic digestion process.

However, the treatment of biodegradable plastics under anaerobic digestion is only in its infancy with notably no specification standard considering the end-of-life of biodegradable plastics in anaerobic digestion system as a stand-alone (Lagnet et al., 2020). Up to date, not all polymers and reactor typologies have received the same attention (Abraham et al., 2021; Bátori et al., 2018a). Furthermore, there have been few investigations of the anaerobic biodegradability of commercial blends (except Mater-Bi®) (Cho et al., 2011a; Narancic et al., 2018; Russo et al., 2009). According to previous literature data, it seems that the biodegradability of biodegradable plastics is enhance by thermophilic conditions in comparison with mesophilic ones (Bátori et al., 2018a; Narancic et al., 2018). As the degradation rate of most biodegradable plastics is low, especially under mesophilic conditions, only a low level of biodegradation is achieved after a period similar to a hydraulic residence time of a full scale plant. Several strategies could be applied to increase the rate of biodegradation such as application of pretreatment, the incorporation of additives into the polymers (*e.g.*, fibers, enzymes, or calcium carbonate), and inoculum acclimation (Calabro et al., 2019; Ryan et al., 2017a, 2017b). Furthermore, most of the data regarding biodegradability performances from biodegradable plastics come from studies based on biochemical methane potential (BMP) test experiments, which, even if very useful, may not provide a true picture of the performance of full-scale anaerobic reactors. To date, there is only little information available regarding continuous anaerobic digestion experiments at pilot or industrial scales. Moreover, little is known about the microorganisms involved in the anaerobic digestion of biodegradable plastics and on the biodegradation mechanisms. A better understanding of the microbial taxa involved in the process could improve the knowledge of biodegradable plastics biodegradability in AD process

and open the way to the use of bioaugmentation to improve the performance of the process. Finally, there is a grey area surrounding the quality and potential toxicity of the digestate from anaerobic digestion of biodegradable plastics.

The thesis outline is presented in **figure 1**.

In that context, this work intend to provide in **chapter 1**, a global state of the art covering the main aspects of the anaerobic digestion of the biodegradable plastics. A comprehensive compilation of the different anaerobic digestion experiments using biodegradable plastics as substrate is given. The microorganisms involved in the process and the strategies developed to improve the biodegradability of these plastics in anaerobic digestion systems are also presented. Additionally, the future developments required to introduce and to enhance the treatment of biodegradable plastic wastes in anaerobic digesters are discussed.

Then, in **chapter 2**, the influence of operational parameters (mainly inoculum to substrate ratio) on the biodegradation measured using BMP test and on microbial communities is explored during the digestion of two selected biodegradable plastics: poly(lactic acid) (PLA) and Poly(3-hydroxybutyrate). PLA and PHB were selected for this study, the first for being recalcitrant to mesophilic anaerobic digestion and the second, by contrast, for being readily biodegradable. Additionally, both polymers are among the most studied polymers to replace petroleum-derived plastics (Boey et al., 2021; Naser et al., 2021)

Based on the methodology developed in the previous chapter, biochemical methane potential test were performed on the main biodegradable polymers (PLA, PHB, Thermoplastic starch, Poly(butylene adipate/terephthalate), Poly(butylene succinate), Poly(ϵ -caprolactone)), and on three commercial polymer blends (purchased in the form of coffee capsules certified biodegradable under industrial composting conditions), under both mesophilic and thermophilic conditions, and the active microbial communities implicated in the process were examined (**chapter 3**).

Chapter 4 focus on the strategies that can be employed (mechanical, thermal and thermochemical pretreatments) in order to improve the biodegradation performances of PLA in mesophilic anaerobic digestion. PLA is one of the most abundant biodegradable polymer (representing 25%

of the production), its biodegradation in mesophilic conditions is problematic because of its slowness.

Finally, **chapter 5** is dedicated to the investigation of the stability and performances of the co-digestion of two selected biodegradable plastics (PLA and PHB) with food wastes fed semi-continuously under mesophilic conditions. Moreover, the PLA is introduced in two form: as powder (1mm) or as thermo-alkaline pretreated granule (70°C, 48h with 2.5% w/w Ca(OH)₂) to assess the impact of the pretreatment on the stability of the process and on the conversion of PLA into methane. Finally, the active microbial diversity of the different reactors is described.

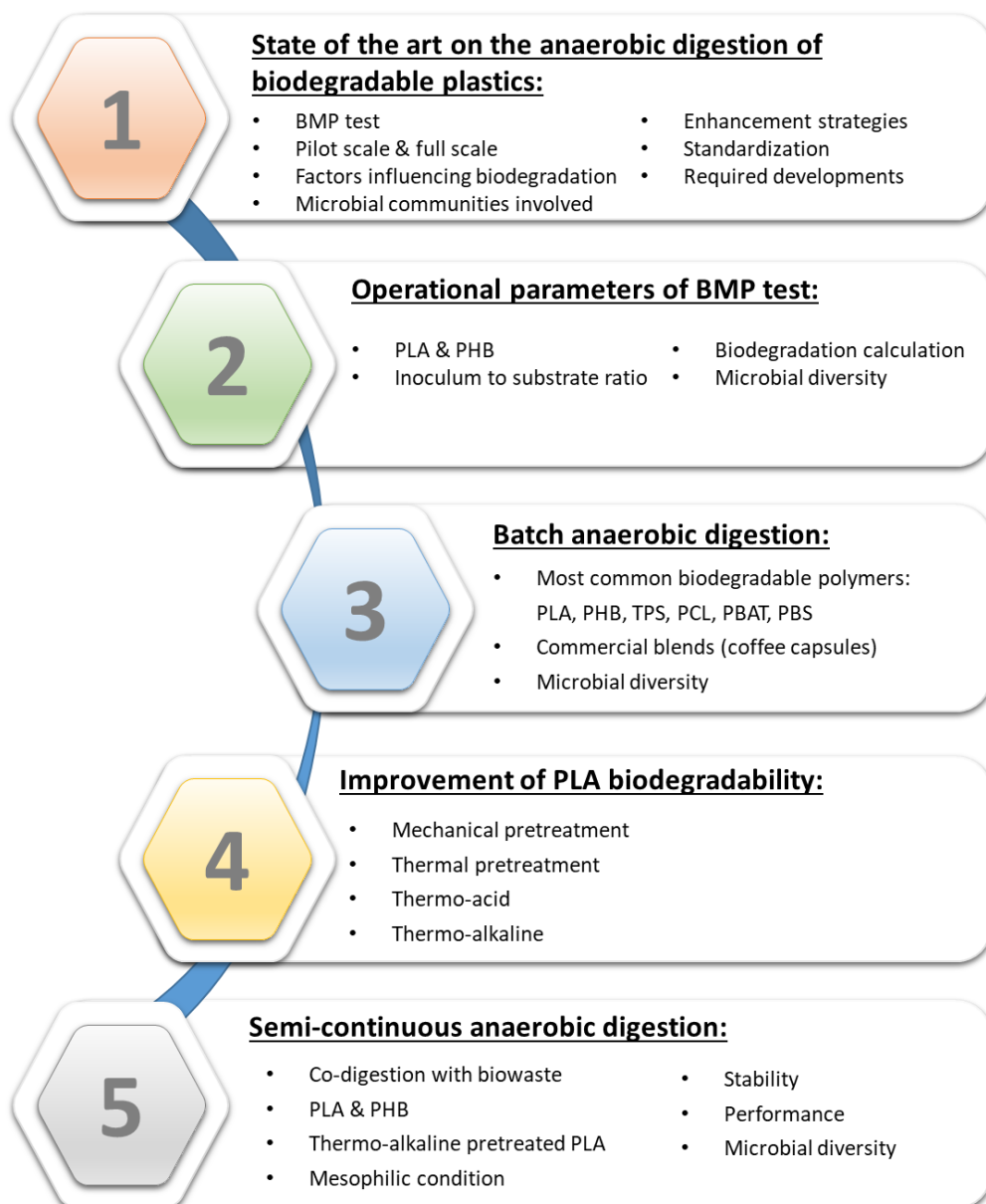


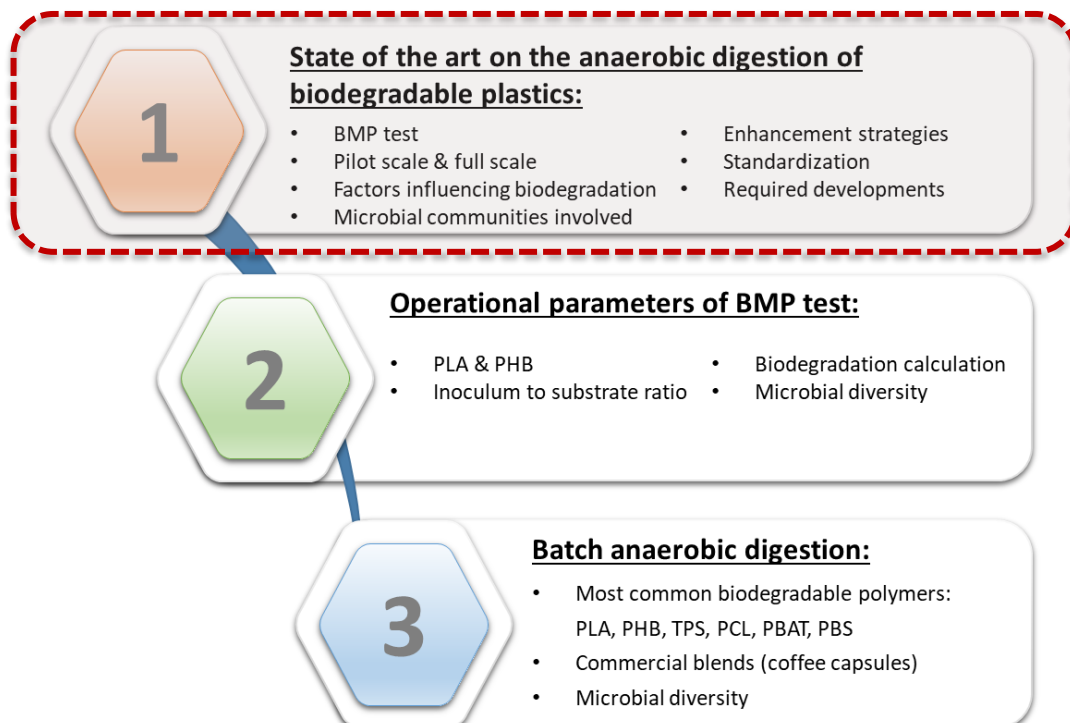
Figure 1. Thesis outline

CHAPTER I

Literature review

This chapter is based on a modified version of a scientific paper submitted to Bioresource Technology:

Cazaudehore, G., Guyoneaud, R., Evon, P., Martin-Closas, L., Pelacho, A.M., Raynaud, C., Monlau, F., 2022. Can anaerobic digestion be a suitable end-of-life scenario for biodegradable plastics? A critical review of the current situation, hurdles, and challenges (under revision). Bioresource Technology.



In this chapter, a global state-of-the-art covering the various aspects of the end-of-life of biodegradable plastic wastes in anaerobic digesters is proposed (**Figure I.1**). For this purpose, we first provide a brief description of the anaerobic digestion process, bioplastics and biodegradable plastics. Plastics biodegradability under mesophilic and thermophilic conditions, alone or in co-digestion, in batch, or in continuous assays is further described based on the data in the literature. Several pathways to enhance the biodegradability of plastics under anaerobic conditions are then presented, such as pretreatments, additive incorporation, and inoculum acclimation. In parallel, a specific section is dedicated to the developments required to make the introduction of biodegradable plastic wastes in anaerobic digesters possible: determination of the impact of plastic addition on the quality/safety of the digestate, and the establishment of international standards and labels to evaluate the biodegradability of a specific plastic by AD. Finally, a section is dedicated to suggestions and recommendations regarding the biodegradability of plastics and their integration into the waste management chain.



Figure I.1. General overview of the main issues addressed in this bibliographic review.

1) Bioplastics and biodegradable plastics

The European Bioplastics Association defines bioplastics as “*plastics that are bio-based, biodegradable or both*” (European Bioplastics, 2019). The first concept, *i.e.*, “*bio-based*”, relates to the origin of the materials used in the plastic production. A plastic can be classified as bio-based if it is wholly or partly derived from renewable carbon sources. In order to meet the requirements of the “OK bio-based” (TÜV Austria®) and “Din Geprüft bio-based” (DIN CERTCO) labels, at least 20% of the carbon should be derived from renewable sources according to the EN 16785-1 or the ASTM D6866 standard test method (TÜV Austria, 2021; TÜV Rheinland, 2021). There are several ways to produce bioplastics. They can be directly extracted from biomass (starch, cellulose, etc.), they can be chemically synthesized from bio-derived monomers (*e.g.*, PLA, bio-PE, bio-PET, bio-PP), or they can be produced by microorganisms (*e.g.*, PHAs) (Avérous and Pollet, 2012; Song et al., 2011).

The other aspect of this definition is related to the end-of-life of the plastic and its biodegradable nature. A biodegradable plastic can be defined according to ISO 472 (2013) as a plastic that undergoes a significant degree of mineralization (conversion into water, CH₄, and/or CO₂) under specific environmental conditions due to the action of naturally occurring microorganisms in a given period. The biodegradation has to be measured by standard test methods appropriate to the plastic and to the application (ISO 472, 2013). Nonetheless, oxo-degradable plastics (*i.e.*, conventional plastics that contain specific additives designed to promote the oxidation of the material at its end-of-life) cannot be considered to be biodegradable plastics (Deconinck and De Wilde, 2013; Ettliger et al., 2016). The pro-oxidant additives allow faster cleavage of the macromolecules induced by environmental factors (*e.g.*, UV light, heat, oxygen) (L. Dilkes-Hoffman et al., 2019; Sivan, 2011). However, the small size/molecular weight plastics that are produced are not necessarily subsequently mineralized (Deconinck and De Wilde, 2013; Ettliger et al., 2016). The biodegradation of a plastic is tightly linked to the environmental conditions in which it occurs. Currently, industrial composting is the favored organic valorization route for biodegradable plastics; thus, the polymers commonly referred to as biodegradable are certified under industrial composting conditions, *i.e.*, according to the EN 13432 standard.

Based on the bioplastic definition, there are three families of bioplastics: **1) Bio-based and non-biodegradable plastics**, also called “drop-in”, which share the same properties as their petrochemical counterparts (e.g., bio-PE and PE, bio-PET and PET, bio-PP and PP), except that they are partially produced from renewable carbon sources and, therefore, help to reduce the dependency on fossil resources (Álvarez-Chávez et al., 2012; Bátori et al., 2018a). They represented 44.5% of the global bioplastic production capacity in 2018 (**Figure I.2**). **2) Petrochemical and biodegradable plastics**, such as PBAT, PBS, and PCL. These have an additional potential waste management option by means of organic recycling (composting or anaerobic digestion). They accounted for 19.1% of the global bioplastic production capacity in 2018 (**Figure I.2**). **3) Bio-based and biodegradable plastics**, such as starch blends, PLA, and PHAs (accounting for 36.4% of the global bioplastic production capacity in 2018; **Figure I.2**). These have the benefit, compared to the two bioplastic families mentioned above, of reducing the fossil fuel dependency while also being suitable for an additional waste management option. Thus, the word “bioplastic” is ambiguous and can be confusing about its ability to biodegrade.

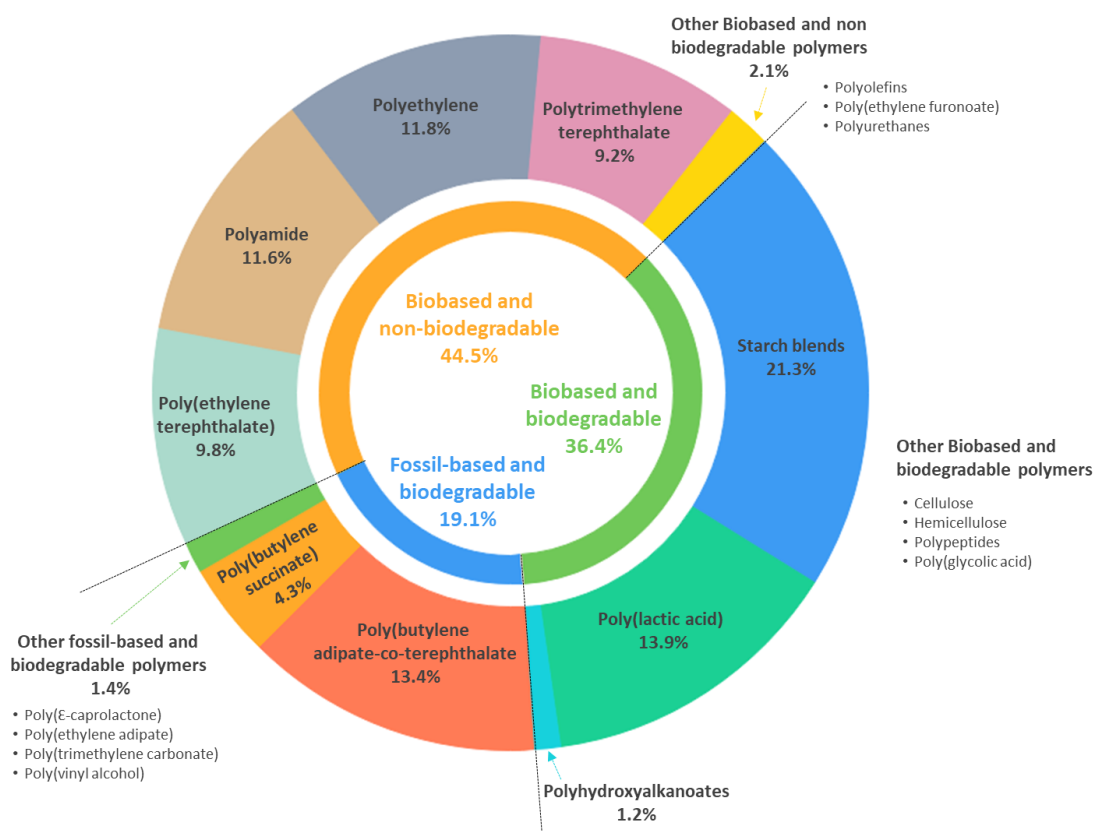


Figure I.2. Global production capacities of bioplastics in 2019 (Source: European Bioplastics, 2019).

Commercially available plastics are often blends of different polymers in order to meet the intended technical requirements (Long and Chen, 2009). Polymers or co-polymers can also be compounded with organic or inorganic fillers to enhance certain properties, thereby contributing to a reduction in the cost of the plastic (Meereboer et al., 2020; Shahlari and Lee, 2012; Song et al., 2011). A wide range of additives are included in the plastics for various purposes. In 2014, these comprised, in decreasing quantities, plasticizers, flame-retardants, coupling agents and impact modifiers, antimicrobials, UV stabilizers, and antioxidants (Global Plastic Additives Market Size & Share - Industry Report, 2016). A plasticizer is a molecule, generally of low molecular mass, that is interposed between the polymer chains, thereby reducing the inter- and intra-molecular bonds and thus increasing the free volume between these chains. The addition of a plasticizer decreases the polymer's viscosity by promoting sliding of the chains relative to each other. At the same time, the crystallinity of the material decreases and its flexibility increases (Rahman and Brazel, 2004; Xiao et al., 2009). In the case of composites combining a polymer with an organic filler (*e.g.*, a vegetable filler added to mechanically reinforce the polymer), the addition of a coupling agent to the mixture is frequently used with the intention of improving the interface between the polymer and the filler (Muthuraj et al., 2015). These are either bifunctional products capable of reacting with the functional groups of the two phases and thereby resulting in the creation of covalent bonds between them, or amphiphilic compounds that have an affinity for each of the two phases (Mittal and Chaudhry, 2015).

The main biodegradable polymers, whether bio-based or based on fossil resources, are listed below.

- **Thermoplastic starch (TPS):** Starch is one of the most abundant and cheapest biodegradable polymers as it represents the main energy reserve of plants (Kaseem et al., 2012). In plants, it is mainly stored in tubers and seeds. The main sources of starch are corn, wheat, potato, cassava, pea, and rice. Starch is also the main source of carbohydrates in the human diet. Chemically, it is a mixture of two homopolymers of α -D-glucose units, linked together by α -1,4 glycosidic bonds, *i.e.*, amylose (a linear or a very slightly branched macromolecule) and amylopectin (a branched macromolecule of glucose units based on α -1,6 bonds) (Kaseem et al., 2012). In plastics, starch is not used in its native form but in its plasticized form, which is obtained by the use of plasticizers. Thermoplastic starch (TPS) is

a native starch that has been subjected to a thermomechanical treatment that allows thermoplastic transformation (Avérous and Pollet, 2012; Song et al., 2011). In most cases, water is used to make the starch a thermoplastic because water plays a dual role as a destructuring agent as well as an efficient plasticizer, due to its particularly low steric hindrance. However, water has a major drawback, namely its volatility. Over time, water migrates and evaporates from the material, promoting the phenomenon of starch retrogradation and thus alteration of its properties. Many other molecules of low molecular mass can, therefore, also be used, either as plasticizers or as destructuring agents. The main entities are glycerol (Chabrat, 2012); sorbitol (Li and Huneault, 2011); ethylene glycol and propylene glycol (Roz et al., 2006); amides including formamide, acetamide, and urea (Ma and Yu, 2004); and citric acid (Chabrat et al., 2012). Thermoplastic starch is, in most cases, blended with other polymers, *e.g.*, polyesters such as PLA, PBAT, PCL, PHAs, etc. (Bátori et al., 2018a; Bulatović et al., 2021).

- **Poly(lactic acid) (PLA):** PLA is a linear aliphatic polyester made from renewable resources. It is synthesized by direct polycondensation of lactic acid or by ring-opening polymerization of lactide (Long and Chen, 2009). Lactic acid is commonly produced by fermentation of various biomasses (*e.g.*, corn, wheat, sugar cane, and sugar beet) (Song et al., 2011). As the carbon of lactic acid bearing the hydroxyl group is asymmetric, lactic acid can exist as two enantiomers (L or D). PLA can, therefore, have two different chemical structures depending on whether the chain of monomers is isotactic (L-PLA) or syndiotactic (D,L-PLA) (Pang et al., 2010). The stereoisomeric L/D ratio of the lactate monomers influences the properties of the PLA produced (*e.g.*, crystallinity, thermal and mechanical properties) (Pang et al., 2010; Zhang and Sun, 2005). Thus, the L-PLA obtained by polymerization of a single isomer has the capacity to crystallize while the D,L-PLA obtained from a mixture of the two isomers is amorphous (Iannace et al., 2014a).
- **Poly(butylene adipate/terephthalate) (PBAT):** PBAT is an aromatic co-polyester made by co-polymerization (condensation reaction) of 1,4-butanediol with adipic and terephthalic acids (Avérous and Pollet, 2012; Long and Chen, 2009). PBAT is produced from non-renewable carbon resources. Its applications are diverse (Avérous and Pollet, 2012). With a low density and good resistance to humidity, stretching, and impact, it is suitable for

applications such as rigid packaging for food or beauty products. Aliphatic aromatic copolyesters, which contain small amounts of aromatic entities, are able to decompose. The breakdown of PBAT depends on the quantity of aromatic ester functions (Witt et al., 1996).

- **Poly(butylene succinate) (PBS):** PBS is a petroleum-based aliphatic polyester synthesized by polycondensation of 1,4-butanediol and succinic acid (Avérous and Pollet, 2012; Bátori et al., 2018a). It is a soft and flexible semi-crystalline thermoplastic. Both building blocks (*i.e.*, 1,4-butanediol and succinic acid) can be produced from renewable raw materials such as starch (mainly from corn), glucose, and sucrose by fermentation, or from petrol. However, for the main commercial grades, the 1,4-butanediol block is of fossil origin, making these grades partially bio-based (approximately 50%). PBS is a very promising material because its mechanical properties are comparable to those of high-density polyethylene and isotactic polypropylene. Compared to PLA, it is much more flexible, and its melting point is much lower (approximately 115 °C). It can be used as a matrix polymer or in combination with other biodegradable polymers such as PLA (Su et al., 2019).
- **Poly(ϵ -caprolactone) (PCL):** PCL is another fossil-based aliphatic polyester that is usually produced by ring-opening polymerization of ϵ -caprolactone (Avérous and Pollet, 2012; Mohamed and Yusoh, 2015). Two types of PCL can be distinguished according to their molar mass. While the first group (molar masses of a few thousands of g/mol) is in a liquid form (used as precursors for polyurethanes, thinners for paints, or plasticizers for vinyl resins), the second type of PCL comprises molecules with higher molar masses (greater than 20,000 g/mol) (Jiang and Zhang, 2013). These latter PCL grades are semi-crystalline, and they exhibit interesting mechanical characteristics. They are generally used to modify the properties of other biodegradable plastics, including TPS (Arakawa and DeForest, 2017).
- **Polyhydroxyalkanoates (PHAs):** PHAs are a family of aliphatic polyesters produced from renewable carbon sources. They are synthesized (*i.e.*, naturally accumulated) by some microorganisms as energy and carbon reserves (Bugnicourt et al., 2014). Bioengineering methods that take advantage of this ability are used to produce commercial grades of PHAs (Bugnicourt et al., 2014; Song et al., 2011). PHAs are generally classified based on the number of carbon atoms in their monomer units: short-chain-length (scl) PHAs (or scl-

PHAs) with 3-5 carbon atoms per monomer and medium-chain-length (mcl) PHAs (or mcl-PHAs) with 5-14 carbon atoms per monomer are the two main forms of PHA compounds (Kynadi and Suchithra, 2014). Poly(3-hydroxybutyrate) (PHB) is the most widespread member of the PHAs family, followed by poly(3-hydroxyvalerate) (PHV) and their copolymer poly(3-hydroxybutyrate-cohydroxyvalerate) (PHBV) (Bugnicourt et al., 2014). PHB is a partially crystalline and biocompatible homopolymer, and it has mechanical properties close to those of isotactic PP, except for its elongation at break, which is lower (Thiré et al., 2006). Depending on their nutrition, bacteria can also produce other PHAs, e.g., PHBV (Albuquerque et al., 2011). PHBV is the second most common commercial PHA. The substituent group is randomly either a methylbutyrate or an ethylvalerate in the PHBV copolymer. The amount of valerate in the copolymer influences the final mechanical properties of PHBV. In particular, an increase in toughness and a decrease in modulus are observed with an increase in valerate content (Savenkova et al., 2000). Indeed, lengthening of the aliphatic chain reduces the glass transition temperature and the degree of crystallinity (Chan et al., 2019). Depending on the composition, the PHBV material changes from a brittle and hard polymer to a flexible and elastic gum (Savenkova et al., 2000).

Table I.1. The main biodegradable plastics commercially available, and their most important physicochemical properties

Polymer type	TPS	PLA	PBAT	PBS	PCL	PHB	PHBV
Main producers	- Vegeplast (France) - Novamont (Italy) - Biotec® (UK)	- NatureWorks (USA) - Total Corbion PLA (NL) - Futerro (Belgium) - BASF (Germany)	- BASF (Germany) - Jinhui Zhaolong (China) - Biofed (Germany) - Eastman (USA)	- PTT MCC Biochem Company Ltd (Thailand) - Succinity (Germany) - MCPP (Japan) - Showa Denko (Japan)	- Perstorp (UK) - BASF (Germany)	- TianAn (China) - Yield10 Bioscience (USA) - Bio-Fed® (Germany) - Biomer® (Germany)	- TianAn, (China) - Yield10 Bioscience (USA) - Biomer (Germany)
Melting point (°C)	n.m.	155 ^b	110-120 ^c	115 ^d	58-60 ^e	170-185 ^f	170-176 ^g
Glass transition (°C)	n.m.	60 ^b	-30 ^h	-32 ^h	-61 ^h	-5 to 5 ^h	-10 to 5 ^h
Molar mass (g/mol)	n.m.	n.m.	n.m.	n.m.	20,000-80,000 ^e	n.m.	n.m.
Density (g/cm ³)	n.m.	1.24 ^b	1.25-1.27 ^c	1.26 ^d	1.1 ^e	1.26 ^f	1.25 ^g
Cristallinity rate (%)	n.m.	0-40 ^h	n.m.	34-45 ^h	67 ^h	60-80 ^h	30-80 ^h
Maximal tensile strength (MPa)	1-23 ^a	45 ^b	35-45 ^c	30 ^d	14	16 ^f	39 ^g
Young's modulus (MPa)	0.1-3.5 ^a	n.m.	n.m.	720 ^d	0.19 ^c	n.m.	2.8-3.5 ^g
Elongation at break (%)	0,5-80 ^a	<5 ^b	560-710 ^c	330 ^d	> 500 ^c	6 ^f	2 ^g

n.m., not mentioned.

^a TPS from corn whole plant or from wheat flour (T55 type), with glycerol (from 0 to 25% w/w) and/or citric acid (from 0 to 14% w/w) used as plasticizer(s).

^b Value for Luminy LX 175 grade (Total Corbion).

^c Value for Ecoflex® F Blend C1200 grade (BASF).

^d Value for PBE003 (Natureplast)

^e Value for Capa™ 6800 grade (Perstorp).

^f Value for M VERA GP1012 (Biofed)

^g Value for ENMAT Y1000P (Tianan)

^h Value from Shruti, V.C., Kutralam-Muniasamy, G., 2019. Bioplastics: Missing link in the era of Microplastics. *Science of The Total Environment* 697, 134139. <https://doi.org/10.1016/j.scitotenv.2019.13413>

2) Anaerobic digestion process

Anaerobic digestion (AD) is a biological process by which, in an oxygen-free environment, organic matter is converted into biogas (mainly composed of carbon dioxide and methane) and digestate (the non-degraded fraction, which is rich in nutrients). AD has turned out to be a promising method for the valorization of organic materials such as agricultural wastes (manure, crop residues, and winery wastes) (Monlau et al., 2013b), food wastes (Morales-Polo et al., 2018), and sewage sludges (Hanum et al., 2019).

The biogas produced can be converted into heat and electricity through a cogeneration heat and power (CHP) system, or after biogas upgrading, biomethane can be introduced into the national gas grid or used as transport biofuel (Miltner et al., 2017; Sahota et al., 2018). The electricity produced can be sold providing economic benefits to the AD plant operator. The price of the electricity is dependent of the national policy and can vary among the various European countries (Monlau et al., 2012; Sambusiti et al., 2013). In parallel, digestate corresponding to the anaerobically non-degraded fraction composed of recalcitrant fibers (*e.g.*, lignin), and which is rich in mineralized nutrients, is also generated. To date, digestates have mostly been used at the farm scale as fertilizers or soil amenders (Monlau et al., 2015; Nkoa, 2014; Sheets et al., 2015). At the industrial scale, digestates are generally mechanically separated (belt press, sieve drum, screw press, sieve centrifuge, rotary screen, or decanter centrifuge) into liquid and solid fractions that are stored separately for ease of handling and transport (Monlau et al., 2015).

Anaerobic digesters are mainly operated at two temperature ranges, namely mesophilic (35-38 °C) or thermophilic (55-58 °C). There are three main full-scale reactor configurations. These are designed to treat feedstocks with different total solids (TS) contents. Upflow anaerobic sludge blanket (UASB) or anaerobic fluidized bed technologies are used to treat liquid feedstocks (< 3% TS), especially from urban wastewater and agro-food sectors. Continuous stirred-tank reactors (CSTR) are used to process feedstocks with a TS content between 8 and 15%. Solid-state AD (SS-AD) are designed for feedstocks with TS contents higher than 15% and are classified as dry batch AD and dry plug-flow AD. For the treatment of organic wastes, dry technologies currently represent approximately 75% of all AD plants in Europe (Mattheeuws, 2015). Unlike CSTR and SS-

AD reactors, UASB reactors cannot be considered to be a consistent option for the treatment of solid organic wastes and biodegradable plastics as they are designed for liquid sewage.

To evaluate the biodegradability of organic products, two tests are generally applied to assess anaerobic performances: a biochemical methane potential (BMP) test and continuous (or semi-continuous) pilot-scale experiments. The BMP test is a procedure developed to determine the methane production of a given organic substrate during its anaerobic digestion at a lab scale. It is a reliable method for obtaining the extent and rate of organic matter conversion into methane. Pilot-scale experiments use more realistic conditions than the BMP test: the feeding is continuous or semi-continuous, and several parameters (biological, operational, performances) are monitored during the assay. Pilot experiments provide precious insights regarding the process performance and stability over a long period of time. An important parameter that can influence AD performances is the C/N ratio of the feedstock. Hawkes (1980) suggested a carbon to nitrogen ratio for anaerobic digestion ranging from 20:1 to 30:1 for preventing both nutrient limitation and ammonia toxicity. Protein-rich wastes such as food wastes or municipal sludges have C/N ratios ranging from 6:1 to 16:1. By contrast, most biodegradable plastics contain carbon but no nitrogen. Thus, co-digestion of biodegradable plastics with proteinaceous substrates can increase the C/N ratio to the suggested values and result in a more robust process (Benn and Zitomer, 2018; Rabii et al., 2019; Rajagopal et al., 2013).

Finally, the extent of biodegradation of a substrate can be estimated by comparison of the experimental methane production to the theoretical methane production (Eq.1). The theoretical methane production can be calculated using the Buswell formula (Eq. 2). The estimation of the biodegradation by such method can rarely reach 100%; indeed a fraction of the substrate's carbon is not converted into biogas and is assimilated by the microbial biomass during the AD process (Shah et al., 2008).

$$\text{Eq.1: Biodegradation (\%)} = \frac{\text{Experimental methane production}}{\text{Theoretical methane production}}$$

Eq 2: (Boyle, 1976; Buswell and Mueller, 1952)

$$\text{Theoretical methane production (L CH}_4 \text{ g}^{-1} \text{ C}_x\text{H}_y\text{O}_z\text{N}_n\text{S}_s\text{)}^* = \frac{22.4 \times \left(\frac{x}{2} + \frac{y}{8} - \frac{z}{4} - \frac{3n}{8} - \frac{s}{4} \right)}{12x + y + 16z + 14n + 32s}$$

*at standard temperature and pressure (0°C, 1 atm)

3) Anaerobic digestion of biodegradable plastics

3.1) Lab-scale anaerobic digestion by BMP

Most of the studies to date have investigated the biodegradability of biodegradable plastics in batch lab-scale anaerobic digestion experiments (BMP). **Tables I.2 and I.3** present the main results obtained under both mesophilic (35-38 °C) and thermophilic (55-58 °C) conditions. A detailed survey is provided below, first for mesophilic and then thermophilic conditions. It is important to keep in mind that the experimental conditions differ between studies; that the exact formulation of the tested plastics is not precisely known, and that the properties of different plastics made from the same polymer can differ significantly. Therefore, this implies that there is a degree of variability in the results obtained in the various studies.

3.1.1) Mesophilic conditions

First of all, the anaerobic digestion performances of biodegradable plastics under mesophilic conditions are presented in **Table I.2**.

Poly(3-hydroxybutyrate) is the most widespread member of the polyhydroxyalkanoates family. Complete or near-complete biodegradation of PHB samples in a short time period have been reported for mesophilic digesters. For example, Abou-Zeid et al. (2001) observed that a 19 mm film made of PHB Biopol® BX G08 (ICI, United Kingdom) was fully converted into methane after only 9 days of incubation in various microbial inocula. PHB is a very promising polymer given its ability to be biodegraded in non-harsh environments such as mesophilic anaerobic digestion, home composting, soil, etc. (Narancic et al., 2018). The short time needed to fully biodegrade PHB makes it compatible with the conventional hydraulic retention time used in industrial anaerobic digestion plants (Narancic et al., 2018). However, the methane conversion differed significantly depending on the grade of PHB used. Benn and Zitomer, (2018) found near-complete

biodegradation of two PHB grades, Mirel M2100 (Metabolix) and methane-derived PHB from Mango Materials, while only 50 to 59% of ENMAT Y3000 (TianAn) and Mirel F1006 (Metabolix) were degraded at the same time. Poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) is a co-polymer of poly(3-hydroxyvalerate) (PHV) and poly(3-hydroxybutyrate), and it is also one of the main members of the PHA family. Similar to PHB, PHBV exhibited a very good level of biodegradation in a short time (Budwill et al., 1992; Day et al., 1994; Reischwitz et al., 1997; Ryan et al., 2017a, 2017b; Shin et al., 1997). For example, Reischwitz et al. (1997) reported a biodegradation level of 95% in 30 days for PHBV powder (420 μm) with 8.4% hydroxyvalerate (HV). A lower conversion into methane was reported by Abou-Zeid et al. (2004, 2001) and Day et al. (1994), between 29 and 55% in 40 and 42 days, respectively. Nunziato et al. (2018) reported a low degree of biodegradation for polyhydroxyoctanoate (PHO), which is a medium-chain PHA, with only 12% of the material being converted into methane after 56 days in a mesophilic digester. On the other hand, Federle et al. (2002) reported near-complete biodegradation (88%) for PHBO, a co-polymer of PHB and PHO (10% PHO), after 60 days of anaerobic digestion.

PLA has been one of the most investigated biodegradable plastics to date. According to Vargas et al. (2009) and Vasmarra and Marchetti, (2016), rigid pieces of PLA (3 mm and $< 1 \text{ cm}^2$) did not biodegrade in 60 and 90 days, respectively. Similar observations have been reported for smaller pieces of PLA. For example, 0.15 mm particles and 20 x 40 mm film did not exhibit any significant biodegradation in 40 and 100 days, respectively (Benn and Zitomer, 2018; Shin et al., 1997). However, other authors have reported methane production during the digestion of PLA at $35 \pm 2 \text{ }^\circ\text{C}$ (Day et al., 1994; Greene, 2018a; Itävaara et al., 2002; Massardier-Nageotte et al., 2006; Yagi et al., 2014a). For Day et al. (1994), Greene, (2018) and Massardier-Nageotte et al. (2006), minor biodegradation levels were observed, with between 10 and 23% of the PLA being converted into methane in 20 to 40 days. Yagi et al., (2014) investigated anaerobic digestion of PLA ground to 125-250 μm over a long period of time (277 days). At the end of the test, the PLA was biodegraded between 29 and 49% (depending on the run) but the methane production did not reach a plateau as methane production was still increasing. They explained the low biodegradation rate of PLA by the fact that the bacteria present in the mesophilic digesters did not have the ability to biodegrade higher molecular weight PLA. The microorganisms were only able to use PLA after a reduction of its molecular weight caused by a random hydrolytic chain scission of the ester

linkages (Ali Akbari Ghavimi et al., 2015). Bernat et al. (2021) also performed mesophilic anaerobic digestion of PLA over a long period of time (280 days). After 40 days of lag phase, there were two phases of constant biogas production. Firstly, $1.4 \text{ L kg}^{-1} \text{ VS d}^{-1}$ of biogas was produced between the 40th and 90th day, and then, between the 90th and 280th day, the biogas production increased to $2.6 \text{ L kg}^{-1} \text{ VS day}^{-1}$. Finally, after 280 days, the biogas production reached 66% of the theoretical value, although the plateau phase was nonetheless not reached.

Thermoplastic starch exhibited a very high level of biodegradation in a short time in mesophilic digesters, according to Narancic et al. (2018). Indeed, TPS from BIOTEC (TPS Bioplast®) was fully biodegraded in 56 days at 35 °C. However, TPS is rarely used on its own, instead, it is usually blended with other biodegradable polymers. PCL is often used in blends with TPS (Ali Akbari Ghavimi et al., 2015). Nunziato et al., (2018) showed that the addition of TPS increased the biodegradation rate of PCL in mesophilic and thermophilic anaerobic digesters. The biodegradation of PCL was reported to be very slow and in all cases, with the degradation level between 0 and 22% at the end of the tests (Abou-Zeid et al., 2004, 2001; Day et al., 1994; Federle et al., 2002; Massardier-Nageotte et al., 2006; Narancic et al., 2018; Nunziato et al., 2018; Puechner et al., 1995). Yagi et al. (2014) performed mesophilic anaerobic digestion tests on PCL powder (125-250 μm) over a very long period of time (277 days). They found a very low biodegradation rate. Only 3 to 22% of the PCL was converted to methane, and they assumed that the same biodegradation mechanisms as with PLA were involved. The microorganisms were only able to degrade lower molecular weight PCL resulting from the random hydrolytic chain scission of the ester linkages.

Other plastics that were found to be biodegradable under industrial composting conditions such as poly(butylene succinate) and poly(butylene adipate-co-terephthalate) did not undergo significant biodegradation in mesophilic anaerobic digesters (Cho et al., 2011b; Massardier-Nageotte et al., 2006; Narancic et al., 2018; Shin et al., 1997; Svoboda et al., 2018). PBS was not degraded at all, even with the very long digestion time (277 days) used by Yagi et al. (2014). According to Shin et al. (1997), a co-polymer of PBS and polyethylene (PBES) showed no sign of biodegradation after 100 days in anaerobic media. The biodegradability of a number of other minor biodegradable plastic was also measured. Calabro et al. (2019), Puechner et al. (1995) ,and Shin et al. (1997) digested plastics derived from cellulose. Calabro et al. (2019) and Shin et al.

(1997) found a good degree of methane conversion of the polymers (310 L CH₄ kg VS⁻¹ and 85% in 44 and 20 days, respectively) while Puechner et al. (1995) observed a relatively low degree of biodegradation (22% in 60 days) for cellulose acetate (Bioceta[®], Mazzucchelli, Italy). Mesophilic digestion of butanediol/adipic acid and butanediol/adipic acid/terephthalic acid resulted in a low level of methane conversion in 42 days of between 1.1 and 10% (Abou-Zeid et al., 2004).

To date, most of the data available in the literature has been in regard to the biodegradability of the main biodegradable polymers individually. However, different polymers are often blended in order to meet the intended technical requirements (Long and Chen, 2009) and there is a paucity of data available in the literature regarding the biodegradability of commercial plastic blends (*e.g.*, Mater-Bi[®], Ecovio[®]) in anaerobic digestion. For example, in the case of Mater-Bi[®] (Novamont, Italy), which is the most studied biodegradable blend in AD, the composition of the blend has not been clearly established. Mater-Bi[®] is a family of compounds based on TPS and other polymers such as cellulose acetate, poly(vinyl alcohol), poly(ϵ -caprolactone), and poly(butylene adipate-co-terephthalate) (Aldas et al., 2020). Biodegradation of Mater-Bi[®] was reported to be relatively limited in mesophilic AD, with between 6 and 32% of the material being converted into methane over a period of 15-80 days (Calabro et al., 2019; Day et al., 1994; Massardier-Nageotte et al., 2006; Puechner et al., 1995; Scandola et al., 1998; Vasmara and Marchetti, 2016). Cazaudehore et al. (2021) explored AD of three commercial coffee capsules composed of biodegradable blends (Vegemat[®] from Vegeplast, Ecovio[®] from BASF, and Mater-Bi[®] from Novamont). After 100 days, the plateau phase of biodegradation was not reached for any of those blends; they only underwent 12 to 20% biodegradation. Recently, Dolci et al. (2022) have investigated the methane potential of four Mater-Bi[®] bags and biodegradability ranged from 71% to 93% in less than 56 days.

Finally, the degradation of non-commercial plastic blends, produced at a laboratory-scale, has, however, been investigated in several scientific publications (Guo et al., 2011; Lee et al., 2016; Narancic et al., 2018; Russo et al., 2009). Guo et al. (2011) examined anaerobic digestion of different polyvinyl alcohol (PVOH) /starch blends produced from various starch origins (from wheat, maize, and potato). The different blends were converted into methane between 58 and 62% in a relatively short period of time (5-6 days). Similarly, Russo et al. (2009) investigated the degradation of TPS/PVOH blends with different polymer contents (90/10, 75/25, 50/50, and 0/100). The PVOH content significantly affected the rate of the starch biodegradation. The

methane production was higher for the blends with higher TPS contents. Narancic et al. (2018) reported a synergistic effect of blending two biodegradable polymers, with plastic blends having higher methane production levels in some cases than individual polymers. This better biodegradation of the blends could be explained by the lower crystallinity of the blend compared to the individual polymers (Narancic et al., 2018). In addition, the blend could have a better fragmentation capacity like the plastic-fiber composites, which would increase the surface area exposed to hydrolysis and thus the biodegradation rate (Ryan et al., 2017b).

Table I.2. Mesophilic biochemical methane potential and operational parameters described in the literature (1/3)

Biodegradable plastic nature	Size and shape	Temperature (°C)	Operational parameters and supplementary data	Time (days)	Methane potential	Percentage of methane conversion	Reference	
Mater-bi (PCL + Amidon, Novamont)	Pieces of plastic bag <1mm	35	Plastic: 1g. Inoculum: 5mL of pig slurry mixed with synthetic medium for methanogens and acclimated to mesophilic anaerobic condition	90	33 L CH ₄ kg ⁻¹ VS	6%	Vasmara and Marchetti, 2016	
PLA (Ingeo)	Pieces of plastic cup <1mm				0 L CH ₄ kg ⁻¹ VS	0%		
PHBV (0,5% HV, ENMAT Y1000P)	31,25 mm x 6,2 mm x 2,1 mm rectangular prism	37	Plastic to inoculum ratio: 0.5 g VS g ⁻¹ VS. Inoculum: digestate from a mesophilic digester. Method: ASTM D5210-90	42	630 L CH ₄ kg ⁻¹	83 %	Ryan et al., 2017(a)	
PHBV (0,5% HV ENMAT Y1000P)	420-840 µm	37	Plastic to inoculum ratio: 0.5 g VS g ⁻¹ VS. Inoculum: digestate from a mesophilic digester. Method : ASTM D5210-90	20	600 L CH ₄ kg ⁻¹	79%	Ryan et al., 2016	
PLA (Fabri-Kal)	Plastic cup ground to 3mm	37	Plastic: 1 g. Inoculum: 10mL of anaerobic inoculum	60	2 L CH ₄ kg ⁻¹ VS	0%	Vargas et al., 2009	
PHB (ENMAT Y3000, TianAn)	0,15 mm	35	Plastic: 125 mg. Inoculum: 50 mL of lab inoculum fed with nutritive media and powdered milk	40	199 L CH ₄ kg ⁻¹ ThOD	50%	Benn and Zitomer, 2018	
PHB (MIREL F1006, Metabolix)					233 L CH ₄ kg ⁻¹ ThOD	59 %		
PHB (MANGO materials)					316 L CH ₄ kg ⁻¹ ThOD	80%		
PHB (Mirel M2100, Metabolix)					316 L CH ₄ kg ⁻¹ ThOD ¹	80%		
PLA (Ingeo 2003D, Natureworks)					1 L CH ₄ kg ⁻¹ ThOD	0%		
PBS (Sigma-Aldrich)					-	0%		
PCL (Sigma-Aldrich)	125-250 µm	37	Plastic: 10 g. Inoculum: mesophilic digestate from an anaerobic digester (37 °C) treating cow manure and green waste	277	-	3-22 %	Yagi et al., 2014	
PLA (Unitika)					-	29-49%		
PHB (Sigma-Aldrich)					-	90%		
PHB (MW 540 000 g.mol ⁻¹ , Biopol BX G08.)					-	100%		
PHBV (MW 397 000 g.mol ⁻¹ , Biopol BX P027)	25 mm of diameter 100 µm of thickness film	37	Plastic: 0.2 g. Inoculum: sludge from a laboratory anaerobic reactor treating wastewater from a sugar factory. Method: ASTM D 5210-91	42	-	29%	Abou-Zeid et al., 2001	
PCL (MW 50 000 g.mol ⁻¹ , Polyscience Inc.)					-	7.5%		
					-	-		
PHA (PHA-4100, Metabolix USA)	1-2 mm wide pellets	37 ± 2	Plastic to inoculum ratio: 4 g L ⁻¹ . Inoculum: sludge from a semi continuous anaerobic digester fed with food waste, olive and cheese waste. Method: ASTM 5511-02	11	-	102%	Greene, 2018	
					Plastic to inoculum ratio: 8 g L ⁻¹ . Inoculum: sludge from a semi continuous anaerobic digester fed with food waste, olive and cheese waste. Method: ASTM 5511-02	-		95%
					Plastic to inoculum ratio: 4 g L ⁻¹ . Inoculum: sludge from a semi continuous anaerobic digester fed with food waste, olive and cheese waste. Method: ASTM 5511-02	20		-
PLA (Natureworks)	19 mm of diameter film	37	Plastic: 35-40 mg. Inoculum: sludge from an anaerobic laboratory reactor fed with wastewater from sugar industry. Method: ASTM D 5210-91	8	-	101%	Abou-Zeid et al., 2004	
PHB (MW 540 000 g.mol ⁻¹ , Biopol BX G08.)				-	29%			
PHBV (MW 397 000 g.mol ⁻¹ , Biopol BX P027)				-	16%			
PCL (MW 50 000 g.mol ⁻¹ , Polyscience Inc.)				-	1.1%			
1,4-butanediol/adipic acid (MW 40 000,GBF)				-	5.5%			
1,4-butanediol (50 mol %) adipic acid (30 mol %)/Terephtalic acid (20 mol %) (MW 47 600, Hüls AG)				-	100%			
PHB (MW 540 000 g.mol ⁻¹ , Biopol BX G08)				-	31%			
PHBV (MW 397 000 g.mol ⁻¹ , Biopol BX P027)				-	17%			
PCL (MW 50 000 g.mol ⁻¹ , Polyscience Inc.)				-	11%			
1,4-butanediol/adipic acid (MW 40 000, GBF)				-	11%			
1,4-butanediol (50 mol %) adipic acid (30 mol %)/Terephtalic acid (20 mol %)			Plastic: 35-40 mg. Inoculum: sludge from an anaerobic digester of a municipal WWTP. Method: ASTM D 5210-91	42	-	11%		
PHBO (90% PHB, 10% HO)		35	Plastic: 100 mg.L ⁻¹ . Inoculum: digestate from an anaerobic digester treating WWTP sludge.	60	-	88%	Federle et al., 2002	
PCL				122	-	0.2%		
PCL	1cm ² film pieces	37 ± 2	Plastic to inoculum ratio: 0.5 g VS g ⁻¹ VS. Inoculum: digestate from a mesophilic anaerobic digester fed with food wastes and manure	30	15.8 ± 21.1 L CH ₄ kg ⁻¹ VS	6.5%	Nunziato et al., 2018	
PCL 40% TPS 60%					133.3 ± 17,5 L CH ₄ kg ⁻¹ VS	32.3%		
PCL 60% TPS 40%					74.2 ± 15.7 L CH ₄ kg ⁻¹ VS	18.5%		
PHBV (PHB/HV; 92/8, w/w)	5x60 mm film			20	-	85%		
Cellophane					-	80%		
PLA (lab)	20x40 mm film	35	Inoculum: anaerobic digested sludge from a WWTP. Method: ASTM D5210	100	-	0%	Shin et al., 1997	
PBS (Elson Green)					-	0%		
PBES (MW 100 000, Sky Green)					-	0%		

Table I.2. Mesophilic biochemical methane potential and operational parameters described in the literature (2/3)

Biodegradable plastic nature	Size and shape	Temperature (°C)	Operational parameters and supplementary data	Time (days)	Methane potential	Percentage of methane conversion	Reference
PHB	Granular form	?	Plastic to inoculum ratio: 10 g VS g ⁻¹ VS. Inoculum: digestate from a WWTP anaerobic digester. Method: ASTM D 5210-91	23	-	100%	Majone et al., 1995
Mater-bi (Starch + PE, AF08H, Novamont)	2x15 cm strips	35	Inoculum : Mixture of sewage sludge treating domestic sewage and paper sludge (3:1 ratio)	40	-	32%	Day et al., 1994
Mater-bi (Starch + PE, AF10H, Novamont)					-	30%	
PHBV (ICI)					-	55%	
PCL (Tone, Union Carbide)					-	5%	
Ecostarplus (starch + PE)					-	12%	
PLA (Argonne A)					-	10%	
PLA (Argonne B)	6x5 cm film	35	Plastic: 5 mg. Inoculum: anaerobically digested domestic sewage sludge	16	0.14 mmol CH ₄	87%	Budwill et al., 1992
PHB	-				96%		
PHBV (13% HV)	-				83%		
PHBV (20% HV)	-				-		
PLA	Granules	37	Plastic: 30 mg. Inoculum: anaerobic sludge from a WWTP. Method: ASTM D 5210	100	-	60%	Itävaara et al., 2002
Mater-bi (60% starch, 40% hydrophilic resin)	Entire bag	35 ± 0.5	Plastic to inoculum ratio: 0.5 g VS g ⁻¹ VS. Inoculum: liquid digestate from an anaerobic digester fed with manure, agro-wastes and residues	15	144 ± 18.4 L CH ₄ kg ⁻¹ VS	-	Calabro et al., 2019
Cellulosic plates	Plate			44	311 ± 37.6 L CH ₄ kg ⁻¹ VS	-	
PCL-Starch blend (55% PCL, 30% Starch, 15% aliphatic polyester)	?	35	Plastic to inoculum ratio: 2g VS L ⁻¹ , Inoculum: 20 mL digestate from a anaerobic digester treating sewage sludge. Method: ASTM E1196-92	139	554 L CH ₄ kg ⁻¹ VS	83%	Cho et al., 2011
PBS				100	11 L CH ₄ kg ⁻¹ VS	2%	
Mater-Bi (PCL+Starch+Glycerin, ZI01U, Novamont)	Film	35	Inoculum: anaerobic sludge from an anaerobic digester. Method: ASTM D 5511-94	81	-	21%	Scandola et al., 1998
PCL (Tone, Union Carbide)	Pellets			-	10%		
PLA (Natureworks, Cargill)	Powder	35	Inoculum: 2mL of digestate from an anaerobic digester treating sewage sludge. Method: ISO 14853 Supplementary data: Cellulose was only degraded to 66% in 28 days.	28	-	0	Massardier-Nageotte et al., 2006
PBAT	2x2 cm film 20 µm of thickness			-	0		
Mater-bi (Starch + PCL, Novamont)	-			-	23%		
PHBV (8.4% HV, ICI)	46.4 µm			35	Plastic: 1% w/w, Inoculum: 10% w/w anaerobic sludge from a WWTP of a sugar factory	30	
PLA (Biopolymer-4043D, Nature Works)	< 2x2 cm	35 ± 2	Inoculum: sludge from a WWTP. Method: ISO 14853	56	-	0%	Narancic et al., 2018
PCL (CAPA 6500, Perstorp)					-	3%	
PBS (PBE 003, NaturePlast)					-	0%	
PHO (Bioplastech R, Bioplastech)					-	12%	
PHB (ENMAT Y1000, TianAn)					-	102%	
TPS (Bioplast TPS, BIOTEC)					-	98%	
PLA/PCL (80/20)					-	0%	
PLA/PBS (80/20)					-	0%	
PLA/PHB (80/20)					-	0%	
PLA/PHO (80/15)					-	2%	
PHB/PHO (85/15)					-	92%	
PHB/PCL (60/40)					-	38%	
PHB/PBS (50/50)					-	15%	
PCL/PHO (85/15)					-	4%	
PCL/TPS (70/30)					-	36%	
Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) 93% HB, 7% HHx	5x5x1 mm Film	38	Plastic to inoculum ratio: 0.7-0.8 g VS g ⁻¹ VS. Inoculum: Digestate from a mesophilic anaerobic digester fed with sludge and fats	80	483.8 ± 35.2 L CH ₄ kg ⁻¹ VS	77 ± 6%	Wang et al., 2018
Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) 93,5% HB 6,5% HHx	5x5x1 mm Flake			40	337.5 ± 100.3 L CH ₄ kg ⁻¹ VS	54 ± 16 %	
Starch (from wheat)/PVOH	Foam	37	Substrate to inoculum ratio: 1 g VS g ⁻¹ VS. Inoculum: digestate from a mesophilic anaerobic digester	10	270 L CH ₄ kg ⁻¹ VS	72.1%	Guo et al., 2011
Starch (from potato)/PVOH					265 L CH ₄ kg ⁻¹ VS	68.6%	
Starch (from maize)/PVOH					248 L CH ₄ kg ⁻¹ VS	75.4%	

Table I.2. Mesophilic biochemical methane potential and operational parameters described in the literature (3/3)

Biodegradable plastic nature	Size and shape	Temperature (°C)	Operational parameters and supplementary data	Time (days)	Methane potential	Percentage of methane conversion	Reference
Cellulose-based metallised film	1x1 cm film	37	Plastic to inoculum ratio: 0.25 g(VS).g(VS) ⁻¹ . Inoculum: digestate from a mesophilic digester treating municipal wastewater biosolids	65	-	74.3%	Zhang et al., 2018
Cellulose-based heat-sealable film					-	86.6%	
Cellulose-based high barrier heat-sealable film					-	84%	
Cellulose-based non heat-sealable film					-	80.4%	
Cellulose diacetate film					-	8.9%	
Starch-based film blend 1					-	18.3%	
Starch-based film blend 2					-	10.2%	
PLA film					-	18.8%	
PLA blend	Pellets				-	2.6%	
Starch (25% amylose) and PVA blend	Film	35	Plastic: 20g. Inoculum: digestate from a wastewater treatment plant. Method: ASTM D5210-92.	25	-	52 ± 1%	Liu et al., 2016
High-amylose starch (80% amylose)-PVA blend				20	-	54 ± 0.5%	
PBS (Enpol G4560, IRE Chemical Ltd, Korea)	5 x 5 mm thin film (100µm)	37	Plastic: 100 mg. Inoculum: mesophilic anaerobic sludge from a wastewater treatment plant. Method: ISO 11734	113	-	2.2%	Dvorackova et al., 2015
Starch:PVOH blends (90/10%)	5x5x1 mm film	38 ± 0.5	Plastic: 2g. Inoculum: supernatant from the second stage of a laboratory scale digester fed with a mixture of primary domestic sludge and food waste	100	140 L(biogas).kg ⁻¹ COD	-	Russo et al., 2009
Starch:PVOH blends (75/25%)					118 L(biogas).kg ⁻¹ COD	-	
Starch:PVOH blends (50/50%)					60 L(biogas).kg ⁻¹ COD	-	
PVOH (Dupont, Australia)					5 L(biogas).kg ⁻¹ COD	-	
PHA	PHA accumulated in activated sludge	37	Plastic: addition of 1mL of PHA accumulating sludge (30 g TS L ⁻¹). Inoculum: 5mL of sewage sludge from a WWTP	15	250 NmL(CH ₄).g(VS) ⁻¹	53 %	Soda et al., 2016
PLA (plastic cup)	2x2x0.5 mm	37 ± 0.5	Plastic to inoculum ratio: 2-4 kg VS m ⁻³ . Inoculum: mesophilic digestate from a wastewater treatment plant anaerobic digester (37°C). Method: EN ISO 11734:2003	280	564 L(biogas).kg(VS) ⁻¹	66%	Bernat et al., 2021
PHB (MW 539 000, Biopol BX G08)	200 µm powder	35	Plastic: 400 mg L ⁻¹ . Inoculum: domestic sewage sludge	30	-	80%	Puechner et al., 1995
Bioceta (Cellulose acetate)	5x5mm 90 µm of thickness fil				-	22%	
Mater-bi ZF03U (PCL + Amidon, Novamont)	5x5mm 35 µm of thickness				-	28%	
PCL (P787, Union Carbide)	5x5 mm 55µm of thickness and 250µm powder				Plastic: 600 and 400 mg L ⁻¹ . Inoculum: domestic sewage sludge	60	
Mater-bi (Novamont)	0,5-1mm film	35 ± 2	Plastic to inoculum ratio: 0.6-1 g TS g ⁻¹ TS. Inoculum: anaerobic sludge from an anaerobic digestion plant treating effluents from a brewery Method: ASTM D5526-94d.	32	220 L CH ₄ kg ⁻¹ VS	-	Mohee et al., 2008
PBAT 93 000 g/mol (Ecoflex, BASF)	5x5 mm film 70 µm of thickness	37 ± 2	Inoculum: mesophilic anaerobic sludge (37 °C) from a municipal waste water-treatment plant	126	-	2.2%	Svoboda et al., 2018

3.1.2) Thermophilic conditions

The degradation of biodegradable plastics under thermophilic conditions has been studied much less than under mesophilic conditions, with fewer data available in the scientific literature. However, the operating conditions and performances of these tests are listed in **Table I.3**.

Only three studies to date have described the digestion of PHB in thermophilic digesters (around 55 °C). Itävaara et al. (2002) and Yagi et al. (2013) found a very high level of biodegradation (between 73 and 88%) in a short time (between 18 and 20 days). Narancic et al. (2018) also noted near-complete mineralization of PHB but, strangely, reported a very long digestion period (127 days). No data regarding PHBV degradation by thermophilic digestion could be found in the scientific literature. As with mesophilic conditions, PHO was weakly degraded (6%) under thermophilic conditions in 50 days (Nunziato et al., 2018).

The methane conversion of PLA under thermophilic conditions was more effective than under mesophilic conditions. Hegde et al. (2018), Narancic et al. (2018), Šmejkalová et al. (2016) and Yagi et al. (2013, 2009) reported a high level of biodegradation of 82 to 90% in a mean digestion time of 90 days. Other authors have found a lower level of biodegradation, between 40 and 60%, with a similar timeframe for the digestion (Itävaara et al., 2002; Tseng et al., 2019; Vargas et al., 2009; Vasmara and Marchetti, 2016). However, it should be noted that the biodegradation levels reported by Itävaara et al. (2002) and Vargas et al. (2009) (60% and 40%, respectively) were not the final biodegradation levels, as the BMP tests were stopped before they reached the plateau of methane production. Šmejkalová et al. (2016) showed that a decrease in the molecular weight of PLA had a positive effect on the biodegradation kinetics. Shrestha et al. (2020) assessed thermophilic degradation of rigid pieces of PLA (1 x 1, 2 x 2, and 3 x 3 cm) and observed negligible methane production of approximately 10 to 30 L CH₄ kg⁻¹ VS.

Thermoplastic starch was found to be readily biodegradable by thermophilic anaerobic digestion, with (Camacho-Muñoz et al. (2020) and Nunziato et al. (2018) observing a high level of biodegradation (between 77 and 92%) in a short period of time (30 days) for a 70% starch and 30% glycerol polymer. Narancic et al. (2018) recorded a biodegradation level of 81 % after 127 days in a solid-state anaerobic digestion system at 52 °C.

Near-complete mineralization of PCL (87-92%) was reported in thermophilic reactors in a time period of 45 to 127 days (Narancic et al., 2018; Yagi et al., 2013a, 2009). Šmejkalová et al. (2016) tested the biodegradation of PCLs with different molecular weights (from 13 kg mol⁻¹ to 58.1 kg mol⁻¹) in the form of a film or powder, and an increase in the molecular weight was associated with a reduction of the biodegradation kinetics. It took 140 days to reach the ultimate methane production of the higher molecular weight PCLs, while 70 days were required for the lower molecular weight PCLs. Only a small difference in the biodegradation level (54-60%) was noted for the different PCLs. Interestingly, Nunziato et al. (2018) found a low level of conversion into methane (11.3%) of PCL films cut into 1-cm square pieces after 30 days.

Most of the publications to date regarding the degradation of biodegradable plastics under thermophilic conditions have focused on PLA, PHB, or PCL. Thus, only one scientific publication has explored the thermophilic degradation of PBAT (Svoboda et al., 2018), which nevertheless represented nearly 13.4% of the production of bioplastics in 2019. Svoboda et al. (2018) reported a very low level of PBAT biodegradation (8.3%) in 126 days at 55 °C. However, they observed a significant reduction in the molecular weight of PBAT from 93 000 to 9430 g mol⁻¹ (Svoboda et al., 2018). Similarly, only three publications have presented data regarding the degradation of PBS, which was weakly mineralized, between 1 and 22%, over a period of 90-120 days (Dvorackova et al., 2015; Narancic et al., 2018; Yagi et al., 2013a).

As with mesophilic conditions, Mater-Bi® (Novamont) is one of the few commercial grades that has been investigated in terms of biodegradation in thermophilic anaerobic digesters. Thermophilic digestion of small pieces (< 1 mm) of plastic bags made of Mater-Bi® resulted in a 55% conversion into methane over a period of 90 days (Vasmara and Marchetti, 2016). Similarly, Calabro et al. (2019) digested whole plastic bags made of Mater-Bi®, and they reported a methane potential of 186 L CH₄ kg⁻¹VS in 30 days. Similarly, Cazaudehore et al. (2021) performed thermophilic anaerobic digestion on three types of commercial coffee capsules composed of biodegradable plastic blends (*i.e.*, Mater-Bi®, Ecovio®, and Vegemat®). After 100 days of digestion, a plateau phase was reached, and the biodegradation rate varied between 49 and 69% of the blends. In parallel, anaerobic digestion under thermophilic conditions of non-commercial blends produced on a laboratory scale has been investigated (Narancic et al., 2018). A synergistic effect of blending biodegradable polymers was observed in the sense that the methane production of

the plastic blends was reported to be equivalent or higher than the methane production of the individual polymers. For example, PCL-TPS (70/30) and PHB-PCL (60/40) blends produced 37% and 18% more methane, respectively, than expected from the performance of the individual polymers during AD.

Table I.3. Thermophilic biochemical methane potential and operational parameters described in the literature (part 1/2)

Biodegradable plastic nature	Size and shape	Temperature (°C)	Operational parameters and supplementary data	Time (days)	Methane potential	Percentage of methane conversion	Reference
PCL (Mn 58.1 kg.mol ⁻¹)	10x10x0,7mm film	55 ± 1	Plastic to inoculum ratio: 0.38 g COD g ⁻¹ of volatile suspended solid. Inoculum: thermophilic digested sludge from a digester	140	663 L biogas kg ⁻¹ TS	60%	Šmejkalová et al., 2016
PCL (Mn 38. kg.mol ⁻¹)	Powder			80	643 L biogas kg ⁻¹ TS	54%	
PCL (Mn 13 kg.mol ⁻¹)				70	676 L biogas kg ⁻¹ TS	57%	
PLA (Mn 44.5 kg.mol ⁻¹)	10x10x0,7mm film			120	677 L biogas kg ⁻¹ TS	74%	
PLA (Mn 3.4 kg.mol ⁻¹)				90	520 L biogas kg ⁻¹ TS	56%	
PLA (Mn 0,35 kg.mol ⁻¹)	Powder			30	625 L biogas kg ⁻¹ TS	84%	
PHB (Biopol)	2x2 cm pieces	52	Plastic: 3-5 g. Inoculum: Anaerobic solid waste treatment plant	20	-	73%	Itävaara et al., 2002
PLA		40		40	-	60%	
Mater-bi (60% starch, 40% hydrophilic resin)	entire bag	55 ± 0.5	Plastic to inoculum ratio: 0.5 g VS g ⁻¹ VS. Inoculum: liquid digestate from mesophilic anaerobic digester fed with manure, agro-wastes and residues shifted progressively to thermophilic condition	30	186 ± 11.8 L(CH ₄)kg ⁻¹ VS	-	Calabro et al., 2019
Cellulose	1x1 cm film	55	Plastic to inoculum ratio: 0.5. Inoculum: sludge from a waste management company	35	280 L biogas kg ⁻¹ VS	18.3%	Shrestha et al., 2020
	2x2 cm film				260 L biogas kg ⁻¹ VS	17.1%	
	3x3 cm film				250 L biogas kg ⁻¹ VS	16.3%	
PLA	1x1, 2x2, 3x3 cm rigid pieces				20 L biogas kg ⁻¹ VS	0%	
PLA (Luminy L130, Total Corbion)	Pellets	55	Plastic: 3 g. Inoculum: sludge from a thermophilic anaerobic digester treating food waste, plant residues and other organic waste product at 55-60 °C	110	224 ± 4 LCH ₄ kg ⁻¹ PLA	-	Tseng et al., 2019
PLA (Biopolymer-4043D, Nature Works)				80	-	88%	
PCL (CAPA 6500, Perstorp)				127	-	95%	
PBS (PBE 003, NaturePlast)				90	-	12%	
PHO (Bioplastech R, Bioplastech)				50	-	6%	
PHB (ENMAT Y1000, TITAN)				127	-	92%	
TPS (Bioplast TPS, BIOTEC)				127	-	81%	
PLA/PCL (80/20)	< 2x2 cm	55 ± 2	Plastic: 15 g. Inoculum: 1 kg of digestate from a thermophilic reactor treating household waste. Method: High solid Anaerobic digestion (ISO 15985)	121	-	90%	Narancic et al., 2018
PLA/PBS (80/20)				121	-	84%	
PLA/PHB (80/20)				80	-	104%	
PLA/PHO (80/15)				66	-	90%	
PHB/PHO (85/15)				66	-	87%	
PHB/PCL (60/40)				80	-	104%	
PHB/PBS (50/50)				121	-	78%	
PCL/PHO (85/15)				66	-	85%	
PCL/TPS (70/30)				80	-	68%	
PBS (Enpol G4560, IRE Chemical Ltd, Korea)	5x5 mm thin film (100µm)	55	Plastic: 50 mg. Inoculum: mesophilic anaerobic sludge from a wastewater treatment plant acclimated to thermophilic temperature	113	-	20.1%	Dvorackova et al., 2015
	5x5 mm thick film (1,02mm)				-	18.1%	
	Powder (320 µm)				-	23.3%	
	5x5 mm thin film (100µm)				-	22.0%	
	5x5 mm thick film (1,02mm)				-	10.3%	
	Powder (320 µm)				-	10.3%	
	Crushed film (>500µm)				936 L biogas kg ⁻¹	97.5%	
PLA (Unitaka, Japan)	1x1 cm film, 25µm of thickness		Inoculum: mesophilic digestate from an anaerobic digester (37 °C) treating cow manure and green waste acclimated to 55 °C. Addition of 20 mL of acclimated sludge to PLA thermophilic digestion during the pre-incubation	60	880 L biogas kg ⁻¹	94.5%	Yagi et al., 2012
	15x34 cm film, 25µm of thickness				893 L biogas kg ⁻¹	96%	
	39x82 cm film, 25µm of thickness				827 L biogas kg ⁻¹	89%	
	Powder 125-250 µm				827 L biogas kg ⁻¹	89%	

Table I.3. Thermophilic biochemical methane potential and operational parameters described in the literature (part 2/2)

Biodegradable plastic nature	Size and shape	Temperature (°C)	Operational parameters and supplementary data	Time (days)	Methane potential	Percentage of methane conversion	Reference
PCL (Mw 65,000, Aldrich)	125-250µm particule	55	Plastic: 10 g. Inoculum: mesophilic digestate from a anaerobic digester (37 °C) treating cow manure and green waste acclimated to 55 °C and addition of diluted sludge acclimated to PCL and PLA	47	-	92%	Yagi et al., 2009
PLA (H-400, Mitsui Chemical)			Plastic: 10 g. Inoculum: mesophilic digestate from a anaerobic digester (37 °C) treating cow manure and green waste acclimated to 55 °C and addition of undiluted sludge acclimated to PCL and PLA	75	-	91%	
			Plastic: 5 g. Inoculum: mesophilic digestate from a anaerobic digester (37 °C) treating cow manure and green waste acclimated to 55 °C and addition of diluted sludge acclimated to PCL and PLA	100	-	79%	
Mater-bi (PCL + Amidon, Novamont) PLA (Ingeo)	Small piece of plastic bags <1mm	55	Plastic: 1g. Inoculum: 5 mL of pig slurry mixed with synthetic medium for methanogens and acclimated to mesophilic anaerobic condition	90	303 L CH ₄ kg ⁻¹ VS 267 L CH ₄ kg ⁻¹ VS	55% 56%	Vasmara and Marchetti, 2016
PLA (Fabri-Kal, Inc., Kalamazoo) PBS (Sigma-Aldrich)	Plastic cup ground to 3mm	58	Plastic: 1 g. Inoculum: 10 mL of anaerobic inoculum	56	187 L CH ₄ kg ⁻¹ VS	40%	Vargas et al., 2009
PCL (Sigma-Aldrich)				100	-	1-3%	
PCL (Sigma-Aldrich)	125-250 µm	55	Plastic: 10 g. Inoculum: mesophilic digestate from a anaerobic digester (37 °C) treating cow manure and green waste acclimated to 55 °C. Pre-incubation of the inoculum with 20 mL of sludge acclimated to PLA	45	-	84%	Yagi et al., 2013
PLA (Unitika)				80	-	82%	
PHB (Sigma-Aldrich)				18	-	83-88%	
PLA (Naturewoks 4043D)	Sheets	52 ± 2	Plastic to inoculum ratio: 0.5 g VS g ⁻¹ VS . Inoculum: digestate from a mesophilic anaerobic digester fed with food wastes and manure shifted to thermophilic temperature	36	409 L CH ₄ kg ⁻¹ VS	90%	Hegde et al., 2018
PCL TPS (70% starch from MP Biomedicals LLC and 30% glycerol) 80% PCL 20 % TPS	1cm ² film	52 ± 2	Plastic to inoculum ratio: 0.5 g VS g ⁻¹ VS. Inoculum: digestate from a mesophilic anaerobic digester fed with food wastes and manure shifted to thermophilic temperature (10 days)	30	44.4 ± 19.5 L CH ₄ kg ⁻¹ VS	11,3%	Nunziato et al., 2018
					320 L CH ₄ kg ⁻¹ VS	77.1%	
PBAT 93 000 g/mol (Ecoflex, BASF)	5x5 mm film 70 µm of thickness	55 ± 2	Inoculum: mesophilic anaerobic sludge (37 °C) from a municipal waste water-treatment plant acclimated to thermophilic temperature (55 °C) for two weeks	126	-	8.3%	Svoboda et al., 2018
PLA (plastic cup)	2x2x0.5 mm	58 ± 0.5	Plastic to inoculum ratio: 2-4 kg VS m ⁻³ . Inoculum: mesophilic digestate from a wastewater treatment plant anaerobic digester (37 °C) acclimated to 58 °C for 14 days. Method: EN ISO 11734:2003	60	835	90%	Bernat et al., 2021

3.1.3) Summary of the mesophilic and thermophilic AD performances

The biodegradability performances observed in the literature of the main biodegradable polymers available on the market (*i.e.*, PHB, PHBV, PLA, PCL, PBS, PBAT, and TPS) are summarized in **Figure I.3**. The data are represented for both mesophilic and thermophilic conditions, with the number of experiments and studies reported. Not all biodegradable plastics have received the same degree of attention, as the majority of studies have focused on mesophilic degradation of PHB, PHBV, PCL, and PLA, and on thermophilic degradation of PLA. Overall, anaerobic digestion of biodegradable plastics under thermophilic conditions is more efficient than under mesophilic conditions (Nunziato et al., 2018; Vargas et al., 2009; Vasmara and Marchetti, 2016). For example, the degradation plateau of PCL and PLA is generally reached in less than 100 days under thermophilic condition, while this same plateau does not occur in less than 300 days under mesophilic conditions (Bernat et al., 2021; Yagi et al., 2014a, 2013a). According to these observations, biodegradable polymers can be classified into three categories based on their degradation performances in mesophilic and thermophilic AD (**Figure I.3 and Figure I.4**).

- a) Polymers that are readily biodegradable. These reach complete or near-complete biodegradation in a relatively short period of time (10-50 days) under both mesophilic and thermophilic anaerobic digestion conditions. This is the case for PHB and TPS. It can be assumed that PHBV also belongs in this category, despite the absence of data for thermophilic conditions, in light of its rapid degradation (31-95% in approximately 35 days) under mesophilic conditions. These polymers could potentially be processed in biogas plants without increasing the hydraulic retention time used at the industrial scale (generally approximately 30-50 days for system treating biowastes).
- b) Polymers that are biodegraded very slowly under mesophilic conditions and much faster under thermophilic conditions. This is the case for PLA and PCL. It was previously demonstrated that PLA and PCL exhibited low levels of biodegradation under mesophilic conditions (over a period exceeding 280 days) (Bernat et al., 2021; Yagi et al., 2014a), which is much higher than the typical retention time observed in industrial biogas plants (around 30-50 days for biowastes, 50-120 days for agricultural wastes). By contrast, under thermophilic conditions, the hydrolysis is enhanced, resulting in much higher degradation rates. This has been documented very well by Bernat et al. (2021), who reported a biogas

production rate under mesophilic conditions of between 1.4 and 2.6 L kg⁻¹VS d⁻¹ and under thermophilic conditions of around 33 L kg⁻¹ VS d⁻¹. Under mesophilic conditions, hydrolysis is the limiting step, and pretreatment strategies are a promising way to enhance degradation under mesophilic conditions (Brémond et al., 2018; Carrere et al., 2016).

- c) The polymers that are biodegradable under industrial compositing conditions according to the EN 13432 standard and poorly or not at all biodegraded by either mesophilic or thermophilic AD. This is the case for PBAT and PBS. The operational conditions and particularly the microorganism diversity between anaerobic and aerobic processes can probably explain such observations.

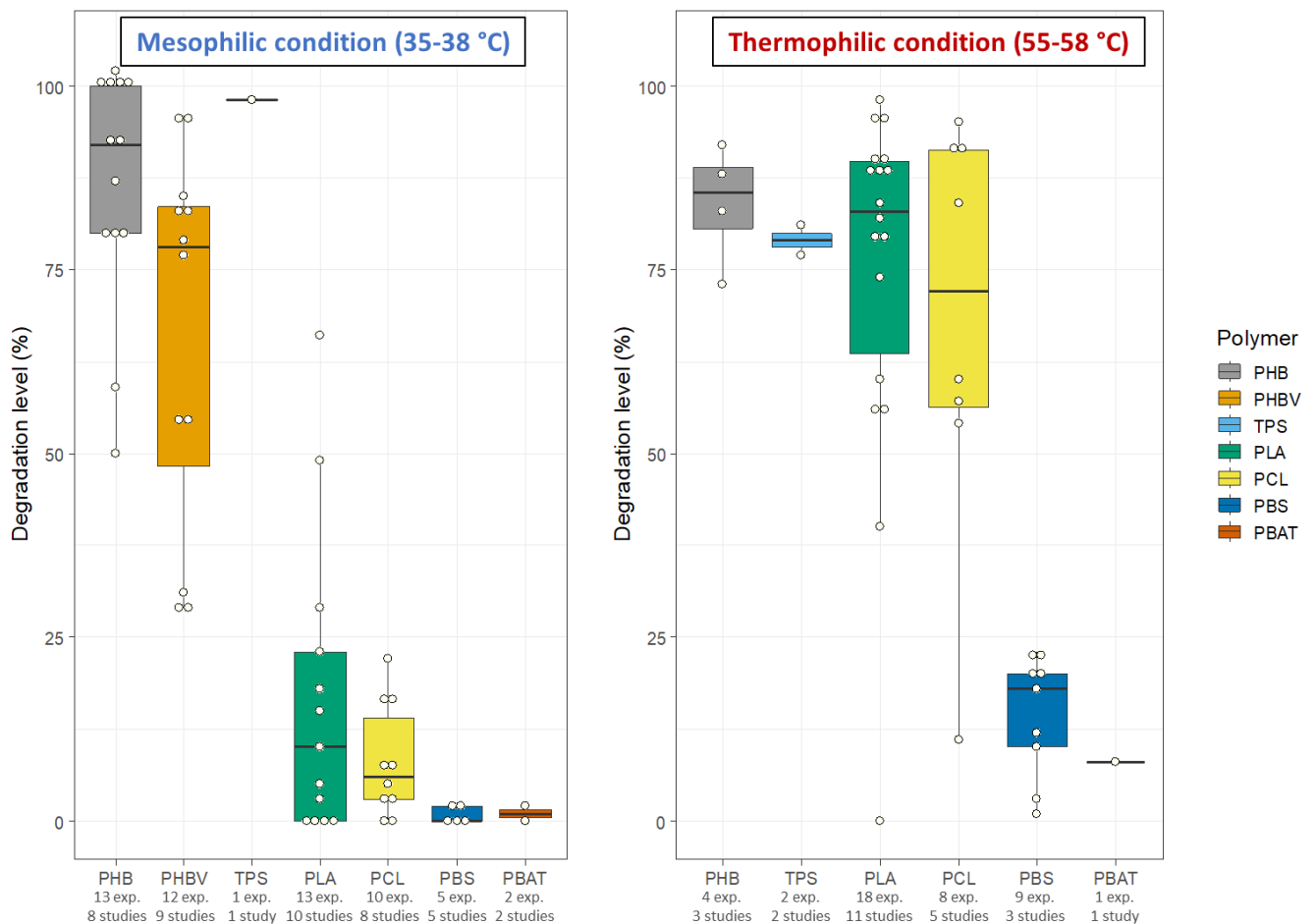


Figure 1.3: Biodegradation (%) reached at the end of the BMP tests under mesophilic and thermophilic condition. The number of experiments (noted exp.), and the number of studies per biodegradable polymers are also presented. White circles correspond to each experiment reported. The Figure is based on data provided in Table I.2 and Table I.3.

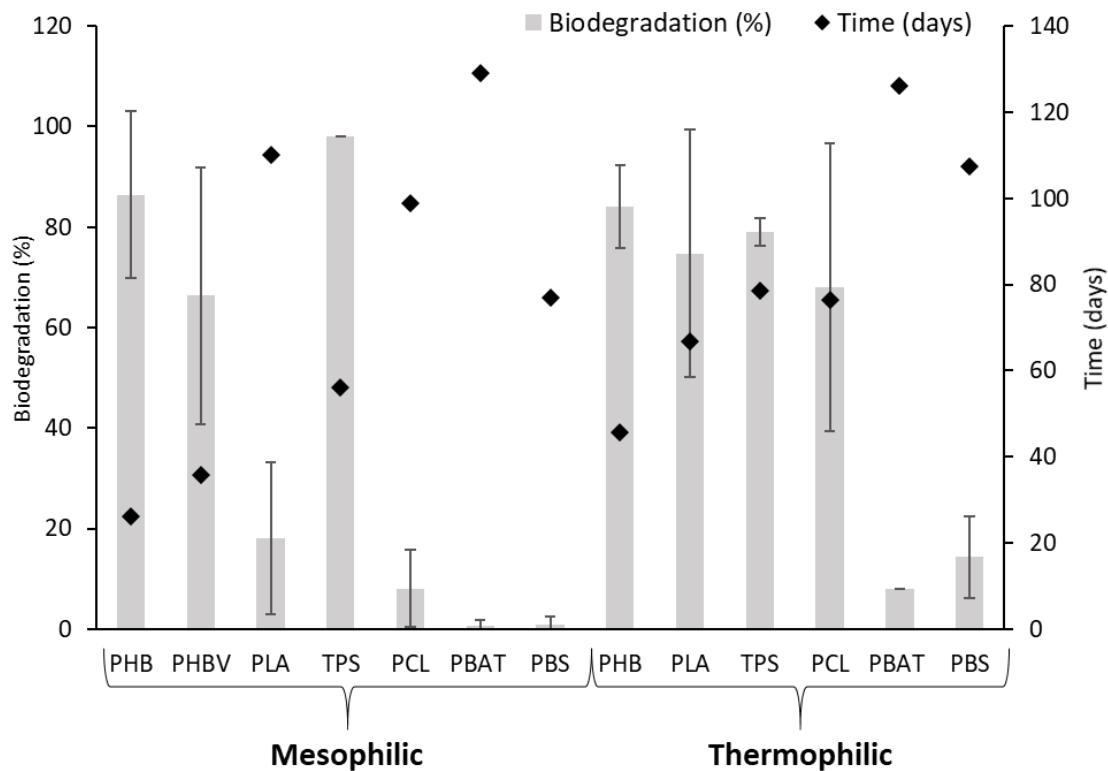


Figure I.4. Mean biodegradation (%) vs mean time of incubation (days) for the various data on biodegradable plastics found in literature for both mesophilic and thermophilic conditions. The Figure is based on data provided in Table I.2 and Table I.3.

3.2) Pilot-scale and full-scale experiments

Although BMP is a reliable, quick, and powerful tool to evaluate the biodegradability of biodegradable plastics, it cannot be used to simulate the future performances of industrial biogas plants. Indeed, biodegradation tests in BMP do not sufficiently reflect the industrial process and this can lead to overestimation of the biodegradation level. For this, pilot experiments with feeding under continuous or semi-continuous conditions are necessary. Pilot experiments provide valuable insights regarding process performances and stability over long periods of time. To date, few studies have investigated the anaerobic digestion of biodegradable plastics on a pilot scale (Benn and Zitomer, 2018; Venkiteshwaran et al., 2019; W. Zhang et al., 2018). Benn and Zitomer, (2018) performed a continuous anaerobic co-digestion experiment on untreated and pretreated PHB (ENMAT™ Y3000 and Mirel™ F1006). Pretreatment consisted of hydrolysis at pH 1 and 55 °C for 24 or 48 h. Eight continuously stirred tank reactors (CSTR) with 2.5 L working volumes were operated at 35.7 °C. Firstly, these reactors were fed for 115 days with synthetic municipal primary sludge (a

mixture of dog food, basal nutrients, and alkaline media) at an organic loading rate of 3.6 g COD d⁻¹ L⁻¹. The reactors were then fed, in duplicate, with a mixture of synthetic municipal primary sludge and PHB samples (two PHB grades, pretreated and untreated), with plastics corresponding to 20% of the initial organic loading rate. When plastics were co-digested, the methane production increased by 17% compared to synthetic sludge alone. Degradation of the untreated PHB was estimated to be between 78 and 92%. Pretreatment resulted in a 5% increase in the conversion of PHB into methane. Synergistic effects of co-digestion of biodegradable plastics with food wastes or pig slurry have also been reported elsewhere (Hegde et al., 2018; Vasmara and Marchetti, 2016). The addition of biodegradable plastics, which are substrates with a high carbon content, can help adjust the carbon-nitrogen ratio to the recommended values and prevent both nutrient limitation and ammonia toxicity (Benn and Zitomer, 2018; Esposito et al., 2012; Hawkes, 1980b). Similarly, Zhang et al., (2018) looked at anaerobic biodegradation of nine different biodegradable plastics (according to EN 13432 standard) in batch-scale and semi-continuous pilot co-digestions. Eight of the plastics (three cellulose-based films, one cellulose diacetate film, two starch-based films, and one PLA film) were digested in the form of 1 x 1 cm square films, while a PLA blend was digested in pellet form. The feedstock in the semi-continuous trial was synthetic food waste, cardboard packaging, and bioplastics at volatile solids (VS) ratios of 80:18:2. The digesters exhibited stable performances and there was no evidence throughout the 144-day experimental period of potential inhibition of the feeding with biodegradable plastics. Cellulose-based films exhibited a high weight loss of 57.4 to 93.4% during the test, whereas starch-based films only lost a small part of their weight ranging from 2.1 to 7.9%. A PLA film lost 57.5% of its mass, while PLA pellets only lost 3.1% of their mass, thus demonstrating the impact of the initial morphology of the polymer. In parallel, Wang et al. (2012) investigated co-digestion of a mixture of kitchen garbage and PLA in two different reactor configurations. A two-phase anaerobic system consisting of a hyperthermophilic (80 °C) reactor and a thermophilic (55 °C) reactor was compared with a single-phase thermophilic reactor (55 °C). Two types of plastic bags (100% PLA and 70% PLA) were used as pieces that were 2 x 2 x 0.1 mm in size. The methane conversion ratios of the two-phase systems were 82.9% and 80.8%, respectively, which was higher than the 70.1% conversion ratio of the single-phase system (Wang et al., 2011). Dolci et al., (2022) have also recently investigated the semi-continuous co-digestion tests of two MaterBi® biodegradable bags together with food wastes

(11/91 % in term of COD). Low degradability was observed as regards bioplastic bags residues, the overall mass of undigested pieces resulted equal to 93% (bag 1) and 69% (bag 2) of the inserted weight suggesting that an aerobic composting phase is necessary to achieve satisfactory biodegradation yield.

While the compostability of biodegradable plastics at an industrial scale has been well documented, only one test has been carried out at the European level on real biogas plants (Kern et al., 2018, European Bioplastics, 2015). Recently, Kern et al. (2018) performed real tests on various biogas plants on Mater-Bi[®] resin (Novamont). The biodegradation was performed in four German AD biogas plants: two discontinuous batch processes operated at thermophilic or mesophilic temperature and two horizontal plug-flow technologies operated at thermophilic temperature. All of the technologies tested resulted in a reduction of the biodegradable plastics content in terms of dry matter by 40% to 58%, except for the mesophilic process, for which no significant degradation was observed. For all the AD plants, the process was coupled with a composting process followed by maturation. After maturation of the compost, full degradation of the plastics in terms of dry matter was measured.

3.3) Microbial communities involved in biodegradable plastics AD

The microorganisms involved in the AD process have undergone intensive study in recent years (Azizi et al., 2016; Castellano-Hinojosa et al., 2018; De Vrieze et al., 2018; Guo et al., 2015; Levén et al., 2007; Li et al., 2015; Liu et al., 2018; Sundberg et al., 2013). However, little is known about the specific microorganisms involved in AD of biodegradable plastics. In the reviews by Shah et al. (2014, 2008), Emadian et al. (2017), and Pathak and Navneet (2017), several microorganisms were reported to degrade biodegradable plastics, although most of them were not from an anoxic environment.

Yagi and co-workers have produced several publications regarding the detection of microorganisms that participate in anaerobic digestion of biodegradable plastics. For this, they performed denaturing gradient gel electrophoresis of the 16S rDNA amplicons (RT-PCR-DGGE). In their first two papers, they did not successfully identify the microorganisms responsible for

thermophilic digestion of PLA (Yagi et al., 2011a, 2010). However, they highlighted that a number of the microorganisms participating in anaerobic digestion of cellulose and PLA at 55 °C were different. Subsequently, Yagi and co-workers carried out the same experiment under mesophilic and thermophilic conditions for PLA, PCL, and PHB (Yagi et al., 2014a, 2013a). This time, they successfully identified several microorganisms involved in the anaerobic digestion process of these polymers, but their precise involvement in the process remains unknown. Under mesophilic conditions, *Arcobacter thereius* participated in the degradation of PHB and PCL, while *Xanthomonadaceae bacterium* and *Mesorhizobium* sp. participated in the digestion of PLA. Under thermophilic conditions, they successfully identified *Bacillus infernus* and *Propionibacterium* as microorganisms participating in the anaerobic degradation of the three biodegradable plastics. They then found that *Peptococcaceae bacterium Ri 50*, *Bacteroides plebeus*, and *Catenobacterium mitsuokai* were specific to anaerobic digestion of PHB. Similarly, *Ureibaccillus* was specific to PLA digestion. Finally, Venkiteshwaran et al. (2019) examined the microbial community shift during anaerobic co-digestion of PHB and synthetic primary sludge (dog food and basal nutrients) by Illumina sequencing. No previously known PHB degraders were observed in the co-digesters. OTUs associated with *Deferribacter*, *Geobacter*, *Kosmotoga*, and *Ruminococcus* were found to correlate positively with increased methane production resulting from PHB co-digestion. Peng et al. (2022), examined the microbial populations during the digestion and the co-digestion of PLA/PBAT blend with food waste under both mesophilic and thermophilic condition. Under mesophilic condition, no significant differences were noted between microbial communities from reactors fed with or without biodegradable plastics. However, under thermophilic conditions, the three genera: *Clostridium* (sensu stricto), *Streptococcus* and *Caldicoprobacter* were highlighted for being responsible for the difference between communities from the reactor fed with a blend of PLA/PBAT and other reactors (i.e., blank reactor, reactor fed with food waste and the reactor performing co-digestion of food waste and PLA/PBAT). Tseng et al. (2019) examined the microbial populations involved in anaerobic digestion of PLA under thermophilic conditions by PCR-DGGE. They pointed out the importance of the genus *Tepidimicrobium* as a key genus in the anaerobic degradation of PLA. Subsequently, (Tseng et al., 2020a) isolated and characterized a strain of *T. xylanilyticum* from this digester. Unlike the type strain of *T. xylanilyticum*, that strain was able to consume lactate and it could produce H₂, CO₂, and acetate. They also found that the

physicochemical depolymerization of PLA to lactate was inhibited by the accumulation of lactate. *T. xylanilyticum* promoted PLA degradation by scavenging the lactate produced, thereby providing methanogens with CO₂, H₂, and acetate (Tseng et al., 2020a). More recently, Cazaudehore et al. (2021) identified a member of the *Tepidimicrobium* genus as key microorganisms involved in thermophilic anaerobic digestion of three coffee capsules composed of biodegradable plastics (*i.e.*, Mater-Bi®, Ecovio®, and Vegemat®). These coffee capsules are made of plastic blends and their exact composition is not known, so the genus *Tepidimicrobium* may be involved in anaerobic digestion of polymers other than PLA. More studies on *Tepidimicrobium* are needed to gain a better understanding of their involvement in the biodegradation of other biodegradable polymers. Unfortunately, Cazaudehore et al. (2021) were unable to identify the key microorganisms involved in mesophilic anaerobic digestion of these biodegradable blends, probably due to the low efficiency of the mesophilic process on such biodegradable plastics blends.

3.4) Factors influencing the anaerobic biodegradability of biodegradable plastics

The biodegradation of polymers is generally summarized as occurring in three main stages, during which a combination of biological and chemical mechanisms are involved. In the first step, called deterioration or biodeterioration, the physical and the chemical properties of the plastic are altered. Then, during the fragmentation or disintegration step, the polymer is cleaved into a simpler form. Finally, the fragmented plastic is assimilated and degraded by microorganisms (Abraham et al., 2021; Artham and Doble, 2008; Meereboer et al., 2020). Abiotic (*i.e.*, pH, temperature, and moisture content) and biotic (*i.e.*, the presence of specific microorganisms or enzymes) parameters have a significant impact on biodegradation performances. In parallel, several factors related to the physical and the chemical properties of the polymer can influence the biodegradation rate (Ahmed et al., 2018; Artham and Doble, 2008). Parameters associated with the surface condition (*e.g.*, surface area, morphology, and hydrophilic and hydrophobic properties), the first-order structures (*e.g.*, chemical structures, molecular weight, and molecular weight distribution), and the higher-order structures of polymers (*e.g.*, glass transition temperature, melting temperature, modulus of elasticity, crystallinity, and crystal structure) have a pronounced impact on the biodegradation process (Tokiwa et al., 2009). Similarly, the exposition to specific condition before the introduction in an anaerobic digestion reactor has been investigated and will be further discussed in the next section. Among the various biotic

parameters, the specific microorganisms present in the inoculum can influence the rate of biodegradation. The impact of the inoculum origin, composition, and potential acclimation will be discussed more in-depth in Section 5.3. The impacts of some of factors influencing the anaerobic biodegradability have been investigated and reported in the literature.

An increase in the temperature of the anaerobic digestion process promotes the conversion of biodegradable polymers into methane (Nunziato et al., 2018; Vargas et al., 2009; Vasmara and Marchetti, 2016; Yagi et al., 2013a, 2012a). This is demonstrated by the fact that thermophilic AD provides better results than mesophilic AD. The mobility of the polymeric chains are increased with temperature, above the glass transition temperatures the disorganization of the chain facilitate the accessibility to chemical and biological degradation (Lucas et al., 2008; Maity et al., 2021). Moreover, faster biochemical reaction rate are found at higher temperatures (Gebreeyessus and Jenicek, 2016). Shi and Palfery, (2010) have highlighted the impact of the glass transition temperature on the anaerobic biodegradability of PLA. The glass transition temperature of PLA was reduced from 60.7 °C to 49.4 °C by the use of different plasticizers. Both the original and the modified PLA were then digested at 50 °C. Interestingly, the time to fully degrade the modified PLA was approximately 5 months, while 9 months were required for the untreated PLA. These authors have assumed that when the degradation temperature is higher than the glass transition temperature of the plastic, the amorphous part of the polymer becomes readily accessible to microorganisms, which then accelerates biodegradation of the plastic (Shi and Palfery, 2010). Ryan et al. (2016) and Yagi et al. (2009) investigated the impact of size reduction (increase in the surface area) of rigid plastics composed of PHBV and PCL. Both studies found that a smaller particle size correlated with faster degradation; however, the final methane potential was not increased. By contrast, Yagi et al. (2012) investigated the impact of size reduction of PLA film in thermophilic anaerobic digestion. They observed that the anaerobic biodegradation rate of crushed or small pieces of PLA film was lower than with large pieces of film or uncut film material. They assumed that the small pieces of PLA floated on the sludge, thereby reducing the total surface in contact with the methanogenic sludge. Crystallinity is another important physical parameter that can affect anaerobic digestion performances, and amorphous parts are more readily degraded than the crystalline fraction (Meereboer et al., 2020; Monlau et al., 2013a). In polymers, the crystalline fractions are more ordered, while the amorphous fractions are more flexible, thereby

making them more vulnerable to microorganism-mediated hydrolysis (Abraham et al., 2020). The amorphous region is known to allow permeation of moisture and microorganisms. Its degradation, therefore, increases the surface area of the available crystalline region (Meereboer et al., 2020). Molecular weight is another important parameter, and it has been reported that the molecular weight was negatively correlated with the biodegradation rate (Abraham et al., 2021). Šmejkalová et al. (2016) tested the biodegradation of different molecular weight PLAs and PCLs by thermophilic anaerobic digestion. They observed that a reduction in the polymer molecular weight was associated with an enhancement of the biodegradation kinetics (Šmejkalová et al., 2016). An additional complicating factor is the complexity of the biodegradable plastic materials, which can affect their physicochemical properties and thus their rates of biodegradation (Artham and Doble, 2008). Indeed, commercial plastics are not merely composed of a single chemically homogenous component. Rather, they can contain different polymers (blends) or low molecular weight additives (Artham and Doble, 2008; Meereboer et al., 2020).

4) Strategies to enhance the biodegradability of biodegradable plastics

As previously described, especially under mesophilic conditions, most biodegradable plastics except starch and PHAs exhibit a very low degree of biodegradability by anaerobic digestion (Bátori et al., 2018a; Narancic et al., 2018). Furthermore, most biodegradable polymers have slower biodegradation kinetics than the conventional substrates (manures, biowastes, sludges, etc.) that are fed into biogas plants. This point is important for the development of efficient organic waste management systems, as it implies that the residence time of biodegradable plastics in AD is longer than the current residence times of industrial AD reactors (Narancic and O'Connor, 2019). In order to enhance the biodegradability kinetics and ideally the methane potential, three main strategies can be implemented: pretreatment technologies, the incorporation of additives into the polymers (fibers, enzymes, calcium carbonate, etc.), and inoculum acclimation or bioaugmentation (Calabro et al., 2019; Ryan et al., 2017b, 2017a).

4.1) Pretreatment technologies

Pretreatments have been widely investigated in the past several decades to enhance the biodegradability of various substrates (*e.g.*, sludges, manures, animal by-products, municipal solid wastes, algae) by anaerobic digestion (Brémond et al., 2018; Carlsson et al., 2012; Carrere et al.,

2016). Pretreatment is seen as a strategy to enhance AD in terms of increased methane yield, faster kinetics, and solids reduction (Carlsson et al., 2012; Monlau et al., 2012). Pretreatment can be applied at different levels of a biogas plant: at the entrance of the digester, between the digester and the post-digester, or in recirculation of the solid digestate, in which case it is called “post-treatment”. Four main categories of pretreatment can be distinguished: mechanical, thermal, chemical, and biological (Carlsson et al., 2012; Carrere et al., 2016). Mechanical pretreatments generally consist of grinding, shredding, extrusion, or cavitation, and they are widely applied in current biogas plants (Carrere et al., 2016; Monlau et al., 2012). Regarding chemical pretreatments, for the time being, they are limited to lab-scale applications due to their high costs and their environmental consequences, even though some alkaline treatments (calcium hydroxide notably) have displayed promising results, especially with lignocellulosic substrates and animal by-products (Brémond et al., 2018). Thermal pretreatment can be applied at low temperatures (50 to 70 °C for a few hours or days) or high temperatures (170 to 190 °C for a few minutes with or without pressure). The most used commercial processes are: THP from Cambi®, with more than 30 operating facilities; and Biothelys® or Exelys® from Veolia, with approximately 10 facilities constructed to date (Carrere et al., 2016). Biological pretreatments can be divided into three parts: enzymatic, anaerobic, and aerobic (Brémond et al., 2018). For enzymatic pretreatment, which appears to be one of the most promising biological options, the three key players in the enzyme market are Novozymes (Denmark), DSM (Netherlands), and Dupont (USA) (Brémond et al., 2018).

Few studies to date have investigated the use of pretreatments to enhance the biodegradability of biodegradable plastics by AD (Benn and Zitomer, 2018; Vargas et al., 2009; Yagi et al., 2009). The main results obtained from the data in the literature are presented in **Table I.4**. First of all, some authors have investigated the impact of mechanical size reduction on the anaerobic biodegradability of various biodegradable plastics (Ryan et al., 2016; Yagi et al., 2012a, 2009). Yagi et al., (2009) investigated the impact of particle size reduction on the anaerobic biodegradability of PCL. Interestingly, no differences in the biodegradation of PCL powder (after 60 days of incubation) for the various particle size distributions (0–250 µm, 250–500 µm, 0–125 µm, and 125–250 µm) were observed, and approximately 80-90% biodegradability was achieved for all conditions. However, particle size reduction has a positive effect on the kinetics of biodegradation,

as small particle sizes result in faster degradation kinetics. Similarly, Ryan et al. (2016) investigated the impact of particle size (from 10 μm to 3 900 μm) on the anaerobic biodegradability of PHBV. For all of the granulometry tested, the methane production was approximately $580 \pm 12 \text{ mL/g}$, corresponding to a biodegradability of $86 \pm 2\%$. Nonetheless, with particle sizes below 840 μm , longer lag phases were observed. That observation could be explained by a faster hydrolysis of the small-sized plastic particles leading to a temporary inhibition of methanogenesis due to VFAs accumulation. On the other hand, Yagi et al. (2012) observed a slower rate of anaerobic digestion with crushed or small pieces of PLA film than with large pieces of film or with uncut film under thermophilic conditions. They assumed that the small pieces of PLA floated on top of the sludge, thereby reducing the total surface area in contact with the methanogenic sludge and thus negating the advantage of size reduction.

The data in the literature indicates that thermal and thermo-chemical pretreatments have also been investigated for the degradation of plastics such as PLA (Battista et al., 2021; Hobbs et al., 2019; Vargas et al., 2009) and PHB (Benn and Zitomer, 2018). For instance, Vargas et al. (2009) investigated the impact of steam pretreatment (3 h, 120 $^{\circ}\text{C}$) on the anaerobic digestion of PLA under both mesophilic and thermophilic conditions. Under mesophilic conditions, the methane potential was enhanced from 2 to 90 $\text{L CH}_4 \text{ kg}^{-1}\text{VS}$, whereas under thermophilic conditions, the methane potential was enhanced from 187 to 225 $\text{L CH}_4 \text{ kg}^{-1}\text{VS}$. Hobbs et al. (2019) investigated the impact of alkaline pretreatment (21 $^{\circ}\text{C}$, $\text{pH} > 11$, 15 days) to enhance the methane potential of crystalline and amorphous PLA in co-digestion with food wastes. Amorphous and crystalline PLA reached near-complete solubilization at 97% and 99%, respectively, when alkaline pretreatment was applied (Hobbs et al., 2019). The treated PLA BMP test in co-digestion with food wastes did not exhibit a lag phase and it produced 1021 NmL of CH_4 at day 70 compared to 756 NmL of CH_4 for untreated PLA (Hobbs et al., 2019). However, a 15-day pretreatment period does not seem suitable for industrial purposes. Benn and Zitomer, (2018) have also investigated the impact of alkaline pretreatment (35-55 $^{\circ}\text{C}$, $\text{pH} = 10-12$, 24-48 h) to enhance the methane potential of various PHAs and PLAs under mesophilic conditions. For all the polymers tested, alkaline pretreatment allowed enhancement of the methane potential, especially for PLA, for which the methane potential increased from 1 $\text{L CH}_4 \text{ kg}^{-1}\text{VS}$ to 86 $\text{L CH}_4 \text{ kg}^{-1}\text{VS}$ (Benn and Zitomer, 2018). Furthermore, Benn and Zitomer, (2018) reported a reduced lag time for pretreated PLA compared to untreated

PLA. Calabro et al. (2019) have also investigated the impact of pretreatments (pre-digestion, alkaline, mechanical) on the anaerobic digestion of Mater-Bi®-compostable bags (Calabro et al., 2019). In one of their experiments, they reported a positive impact of alkaline pretreatment (NaOH 5% w/w, 24 h) on the methane potential, resulting in a 70% enhancement, but pre-digestion and mechanical pretreatment did not result in a discernible enhancement. Wang et al. (2011) also investigated a novel combination of anaerobic hyperthermophilic treatment in the presence of ammonia followed by thermophilic anaerobic digestion of two grades of PLA. Such a combination allowed final methane conversion ratios of 82% and 77% to be achieved after 22 days with two types of PLA. Nonetheless, some other studies have also highlighted the absence of an impact, or even a negative impact, when thermo-chemical pretreatments were used with biodegradable plastics (Battista et al., 2021; Endres and Siebert-Raths, 2011). For instance, Endres and Siebert-Raths, (2011) attempted pretreatment of PLA at 70 °C for 1 h with no pH control, but this resulted in less biomethane production than with untreated PLA. More recently, Battista et al. (2021) also investigated the impact of thermo-acid (48 h, pH = 2, HCl) and thermo-alkaline (48 h, pH = 12, NaOH) pretreatments on the AD performances of PLA. Interestingly, Battista et al. (2021) demonstrated that both of these pretreatments did not enhance the methane potential of the PLA.

No studies to date have reported regarding the use of biological pretreatments (*e.g.*, aerobic, fungal, enzymes) to enhance the anaerobic degradation of biodegradable plastics. Nonetheless, such pretreatment technologies have already been shown to improve the biodegradability of organic wastes (Brémond et al., 2018; Carrere et al., 2016; Mahdy et al., 2015; Yu et al., 2013). Recently, several enzymes from fungal strains have been found to increase the solubilization and degradation of various biodegradable plastics such as PLA, PHA, and PCL (Emadian et al., 2017a; Oda et al., 2000; Panyachanakul et al., 2019; Roohi et al., 2017; Tokiwa et al., 2009). Among the enzymes that have been found to enhance the degradation of biodegradable plastics are esterases, proteases, lipases, cutinases, and PHA depolymerases (Narancic and O'Connor, 2019; Numata et al., 2009; Oda et al., 2000; Tokiwa and Calabia, 2006). For instance, Oda et al. (2000) tested the polylactide-degrading activity of 56 commercially available proteases. Purafect 4000L, Protin A, and Savinase® 16.0L were the most efficient enzymes for PLA degradation, with polylactide-degrading activities of 15.8, 23.4, and 41.9 U/mg protein,

respectively. The application of enzyme products in anaerobic digestion can involve three digestion configurations: (1) pretreatment with enzymes followed by a single-stage anaerobic digestion, (2) direct enzyme addition to a single-stage digester, and (3) direct enzyme addition at the first stage (hydrolysis and acidification) of a two-stage digestion system (Romano et al., 2009).

4.2) Addition of additives into plastic composition to enhance biodegradable plastics biodegradability

Another strategy to enhance the biodegradability kinetics and rate is direct addition of co-product fillers in polymers, such as fibers (Ryan et al., 2017b, 2017a), calcium carbonate (Hegde et al., 2018), lactic acid monomer (Lee et al., 2016), eggshells (González Petit et al., 2015), or enzymes (<https://www.carbiolice.com/>). It has been shown that the addition of fillers in plastics not only reduces the cost but also makes use of recycled waste streams and enhances properties such as fluid barrier properties and thermal stability (Ryan et al., 2017b, 2017a; Syafri et al., 2017). Ryan et al. (2017a, 2017b) recently investigated the addition of oak wood flour (WF) as a filler in raw PHBV and maleated PHBV. As shown in **Table I.4**, Ryan et al. (2017b) investigated the biodegradation of PHBV with different ratios of WF (0%, 20%, and 40%). The lag time for CH₄ production decreased linearly with increasing WF fraction. The decrease in lag time can be attributed to increased surface accessibility for microbial enzymatic hydrolysis as a result of fibers being present at the composite surface. Ryan et al. (2017b) have reported the main mechanisms involved in anaerobic degradation of biodegradable plastics in the presence of fillers such as wood fibers. When exposed to moisture, the WF becomes increasingly saturated, leading to cracking, which exposes additional surface area to enzyme hydrolysis (Ryan et al., 2017a). By contrast, the extent of the composites biodegradation (*i.e.*, the ultimate CH₄ production) decreased linearly with increasing WF content, due to incomplete biodegradation of the lignin-rich WF, which is not degraded during the AD process (Barakat et al., 2012; Ryan et al., 2017a). Ryan et al. (2017a) have also investigated the impact on the AD process of two fiber-matrix compatibilization treatments that are generally applied to enhance the moisture resistance of biocomposites: (1) hydrophobic silane treatment of the wood fiber and (2) grafting of hydrophilic maleic anhydride groups to the PHBV matrix (mPHBV). Neat PHBV and mPHBV produced 94 and 95% of their respective theoretical CH₄ yields. For PHBV mixed with 20% fibers, the maximum rate of anaerobic biodegradation was enhanced by 30% for the maleic anhydride treatment and 92% for the silane treatment relative to

untreated samples. Similarly, Hegde et al. (2018) have investigated the addition of calcium carbonate (5% w/w) as a filler in a PLA/PBS blend. Interestingly, calcium carbonate addition enhanced the degradation rate (45-49% compared to 37% in the control) and the biomethane potential of the PLA/PBS mixture. Such an enhancement of the biodegradation rate can be explained by an increase in the permeability to water as a result of the incorporation of natural fillers. As the hydrolytic chain scission mechanism can be expected to increase with the water permeability of the materials, this could contribute to enhancement of the biodegradation of composite materials compared to the neat matrix (David et al., 2019). Finally, the companies Carbiolice® and Novozymes® have developed an enzyme cocktail called Evanesto® that can be directly incorporated into biodegradable plastics such as PLA to enhance their degradation in home composting and mesophilic anaerobic digestion.

4.3) Inoculum acclimation (biostimulation) or bioaugmentation

Although there have been no studies to date specifically in regard to these techniques for improving the biodegradability of biodegradable plastics, the inoculum origin and acclimation (a type of biostimulation), as well as bioaugmentation, have been shown to enhance the anaerobic biodegradability of organic wastes (De Vrieze et al., 2015; Nzila, 2017; Sambusiti et al., 2014; Yang et al., 2016). Sambusiti et al. (2014) have investigated the impact of four inocula (urban, agricultural, mixture of agricultural and urban, and granular) on the methane potential and methane production rate of ensiled sorghum. Interestingly, the nature of the inoculum did not affect the final methane potential, although it did have a significant influence on the methane production rate. In particular, the fastest biomethanization occurred using urban sludge (hydrolytic kinetic constant $k_h = 0.146 \text{ d}^{-1}$) while the slowest was with agricultural sludge ($k_h = 0.049 \text{ d}^{-1}$). Similar results were shown by Koch et al. (2017) during the anaerobic digestion of different substrate (*i.e.*, sewage sludge, dried whole crop maize, food waste) using three inocula (*i.e.*, from plant treating wastewater, agricultural waste or biowaste). According to these results, it would appear that the nature of the inocula as well as the nature of the biomass has a major impact on the results. It is, therefore, relevant to, in the future, investigate the impact of the inoculum origin on the anaerobic biodegradability performances of biodegradable plastics.

Another strategy to enhance the anaerobic biodegradability of biodegradable plastics is inoculum acclimation prior to the AD process. This has previously been applied successfully to anaerobic digestion of various organic wastes (Gonçalves et al., 2012; Yangin-Gomec et al., 2018). For instance, Gonçalves et al. (2012) demonstrated that the use of an acclimated microbial consortium is a promising way to accelerate the start-up of the digestion process and to enhance the overall anaerobic treatment of a real-life oily wastewater such as olive mill wastewater. These results suggest that changes in the structure of a microbial community can lead to changes in biogas production, and controlling the ultimate methanogenic archaeal community may promote successful methane production in anaerobic reactors (Yangin-Gomec et al., 2018). Aside from acclimation or in combination with biostimulation, bioaugmentation of inocula, consisting of the addition of efficient biomethane-generating single or mixed microorganisms (fungi, bacteria, or enzymes) in bioreactors, can enhance the AD process (Hu et al., 2016; Nzila, 2017). Bioaugmentation, by the addition of specific microbial strains, is a promising technique to accelerate the biodegradation of biodegradable plastics so that they biodegrade in time frames comparable with other organic materials (Castro-Aguirre et al., 2018). This strategy has been used for the past 15 years to increase biomethane production (Mshandete et al., 2005; Weiß et al., 2011; Zhong et al., 2011). Bioaugmentation has been successfully used in composting-mediated aerobic degradation of plastics (Castro-Aguirre et al., 2018; Nair et al., 2016). For instance, Castro-Aguirre et al. (2018) evaluated the effect of bioaugmentation (using *Geobacillus*) on the biodegradation of PLA and PLA bio-nanocomposites (BNCs) under simulated composting conditions. Bioaugmentation with *Geobacillus* increased the production of CO₂ and accelerated the biodegradation phase of PLA and BNCs when tested in compost and inoculated vermiculite with compost mixed culture, probably due to the secretion of esterases from such microorganisms. A better understanding of the key bacteria involved in the biodegradation of plastics is needed to be able to use bioaugmentation strategies in anaerobic digesters.

Table I.4. Strategies tested in the literature for enhancement of the methane potential and the kinetics of biodegradable plastics

Biodegradable plastic nature	Size and shape	Temperature (°C)	Pretreatment		Time (days)	Methane production or biodegradability		Reference
			Condition	Effect		Without pretreatment	With pretreatment	
PHB (ENMAT)			35 °C, addition of NaOH until pH 12 for 24h			199 L CH ₄ kg ⁻¹ ThOD	398 L CH ₄ kg ⁻¹ ThOD	
PHB (Mirel F1006)	0.15 mm	35 °C	35 °C, pH 7 for 48h	Reduced lag phase and improved methane production	40	233 L CH ₄ kg ⁻¹ ThOD	359 L CH ₄ kg ⁻¹ ThOD	Benn and Zitomer, 2018
PHB (Mango materials)			55°C, addition of NaOH until pH=10, 24h			316 L CH ₄ kg ⁻¹ ThOD	322 L CH ₄ kg ⁻¹ ThOD	
PHB (Mirel M2100, Metabolix)			55°C, addition of NaOH until pH=12, 24h			316 L CH ₄ kg ⁻¹ ThOD	357 L CH ₄ kg ⁻¹ ThOD	
PLA (Ingeo 2003D Natureworks)			90°C, addition of NaOH until pH=10, 48h			1 L CH ₄ kg ⁻¹ ThOD	86 L CH ₄ kg ⁻¹ ThOD	
PLA			Commercial items			38 °C 55 °C	Steam exposition, 3h 120 °C	
PLA	Commercial items	37 ± 1 °C	48h, addition of HCl pH= 2 48h, addition of NaOH pH= 12	No benefits	250	130 L CH ₄ kg ⁻¹ VS 130 L CH ₄ kg ⁻¹ VS	125 L CH ₄ kg ⁻¹ VS 101 L CH ₄ kg ⁻¹ VS	Battista et al., 2021
Crystalline PLA	2x2 cm cups	37 ± 1 °C	21 °C, pH > 11, 15 days	After 15 days of alkaline pretreatment, near total solubilisation was reached. No lag phase with pretreatment	70	756 mL(CH ₄)	1021 mL(CH ₄)	Hobbs et al., 2019
Polycaprolactone (Mw 65,000, Aldrich, USA)	<125 µm 125-250 µm 250-500 µm > 500µm	55 °C	Size reduction	Smaller particle size powder degraded faster	25-35	91-98%		Yagi et al., 2009
					50	89-9 %		
					65	81-87%		
PLA film 25 µm of thickness (Unitaka, Japan)	1x1 cm 15x34 cm 39x82 cm	55 °C	Size reduction	The crushed or small pieces of PLA film had slower anaerobic biodegradation rates than large pieces of PLA film due to flotation and static cling		936 L biogas kg ⁻¹ / 97.5%		Yagi et al., 2012
					60	880 L biogas kg ⁻¹ / 94.5%		
						893 L biogas kg ⁻¹ / 96%		
						827 L biogas kg ⁻¹ / 89%		
PHBV (0.5% HV ENMAT Y1000P China)	3900 µm (pellets) 420-840 µm 250-420 µm 150-250 µm 10 µm	37 °C	Size reduction	A temporary inhibition is observed for particle size less than 840 µm, it is attributed to the accumulation of VFAs caused by the rate of hydrolysis and acetogenesis exceeding that of methanogenesis	40	600 mL(CH ₄).g ⁻¹ / 79%		Ryan et al., 2016
					18	600 mL(CH ₄).g ⁻¹ / 79%		
					25	600 mL(CH ₄).g ⁻¹ / 79%		
					20	600 mL(CH ₄).g ⁻¹ / 79%		
					25	600 mL(CH ₄).g ⁻¹ / 79%		
Mater-bi® (60% starch, 40% hydrophilic resin)	Entire bag	35 ± 0.5 °C	5% TS NaOH, 24h	Mechanical pretreatment (reduction to 1x1 cm) and predigestion were also tested but do not show significant improvement	15	144 L CH ₄ kg ⁻¹ VS mass loss = 27.5 ± 1.3%	203 L CH ₄ kg ⁻¹ VS mass loss = 78.2 ± 5.9%	Calabro et al., 2019
Composite of PLA 76%, PBS 19% and CaCO ₃		52 ± 2 °C	Composite production with addition of two type of 5 % of CaCO ₃ (Omya TP39914 and Omya TP39968)		60	190 L CH ₄ kg ⁻¹ VS 37 %	210 L CH ₄ kg ⁻¹ VS 45 %	Hegde et al., 2018
Composite of PLA 76%, PBS 19% and CaCO ₃						190 L CH ₄ kg ⁻¹ VS 37 %	230 L CH ₄ kg ⁻¹ VS 49 %	
PHBV (0.5% HV ENMAT Y1000P China)	Rectangular prism 31,25 mm x 6,2 mm x 2,1 mm	37°C ± 2 °C	Addition of 20% oak wood flour		42	630 L CH ₄ kg ⁻¹ / 83 %		Ryan et al., 2017(b)
80% PHBV and 20% oak wood flour					28	510 L CH ₄ kg ⁻¹ / 73 %		
60% PHBV and 40 % oak wood flour					28	430 L CH ₄ kg ⁻¹ / 60 %		
PHBV (ENMAT Y1000P China)	Rectangular prism 31,25 mm x 6,2 mm x 2,1 mm	37°C ± 2 °C	Maleated PHBV	Maleated PHBV	80	94 %		Ryan et al., 2017(a)
Maleated PHBV					80	95 %		
80% PHBV 20% oak wood flour						84 %		
80% maleated PHBV 20% oak wood flour						88 %		
80% PHBV 20% silane treated oak wood flour						83 %		
PLA					270	90 %		
PLA + 10 % poly(ethylene glycol) and poly(propylene glycol)	Pellets	50 °C	Reduction of the glass transition temperature to 49 °C (bellow the temperature of digestion) by the addition of additives		150	90 %		Lee et al., 2016
PLA + 12 % poly(ethylene glycol) and poly(propylene glycol)					150	90 %		
PLA + 10 % poly(ethylene glycol) and poly(propylene glycol) + 2% additives					150	90 %		

5) Developments required to introduce biodegradable plastic wastes in anaerobic digesters

To make the treatment of biodegradable plastics in anaerobic digestion systems possible, the impact of anaerobic digestion on the quality and safety of the digestate has to be evaluated. Similarly, international standards need to be established in order to facilitate the collection and introduction of plastic wastes in anaerobic digestion.

5.1) Impact of biodegradable plastics AD on the agronomic quality and safety of digestate

Anaerobic digestion (AD) of feedstock or organic matter (OM) results in their biodegradation, thereby producing biogas in addition to the corresponding digestate. Although a number of emerging digestate valorization routes are being explored (Monlau et al., 2015; Sheets et al., 2015), the most common use of digestate is for agronomic purposes (Nkoa, 2014). Prior to being used, digestates need to be compatible with their application in agriculture (Nkoa, 2014). Digestates fulfilling this compatibility criterion can be directly applied to crop fields or they can undergo a solid-liquid separation (*e.g.*, screw press, filter press, centrifugation) resulting in a solid and a liquid digestate fraction (Akhiar et al., 2017; Guilayn et al., 2020; Möller and Müller, 2012). The solid fraction can be used directly as a soil amender or it can be aerobically composted to attain the attributes required for being applied as a soil amendment (Tambone et al., 2010). After being conveniently treated by dilution, filtration, etc., the liquid fraction can be used as liquid fertilizer for plant growth (Möller and Müller, 2012; Tampio et al., 2016a).

Biodegradable plastics are expected to become a significant fraction of biogas plant feedstock, especially in municipal organic wastes, where some items will be packaged or manufactured with biodegradable plastics (Möller and Müller, 2012; Narancic et al., 2018; W. Zhang et al., 2018). To date, the potential impact of biodegradable plastics on the digestate quality is expected to be low, although this has not been specifically investigated (Narancic et al., 2018; W. Zhang et al., 2018). The main contribution of biodegradable plastics to feedstock composition will depend on the composition of the biodegradable plastics: enrichment in C, O, and H, with basically no other elements, including nitrogen or sulfur (W. Zhang et al., 2018). Typically, the quality parameters of digestates are those that affect their agronomic value, mostly as plant nutrients or soil amendments. As soil amendments, digestates contribute to soil organic matter turnover, thus

improving the soil's biological, chemical, and physical characteristics (Tambone et al., 2010). In terms of plant nutrients, the properties associated with the agronomic quality of the digestate are the dry organic matter content; total N; total $\text{NH}_4^+\text{-N}$; total P; water-soluble P, K, Ca, Mg, and S content; C/N ratio; and pH. The attributes of digestates from AD with biodegradable plastics-containing feedstock have not been assessed. However, it is reasonable to assume that the impact of biodegradable plastics on the quality of agronomic digestates (*i.e.*, fertilizers and amendments) is minor. To our knowledge, there is still no information in the literature regarding the effects of biodegradable plastics on the quality and the properties of digestates.

Aside from agronomic evaluation, the safety of digestates should also be monitored. Toxic chemicals and physical contaminants depend on the predominant composition of the feedstock, and the presence of such entities in the original biodegradable plastic should be carefully monitored (Coelho et al., 2018; W. Zhang et al., 2018).

No specific recommendations have been made to date regarding the presence of heavy metals in digestates from AD plants treating biodegradable plastics, but the same threshold level as that in the European Norms for biodegradable wastes (Saveyn et al., 2014) seems appropriate. In the case of industrial compost, the European Norm "EN 13432" specifies that threshold levels should not be exceeded for heavy metals or for the fluorine content in biodegradable plastics treated by composting. In parallel with heavy metals, the content of organic contaminants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls) also needs to be monitored (Briassoulis et al., 2010). It is then of prime importance to evaluate the potential benefits, or otherwise the phytotoxicity, on plants through growth tests. Such tests can be performed in climatic chambers, greenhouses, or ideally in real field-scale assays (Adamcová et al., 2019; Gell et al., 2011; Nkoa, 2014). As for the composting process, the EN13432 Norm specifies carrying out assays with two plant species (one monocotyledon and one dicotyledon) at two compost doses (25% or 50% w/w), according to the protocol defined in the OECD 208 guidelines. It is likely that there will be further developments in ecotoxicity testing in the near future, especially given the increasing amount of biodegradable plastics on the market and the consequent increase in the amount of biodegradable plastics in organic wastes (Ruggero et al., 2019). For ecotoxicity testing, it could be quite pertinent to verify the impact of the digestate on edaphic (bacteria, protozoa, and earthworms) and aquatic

ecosystem organisms (*e.g.* algae, daphnia) (Pivato et al., 2016; Sforzini et al., 2016; Tigini et al., 2016).

In case the biodegradable plastics or their blends undergo substantial biodegradation during the AD, the carbon is converted to CH₄ and CO₂, and only non-biodegraded components will remain in the digestate. The latter will preferentially stay in the solid fraction (*e.g.*, fragments of films and packaging), while some of the residue will remain in both the liquid and solid fractions of the digestate (molecules derived from biodegradable plastics after the AD process, mainly in the form of micro- and nanoplastics). Generally, plastic fragments smaller than 5 mm are considered to be microplastics (Shruti and Kutralam-Muniasamy, 2019). Definition of the term “nanoplastics” is still a matter of debate, and different studies have set the upper size limit at either 1 000 nm or 100 nm (Gigault et al., 2018). High concentrations of microplastics can affect the AD performance and impact the digestate dewaterability and other properties (Li et al., 2020). Furthermore, plastic fragments that remain visible to the naked eye after the AD compromise the acceptability of the digestate as a commercial product (W. Zhang et al., 2018) as they may not meet the farmers’ expectations. Therefore, biodegradable plastics that undergo significant biodegradation under AD are expected to have a minor impact on the digestate quality, and this is especially the case under thermophilic AD conditions, whereby biodegradation is promoted by the temperature. Nevertheless, as the time required to treat biodegradable plastics by AD is longer than the current residence times for conventional organic waste treatment, especially in the case of mesophilic AD, it is paramount that the impact of residuals plastics on the agronomic quality and safety of the digestate is monitored (Accinelli et al., 2020; Adamcová et al., 2019; Narancic and O’Connor, 2019). For this purpose, the occurrence and the impact on terrestrial and aquatic ecosystem of micro- and nanoplastics that may be generated during AD of biodegradable plastics should be taken into account, especially if the digestate is used for agronomic applications (Shruti and Kutralam-Muniasamy, 2019; Zimmermann et al., 2020). Microplastics and nanoplastics are small enough to be ingested by a wide variety of organisms, and thus they also represent a pathway for the introduction of various environmental contaminants (*e.g.*, heavy metals, organic contaminants) into the food chain (Ng et al., 2018; Shruti and Kutralam-Muniasamy, 2019). As previously mentioned, the degradation of plastics during the AD process can be incomplete (especially under mesophilic conditions and if composting is not applied as a post-treatment), and they can further

accumulate in the environment and breakdown into micro- and nanoplastics (Ng et al., 2018; Shruti and Kutralam-Muniasamy, 2019). A number of preliminary studies have demonstrated that biodegradable microplastics have similar ingestion mechanisms, exhibit similar characteristics, and cause analogous effects as those of conventional microplastics (Shruti and Kutralam-Muniasamy, 2019; Straub et al., 2017) even though, due to their biodegradable nature, their persistence in soil and aquatic environments is shorter than that of conventional microplastics.

5.2) International norms and labels to evaluate the biodegradability by AD

To enhance the acceptability of biodegradable plastics among the general public and to facilitate results comparison, it is of prime importance that standards are established to assess the biodegradability of plastics by anaerobic digestion. Generally, the norms are classified as specifications or analyses and tests. Specification norms set the requirements, characteristics, and performance thresholds to be achieved during biodegradation trials, while analyses and test norms harmonize the methods for the quantification of these characteristics and performances.

To date, the major focus of society and policy makers has been in regard to composting as an end-of-life scenario for biodegradable plastics (Briassoulis et al., 2010; Narancic and O'Connor, 2019). Currently, the composting process has two specification norms (EN 13432 Packaging - Requirements relating to packaging recoverable by composting and biodegradation - Test program and criteria for evaluation of acceptance packaging, EN 14995 Plastics - Evaluation of compostability - Test scheme and specifications) that specify the requirements, characteristics, and performance thresholds by addressing four characteristics: 1) initial polymer characterization 2) biodegradability, 3) disintegration during biological treatment, and 4) effect on the quality of the resulting compost. Both EN 13432 and EN 14995 also include a number of specifications for coupling AD with composting. Nonetheless, to date, no specification norms have been devised at the international level for the stand-alone AD process. Only analyses and test norms (ISO and ASTM) have been developed to date to evaluate the biodegradability of biodegradable plastics in the AD process.

Table I.5 shows the main analyses and test norms for the evaluation of the biodegradability of biodegradable plastics in AD process, along with their main operational parameters. Presently, at

the international level, two norms (NF EN ISO 14853 and ASTM D5210) are referenced for the biodegradability in a liquid medium (1-3% TS) under mesophilic conditions. Similarly, one norm (ISO 13975) is designed for wet processes (< 15% TS) and is applicable in mesophilic and thermophilic environments. Finally, two norms (ASTM D551 and ISO 15985) are made for simulating dry processes (> 15% TS) under thermophilic conditions. Covering all of the typologies of anaerobic digesters is difficult and there is presently an absence of norms to assess the biodegradability of plastics under dry mesophilic AD conditions. Up to date the norms ISO 13975 (simulating CSTR under mesophilic or thermophilic conditions), ASTM D5511 and NF EN ISO 15985 (simulating the dry process under thermophilic conditions) appear to be the most useful and representative norms to simulate and assess the biodegradability of plastics in anaerobic digestion for industrial-scale processes. All of these norms are based on the principle of the biochemical methane potential (Holliger et al., 2016; Raposo et al., 2011), with a number of variations for the operational conditions and for the nature of the inoculum (**Table I.5**). For all norms, the biodegradability rate is calculated based on the carbon emitted in gaseous form in the biogas (CH₄, CO₂) compared with the amount of total organic carbon in the tested material. Only the ISO 14853 and ISO 13975 norms take into account the inorganic carbon dissolved in the medium, which increases the accuracy. Failure to account for dissolved inorganic carbon can result in underestimation of the biodegradability.

Finally, at present, there are no specification norms (as is the case for composting, soil, and marine environments) nor analysis and testing norms to assess the disintegration of plastics by AD and the ecotoxicity of the resulting digestate. The recent development of the anaerobic digestion sector, as well as the diversity of the AD processes encountered, may explain this delay in European standardization, even though there have been a number of new initiatives in recent years such as the Open-Bio project (Open-Bio, 2016). The Open-Bio project investigated how markets can be opened for bio-based products through standardization, labelling, and procurement, and there was a specific focus on the biogas sector. Finally, it is important to design and implement test standards representative of industrial reality but that also take into account economic constraints in order to make them accessible to as many stakeholders as possible. The deployment of a biodegradable plastics sector will also require better organization and interactions between the various stakeholders, including public organizations, industrial entities, end-users, and policy

makers.

Table I.5. International standards available and operational parameters to assess the biodegradation of plastics under anaerobic conditions (adapted from Lagnet et al., 2020)

Standard	ISO 14853 (2017)	ISO 13975 (2019)	ISO 15985 (2017)
Equivalent ASTM standard	ASTM D5210	-	ASTM D5511
Process simulated	Liquid process (UASB/fixed bed reactor)	Semi-liquid process (CSTR)	Solid state process
Temperature (°C)	35 (± 2)	35 (± 3) or 55 (± 5)	52 (± 2)
Inoculum	Digested sludge from a wastewater treatment plant (treating mainly household water). Alternatively, anaerobic sludge prepared in laboratory.	Digestate from an AD plant preferably treating domestic sewage. Alternatively, from AD plant treating livestock faeces or garbage	Digestate from a thermophilic dry reactor (>20% TS) treating organic fraction of municipal solid waste. Alternatively, liquid digestate dehydrated (>20% TS) by the use of centrifugation, press or dried at 58 °C
Inoculum preparation	Pre-incubation up to 7 days at 35 °C (± 2) and possible acclimation to the test material	Pre-incubation up to 5 days at 35 (± 3). In case of thermophilic experiment, mesophilic inoculum could be acclimated to 55 °C for one month. Possible acclimation to the test material	Pre-incubation up to 7 days at 52 °C (± 2).
Inoculum quality control	Organic carbon < 20 mg L ⁻¹	TS < 15%, pH between 7.5-8.5	TS ≥ 20%; pH between 7.5 and 8.5; VFAs < 1g kg ⁻¹ ; N-NH ₄ ⁺ between 0.5-2 g kg ⁻¹
Reactors volume	Between 0.1 and 1 L	≥ 1.5 L	≥ 0.75 L
Number of replicate	3	3	3
Positive control	Cellulose, PHB or Poly(ethylene glycol)	Microcrystalline cellulose < 20 µm	Microcrystalline cellulose < 20 µm
Plastic shape and size	Powder (250 µm), film or fragment of items	Powders or films	Powder, pellets, film or fragment with surface ≤ 2x2 cm
Amount of plastic	20-200 mg organic carbon L ⁻¹ of inoculum	7-10 g VS L ⁻¹ of inoculum	15-20 g VS kg ⁻¹ of inoculum
Method of biodegradation calculation	Dissolved inorganic carbon and gaseous carbon are compared to the introduced organic carbon	Dissolved inorganic carbon and gaseous carbon are compared to the introduced organic carbon	Gaseous carbon are compared to the introduced organic carbon
Duration of the test	Maximum of 90 days	Maximum of 90 days	15 days extensible
Validity criteria	Biodegradation yield of the positive control > 70% after 60 days and the difference between the replicates < 20%	Biodegradation yield of the positive control > 70% after 15 days and the difference between the replicates < 20%	Biodegradation yield of the positive control > 70% after 15 days and the difference between the replicates < 20%

6) Conclusion

This review highlights the potential of end-of-life treatment of biodegradable plastics by anaerobic digestion. The main conclusions are as follows:

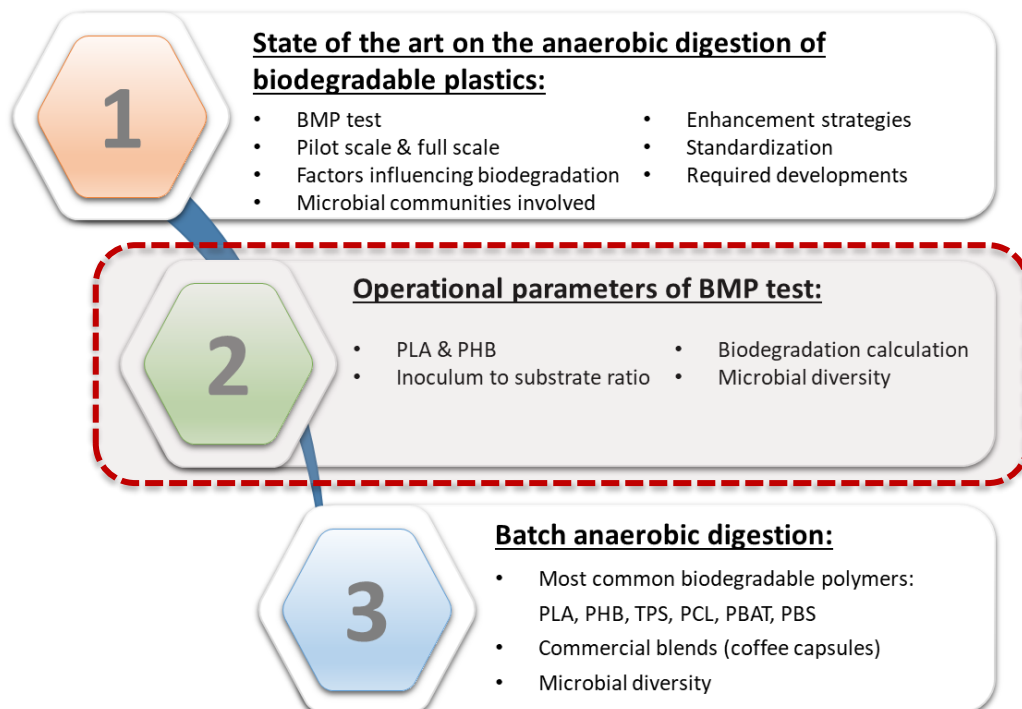
- Not all polymers and reactor typologies have received the same degree of attention; anaerobic digestion under thermophilic conditions has been investigated less than mesophilic conditions. There have been few investigations of the anaerobic biodegradability of commercial blends (except Mater-Bi®), a number of emerging polymers (such as mcl-PHAs and crystallized poly(lactic acid)). Furthermore, most of the data regarding biodegradability performances come from studies based on BMP test experiments, which may not provide a true picture of the performance of full-scale anaerobic reactors. To date, there is little information available regarding continuous anaerobic digestion experiments at pilot or industrial scales.
- Thermoplastic starch and PHAs can reach near-complete mineralization in a relatively short period under both mesophilic and thermophilic conditions. These materials could potentially be treated in biogas plants without alteration of the hydrolytic retention time used. Other polymers such as poly(lactic acid) and poly(ϵ -caprolactone) have been shown to have very low biodegradation rates under mesophilic conditions. More than 280 days appear to be required to reach their methane potential. Under thermophilic conditions, their biodegradation rate is greatly increased. Lastly, some polymers that appear to be biodegradable under industrial composting conditions were very poorly biodegraded even with very long digestion times, such as poly(butylene succinate) and poly(butylene adipate/terephthalate).
- Little is known about the microorganisms involved in the anaerobic digestion of biodegradable plastics. Recently, some publications have reported that *Tepidimicrobium* sp. plays a key role in the anaerobic biodegradation of PLA and of three commercial blends. A better understanding of the microbial taxa involved in the process could open the way to the use of bioaugmentation to improve the performance of the process. In parallel, a number of pretreatment strategies (e.g., mechanical, thermal, and thermo-chemical) and the addition of various additives in the plastic formulation (e.g., fibers, minerals, enzymes) have been shown to enhance the biodegradation rate of the biodegradable plastics in anaerobic digestion, albeit with varying degrees of success. Finally, there is a grey area surrounding the quality and potential toxicity of the digestate from anaerobic digestion of biodegradable plastics.
- Standardization, labelling, and further research on the sustainability (i.e., economic, environmental, and societal) is required in the near future in order to improve the public perception and the social acceptance of these new products.

CHAPTER II

Operational parameters of biochemical methane potential test

This chapter is based on a modified version of a scientific paper submitted to Frontiers in bioengineering:

Cazaudehore, G., Guyoneaud, R., Lallement, A., Gassie, C., Monlau, F., 2022. Influence of the inoculum-substrate ratio on the biochemical methane potential and active microbial communities during anaerobic digestion of biodegradable plastics (under revision). Frontiers in Bioengineering.



Foreword

When considering the use of biodegradable plastics, one of the key parameter is the experimental evaluation of their biodegradability in a given environment and condition. For the evaluation of the biodegradability in anaerobic digestion (AD), the biochemical methane potential (BMP) test has proven to be a straightforward and reliable method to estimate the extent and the rate of conversion to methane of a given organic substrate (Hafner et al., 2020; Holliger et al., 2021, 2016). In very recent years, some research projects have been conducted to improve the reproducibility and comparability of BMP testing (Hafner et al., 2020; Holliger et al., 2021, 2016; Raposo et al., 2011, 2008; Ribeiro et al., 2020). The Inoculum to Substrate Ratio (ISR), *i.e.*, the ratio of the volatile solids from the inoculum (residual organic material and microorganisms) to the volatile solids from the substrate, has been highlighted as a key parameter for BMP test.

Besides, to the operational conditions of the BMP tests, a better knowledge of the microbial communities responsible for the biodegradation of biodegradable plastics is necessary, only very few data are available on that subject (Peng et al., 2022b; Venkiteshwaran et al., 2019; Yagi et al., 2014a, 2013b). The observation of the active microbial communities in the reactors fed with different ISR, allows eventually highlighting the microorganisms involved in the biodegradation of the plastic by comparing the evolution of the active communities in relation to the production of methane. A better understanding these microbial taxa could open the way to the use of inoculum selection or bioaugmentation strategies to improve the performance of the process.

The recommended ISR in the standard test methods for the evaluation of the biodegradability of a plastic in liquid anaerobic digestion (ISO 14853 and ISO 13975) are not clearly expressed nor well defined. In the case of ISO 14853, designed for aqueous media (Total solid (TS) content between 1 and 3%), the recommended amount of added plastic is between 20-200 mg of organic carbon per liter of inoculum. For ISO 13975, designed for media with less than 15% TS content, the amount of plastic to add is between 7-10 g of volatile solid per liter of inoculum. Considering that these methods can be applied to inocula having different TS content, the associated ISR can vary a lot. Additionally, biodegradation of the polymers is calculated in these two standards by considering the carbon of both the gaseous (methane and carbon dioxide) and the liquid (dissolved

inorganic carbon) phase. On the other hand, several scientific publications estimate the biodegradation by comparing the observed methane production and the theoretical methane production (Cho et al., 2011a; Nunziato et al., 2018; Ribeiro et al., 2020; Ryan et al., 2017a).

The first objective of this study is to evaluate, the influence of the ISR and of the method of biodegradation calculation on the evaluation of the biodegradability of two selected biodegradable plastics (PLA and PHB) in an anaerobic digestion system. Then, the second objective of this study is to analyze the active microbial communities during anaerobic digestion of PLA and PHB at different ISRs.

Abstract

The influence of the inoculum-substrate ratio (ISR) on the mesophilic and thermophilic biochemical methane potential test of two biodegradable plastics was evaluated. Poly(lactic acid) (PLA) and polyhydroxybutyrate (PHB) were selected for this study, the first for being recalcitrant to mesophilic anaerobic digestion (AD) and the second, by contrast, for being readily biodegradable. Several ISRs, calculated on the basis of volatile solids (VS), were tested: 1, 2, 2.85, 4, and 10 g VS g VS⁻¹. A high ISR was associated with an enhanced methane production rate (*i.e.*, biodegradation kinetics). However, the ultimate methane production did not change, except when inhibition was observed. Indeed, applying a lower ISR to readily biodegradable plastics such as PHB resulted in inhibition of methane production. Based on these experiments, in order to have reproducible degradation kinetics and optimal methane production, an ISR between 2.85 and 4 is recommended for biodegradable plastics. The active microbial communities were analyzed, and the active bacteria differed depending on the plastic digested (PLA versus PHB) and the temperature of the process (mesophilic versus thermophilic). Previously identified PHB degraders (*Ilyobacter delafieldii* and *Enterobacter*) were detected in PHB-fed reactors. *Thermogutta* and *Tepidanaerobacter* were detected during the thermophilic AD of PLA, and they are probably involved in PLA hydrolysis and lactate conversion, respectively.

1) Introduction

The accumulation of plastic waste in the environment is one of the biggest environmental threats faced by our planet. Research and development efforts are being undertaken to develop more environmentally friendly options, such as biodegradable plastics. These have the added benefit over conventional plastics of having the potential to be treated by organic recycling involving either aerobic (*i.e.*, composting) or anaerobic conditions (*i.e.*, anaerobic digestion) (Abraham et al., 2021; European Bioplastics, 2019). The biodegradation of biodegradable plastics is highly dependent on the environment in which it takes place (Narancic et al., 2018). Therefore, a plastic that is biodegradable under industrial composting conditions is not necessarily biodegradable by other processes (*e.g.*, home composting, anaerobic digestion) and environments (soil, water, or sediment, marine).

Polyhydroxybutyrate (PHB) and poly(lactic acid) (PLA) are among the most studied polymers to replace petroleum-derived plastics (Boey et al., 2021). PLA represent approximately 25% of the biodegradable plastic production in 2018 (European Bioplastics, 2019). However, the biodegradation rate of the PLA is very low for mesophilic AD (*i.e.*, 35-40 °C), to the point that several scientific publications have reported no biodegradation over periods ranging from 40 to 100 days (Benn and Zitomer, 2018; Shin et al., 1997; Vargas et al., 2009; Vasmara and Marchetti, 2016). Bernat et al. (2021) and Yagi et al. (2014) observed better biodegradation (29 to 66%) when very long digestion periods were applied (280 days). Anaerobic digestion of PLA under thermophilic conditions (*i.e.*, 55-60 °C) was much faster. Near-complete conversion into methane has been reported (between 82 and 90%) in a mean digestion time of 90 days (Hegde et al., 2018; Narancic et al., 2018; Šmejkalová et al., 2016; Yagi et al., 2013c, 2009). On the other hand, PHB represents a relatively small portion of the biodegradable plastics market (approximately 2% of the biodegradable plastic production in 2019), but the production capacities are expected to more than triple in the next five years (European Bioplastics, 2020). Furthermore, PHB also has ample potential for being rapidly converted into methane under either mesophilic (Abou-Zeid et al., 2004; Benn and Zitomer, 2018; Narancic et al., 2018) or thermophilic conditions (Itävaara et al., 2002; Narancic et al., 2018; Yagi et al., 2013c).

The rating of the biodegradability of a plastic has to refer to standards that define the test method (method and assay standards) and the criteria (specification standards) appropriate to the environment tested (ISO 472, 2013). The treatment of biodegradable plastics in anaerobic digestion systems is in its infancy, with only methods and standards assays but neither specification standards nor certification processes (Lagnet et al., 2020). The method used to determine the anaerobic biodegradation of a plastic is generally based on the biochemical methane potential (BMP) test. The BMP test has proven to be a straightforward and reliable method to estimate the extent and the rate of conversion to methane of a given organic substrate under AD conditions. In recent years, numerous research projects have been conducted to improve the reproducibility and comparability of BMP testing, including a number of large-scale inter-laboratory studies (Hafner et al., 2020; Holliger et al., 2021, 2016; Raposo et al., 2011, 2008; Ribeiro et al., 2020). The results of these have highlighted that the inoculum to substrate ratio (ISR) is a key parameter for accurate estimation of the BMP of a substrate. The ISR corresponds to the ratio of the volatile solids from the inoculum (residual organic material and actively degrading microorganisms) to the volatile solids from the substrate. A low ISR (*i.e.*, a high substrate level) leads to an increased risk of acidification and inhibition phenomena and, consequently, such ISRs do not allow determination of the actual BMP of a compound or substrate (Holliger et al., 2016). By contrast, a high ISR can result in non-reproducible observations, particularly in case of heterogeneous substrates (Raposo et al., 2006). Holliger et al. (2016) recommend ISRs between 2 and 4 in most cases. The value of the ISR is adjusted based on whether or not the substrate is readily biodegradable. If the organic compound is readily biodegradable, the ISR can be increased (\geq four) to avoid rapid accumulation of intermediates (*e.g.*, volatile fatty acids) and the subsequent inhibition of the process. While for less biodegradable substrates (*e.g.*, lignocellulosic substrates), ISRs less or equal to 1 can be applied. For unknown substrates, such as biodegradable plastics, several ISRs should be tested. In the standard test method, specific to mesophilic anaerobic digestion of biodegradable plastic in liquid media (ISO 14853 and ISO 13975), the recommended ISR has not been clearly expressed or well defined. In the case of ISO 14853, designed for aqueous media (TS content between 1 and 3%), the ISR values are based on the organic carbon content of the plastic and the TS content of the inoculum. Based on the characteristics of the plastics and the inocula used in this study, the ISRs recommended in ISO 14853 are between 1.75 and 50. For ISO

13975, designed for media with less than 15% TS content, the ISR values are based on the volatile solids content of the plastic and the TS content of the inoculum.

Anaerobic digestion relies on complex microbial populations (Castellano-Hinojosa et al., 2018; De Vrieze et al., 2018). The composition of these populations is another fundamental factor that can affect the biodegradability in AD (Peng et al., 2022b). However, little is known about the microbial populations involved in the anaerobic digestion of biodegradable plastics (Bandini et al., 2020; Cazaudehore et al., 2021; Emadian et al., 2017; Shah et al., 2008; Tseng et al., 2019, 2019; Venkiteshwaran et al., 2019; Yagi et al., 2013, 2014). Recently, Tseng et al. (2020, 2019) and Cazaudehore et al. (2021) pointed out the importance of the genus *Tepidimicrobium* during the thermophilic anaerobic digestion of PLA and different biodegradable plastic blends, respectively. Similarly, Peng et al. (2022) identified *Clostridium_sensu_stricto* 1, *Streptococcus*, and *Caldicoprobacter* as the genera responsible for the difference between thermophilic reactors fed with a blend of PLA/PBAT and reactors not fed with plastics.

The first objective of this study was to evaluate the influence of the ISR on the biodegradation behavior of biodegradable plastics in an anaerobic digestion system. For this, two biodegradable plastics were selected as a model: polylactic acid (PLA) and poly(hydroxyl butyrate) (PHB). Then, the second objective of this study was to analyze the active microbial communities during anaerobic digestion of PLA and PHB at different ISRs.

2) Materials and Methods

2.1) Samples and inocula

Two plastics, certified as biodegradable under industrial composting conditions according to the EN 13432 standard, were used for this experiment: semi-crystalline PLA from NaturePlast® (NP SF 141) and PHB from K.D. Feddersen (M-Vera). They were both obtained in the form of pellets that were ground using liquid nitrogen and a centrifugal mill (ZM 100, Retsch, Haan, Germany) to generate a mean particle size of 1.01 ± 0.51 mm (determined by laser granulometry (Mastersizer 3000, Malvern, United Kingdom). Cellulose (a positive control) was purchased from Tembec® (France) in the form of plates and was then ground using a plant shredder (GHE 355, Stihl®, Waiblingen, Germany).

Two laboratory inocula were used for the BMP testing. Both of these were prepared from digestate of a local anaerobic digestion plant and acclimated to either mesophilic (38 °C) or thermophilic (58 °C) anaerobic conditions. They were maintained under conditions of anaerobic digestion for several months before their use in BMP testing, and they were fed twice a week with a mixture of WWTP sludge and green grass. The ability of the laboratory inocula to convert organic compounds to methane is regularly verified by using the BMP test on cellulose. Other parameters such as the pH, redox, ammonia titration, volatile fatty acids (VFAs) content, and alkalinity were measured regularly to ensure that the inocula were of good quality. Before their use in BMP testing, the inocula were sieved at 2 mm in order to remove part of the non-degraded organic fraction and thus reduce the endogenous methane production from the inocula.

The total solids (TS) and the volatile solids (VS) contents of the various samples (biodegradable plastics, inocula, and cellulose) were determined using APHA standard methods (American Public Health Association, 2005). Elemental analyses (carbon, hydrogen, nitrogen, and sulfur) were performed on plastic and cellulose samples using an Elemental Vario Macro Cube analyser (Elementar, Germany). The oxygen content was estimated by the difference between the VS, carbon, hydrogen, nitrogen, and sulfur contents. The characteristics of the inocula and plastics are provided in **Table II.1**. Ammonia titration was performed using a designated kit (Spectroquant® Ammonium Cell Test). The VFA content was determined by gas chromatography (7890B, Agilent, USA) and the FOS/TAC ratio by titration using sulfuric acid (0.1 N) (Lallement et al., 2021).

2.2) Anaerobic digestion experiment

2.2.1) Biochemical methane potential test

Biodegradable plastics (PLA and PHB) and cellulose (a positive control) were tested in batch bottles (0.5 L) under mesophilic (38 ± 1 °C) or thermophilic (58 ± 1 °C) conditions. Although PHB was readily biodegradable under the mesophilic conditions, it was not tested under the thermophilic conditions. The experimental protocol of this test was adapted from the recommendations of Holliger et al. (2021, 2016) and was proven by the French and European inter-laboratory studies (Hafner et al., 2020; Ribeiro et al., 2020). Each set of conditions was run in triplicate. The BMP bottles were filled with 300 mL of inoculum, water, and test material mixture. The amount of inoculum was the same in the various bottles, while the amount of water and the test material

content varied in order to have the same working volume in each bottle and to test different ISRs, respectively. Five ISRs were tested: 10, 4, 2.85, 2, and 1. A blank control, without test material, was also assayed. The positive control (*i.e.*, cellulose) was tested with an ISR of 2.85. Before placing a cap equipped with a gas-tight connector on the bottles, the gas phase was flushed out with nitrogen (Alphagaz™ with SMARTOP™, Air Liquide, France) to ensure anaerobic conditions. The daily biogas production was calculated from the pressure increase in the bottles measured by a manometer (2023P, Digital Instrumentation Ltd, Worthing, United Kingdom). Manual mixing was performed once a day. The biogas composition was determined once a week on the biogas accumulated in the headspace of the bottles using a gas chromatograph (Micro GC 490, Agilent, USA) equipped with two columns. The first column (M5SA 10 m, Agilent, USA) was used at 80 °C and 200 kPa to separate O₂, N₂, and CH₄ using argon as the carrier phase; the second column (PPU 10 m BF) was used at 80 °C and 150 kPa to separate the CO₂ from the other gases using helium as the carrier phase. The injector temperature was 110 °C. Gaseous compounds were detected using a thermal conductivity detector. The calibration was carried out using two standard gases composed of 9.5% CO₂, 0.5% O₂, 81% N₂, and 10% CH₄ and 35% CO₂, 5% O₂, 20% N₂, and 40% CH₄ (special gas, Air Liquide®, France). The biogas production of the negative control, which was endogenous to the inoculum, was subtracted from the production of the other bottles. All of the results are presented for normalized conditions of temperature and pressure (P_{atm}, 0 °C). The pH and redox were controlled at the beginning of the test to verify the initial state of the inocula, and at the end of the test in order to observe potential acidification that would be inhibitory for the methane production.

2.2.2) Calculation of the biodegradation

Biodegradation of the samples was calculated using two methods. The first method was based on the theoretical methane production (BMP_{th}) calculated from the elemental characterization (C_xH_yO_zN_nS_s) according to Equation 1 (Achinas and Euverink, 2016a; Boyle, 1977; Buswell and Mueller, 1952). The biodegradation was calculated by comparing the observed methane production (BMP_{exp}) and the theoretical methane production (Equation 2).

$$\text{BMP}_{\text{th}} (\text{NL}(\text{CH}_4) \cdot \text{g}^{-1}(\text{C}_x\text{H}_y\text{O}_z\text{N}_n\text{S}_s)) = \frac{22.4 \times \left(\frac{x}{2} + \frac{y}{8} - \frac{z}{4} - \frac{3n}{8} - \frac{s}{4} \right)}{12x + y + 16z + 14n + 32s} \quad (\text{Eq.1})$$

where 22.4 is the molar volume of an ideal gas.

$$\text{Biodegradation (\%)} = \frac{\text{BMP}_{exp}}{\text{BMP}_{th}} \times 100 \text{ (Eq. 2)}$$

The second method was based on the total carbon content, considering carbon from the gas phase (methane and carbon dioxide) as well as from the liquid fraction. Dissolved inorganic carbon was measured at the end of the BMP on a subsample (20 mL) of digestate. The vessels were closed and 20 mL of H₂SO₄ (2 M) were added through the ethyl rubber septum. The vessels were incubated for 24 h with magnetic stirring at 35 °C. The amount of carbon dioxide released was determined by a manometric method. The production from the blank reactors was subtracted from the production of the reactors with samples. The biodegradation was then estimated using Equation 3.

$$\text{Biodegradation (\%)} = \frac{m(\text{dissolved inorganic carbon}) + m(\text{carbon from gas})}{m(\text{Carbon introduced})} \times 100 \text{ (Eq.3)}$$

2.3.3) Modeling and statistical analysis

The cumulative methane production curves of the various substrates were modeled according to a modified Monod-Gompertz model (Rakmak et al., 2019), Eq.4. The parameters of the model were determined for each condition using R software (version 3.6.2) and the Nonlinear Least Squares method (nlsLM function of the minpack.lm package). Statistical comparison of the parameters was performed on R using ANOVA and Tukey's HSD at a probability of significance level $P \leq 0.05$.

$$G(t) = G(0) * \exp \left[- \exp \left(\frac{R_{max} * \exp(1)}{G(0)} * (\lambda - t) + 1 \right) \right] \text{ (Eq.4)}$$

where:

- G(t) is the cumulative methane production at time t in L CH₄.kg⁻¹ VS
- G(0) is the ultimate methane produced in L CH₄.kg⁻¹ VS
- λ is the time lag in days
- R_{max} is the methane production rate in L CH₄.kg⁻¹ VS d⁻¹

2.3) Analysis of the active microbial communities

2.3.1) Sampling

Digestates were sampled, in triplicate, at the beginning of the BMP test (Inoculum) and at two different times (T1 and T2) during the anaerobic digestion process of PHB under mesophilic conditions and under both mesophilic and thermophilic conditions for PLA. The sampling days are indicated in **Figure II.1** and the methane production rates at the time of the sampling are presented in **Figure II.3**. Sampling was not performed for all the ISRs tested. Rather, it was performed for extreme ISRs (*i.e.*, 10 and 1) and for an intermediate ISR usually applied in the testing in our laboratory (*i.e.*, 2.85). The digestate samples were mixed with two volumes of RNAprotect Bacteria Reagent® (Qiagen, USA), incubated for 5 min at room temperature, and then centrifuged (5000 x g, 5 min). The supernatants were discarded, and the pellets were stored at –20 °C for less than two weeks before being transferred to – 80 °C until the analysis.

2.3.2) Sequencing of 16S rRNA transcripts

RNA extraction was performed using a FastRNA™ Pro Soil-Direct kit (MP Biomedicals™, USA) according to the manufacturer's instructions. The absence of DNA in the extracts was verified by PCR followed by 1% agarose gel electrophoresis. The amount of RNA extracted was determined using Quant-iT™ RNA HS Reagent (Invitrogen™, USA). Reverse transcription of the RNA was carried out using the instructions provided with the M-MLV reverse transcriptase (Invitrogen™, USA) on 5 ng of extracted RNA. PCR of the V4-V5 region (nucleotides 515-929) of the 16S rRNA gene was performed as described in Cazaudehore et al., (2021). The amplification was confirmed by 1% agarose gel electrophoresis of the PCR samples. Amplicons were sequenced by the PGTB unit (Bordeaux, France) using Illumina MiSeq 250 bp paired-end technology.

2.3.3) Bioinformatics analyses and data processing

Bioinformatics processing of the data was performed using the method described by Escudié et al. (2018) on the Galaxy FROGS pipeline (Afgan et al., 2018). After a pre-processing step (merging, denoising, and dereplications of the reads), the sequences were clustered into Operational Taxonomic Units (OTUs) with an aggregation distance of three bases. OTUs containing less than 0.0005% of the total sequences were deleted, as were chimeric OTUs. Taxonomic assignments

were performed using the Silva database v.128.1 (Pruesse et al., 2007). The number of sequences per sample was normalized based on the minimum number of sequences per sample found (12 933 sequences). The sequence data have been deposited in GenBank under accession number PRJNA757376.

The following statistical analyses were performed using R version 3.6.2 software. Alpha diversity metrics (Richness, Chao1, Exp. Shanon, and Inv. Simpson) were calculated for each sample using the package 'phyloseq'. Alpha diversity indices were statistically compared using the Kruskal-Wallis and Dunn's test (package 'dunn.test'). The relationship between the environmental variables and the microbial community distribution of the samples was explored using redundancy analysis (RDA) as described by Wang et al. (2012). Prior to performing the RDA, the OTUs table (containing many zeros) was transformed using the Hellinger transformation to be suitable for a linear method (such as RDA) (Paliy and Shankar, 2016; Ramette, 2007). Selection of the environmental variables driving the microbial community composition was based on the use of ANOVA on the RDA (p-value < 0.05). Two quantitative environmental variables were selected for the mesophilic samples (methane production from PLA and methane production from PHB) and one qualitative variable (presence or absence of inhibition due to PHB overload). For the thermophilic samples, only two variables were selected, namely the methane production from the PLA and the nature of the sample (samples from inoculum or from BMP reactors). Triplots representing the samples, the OTU, and the environmental variables were produced using the package 'vegan'. Selected variables were subjected to multivariate regression analysis (function `lm` and ANOVA) to determine the OTUs significantly correlated with the variation of the environmental variables (p-value of less than 0.05). At this stage, the magnitude and the direction (positive or negative) of the correlation between the significant OTUs and the different variables were determined (X. Wang et al., 2012). A heatmap showing the significant correlation (p-value < 0.05) between the variables and the OTUs was generated using the package 'heatmap.2'. Only the OTUs with the highest magnitude (the top 10 positively and negatively correlated OTUs for each variable) and representing more than 1% of the sample sequences in at least one sample are depicted. The OTUs were reordered according to the phylogenetic tree produced using the package 'ggtree'.

3) Results and Discussion

3.1) BMP test

Figure II.1 shows the cumulative methane production obtained from the anaerobic digestion of PLA and PHB at the various ISRs studied, under mesophilic (for both biodegradable plastics) and thermophilic (only for PLA) conditions. The properties of the inocula and the substrates used are presented in **Table II.1**. The inocula complied with the quality criteria proposed by Holliger et al. (2021, 2016). Moreover, the BMP test on the positive control (i.e., cellulose) also complied with the validation criteria proposed by Holliger et al. (2021, 2016), reaching a mean biodegradation of $86.1 \pm 1.2\%$ and $87.7 \pm 1.5\%$ under mesophilic and thermophilic AD, respectively (data not shown), thus indicating that the inocula used in this study were suitable for anaerobic digestion experiments. The BMP test on cellulose was performed at the intermediate ISR of 2.85. The methane production from the various reactors was modeled using a modified Monod-Gompertz model (Rakmak et al., 2019). This allowed the methane production to be described by three parameters: the ultimate methane production ($G(0)$); the duration of the lag phase (λ), and the methane production rate (R_{max}) (**Table II.2**). High correlation coefficients were obtained ($R^2 = 0.94\text{--}0.99$), except for reactors with inhibited methane production (PHB with an ISR of 1), indicating that the Monod-Gompertz model accurately described the cumulative methane production.

Figure II.2 shows the ultimate biodegradation estimated by both methods based on the total organic carbon or methane production for PHB at different ISRs under mesophilic conditions. The method based on total organic carbon, considering the carbon of both the gaseous (methane and carbon dioxide) and the liquid (dissolved inorganic carbon) phase, is recommended by standard test methods such as ISO 14853 and ISO 13975. On the other hand, the method based on comparison between the observed methane production and the theoretical methane production has been used in numerous scientific publications (Cho et al., 2011c; Nunziato et al., 2018; Ribeiro et al., 2020; Ryan et al., 2017a). Slightly higher ultimate biodegradations were observed with the method based on methane production. However, the differences in biodegradation between the two methods were not statistically significant. Therefore, the method based on methane

production was used in the remainder of the study because it requires less experiment and the associated experimental errors.

Table II.1. Properties of the inocula and the biodegradable plastics.

Parameters	Unit	Mesophilic inoculum	Thermophilic inoculum	PLA NaturePlast SF 141	PHB K.D. Feddersen
pH	-	7.55 (± 0.10)	7.6 ± 0.12	-	-
Redox	mV	-350 ± 13	-376 ± 5	-	-
Ammonia	gN-NH ₃ .L ⁻¹	1.66 ± 0.10	1.77 ± 0.10	-	-
FOS/TAC	-	0.20 ± 0.02	0.29 ± 0.02	-	-
VFAs	g _{eq acetate} .L ⁻¹	0.08 ± 0.01	0.14 ± 0.01	-	-
TS	% raw mass	3.59 ± 0.01	3.03 ± 0.09	99.8 ± 0.00	99.6 ± 0.01
VS	% raw mass	2.35 ± 0.01	1.94 ± 0.06	99.6 ± 0.01	97.7 ± 0.01
Carbon	% TS	-	-	51.80 (± 0.17)	53.19 (± 0.22)
Hydrogen	% TS	-	-	6.05 (± 0.01)	6.95 (± 0.03)
Nitrogen	% TS	-	-	0.00 (± 0.00)	0.22 (± 0.02)
Sulfur	% TS	-	-	0.00 (± 0.00)	0.10 (± 0.03)
Oxygen*	% TS	-	-	40.19	39.45
Theoretical methane potential	NL CH ₄ .kg VS ⁻¹	-	-	522	552

*Calculated O = 100 - C - H - N - S - Ash

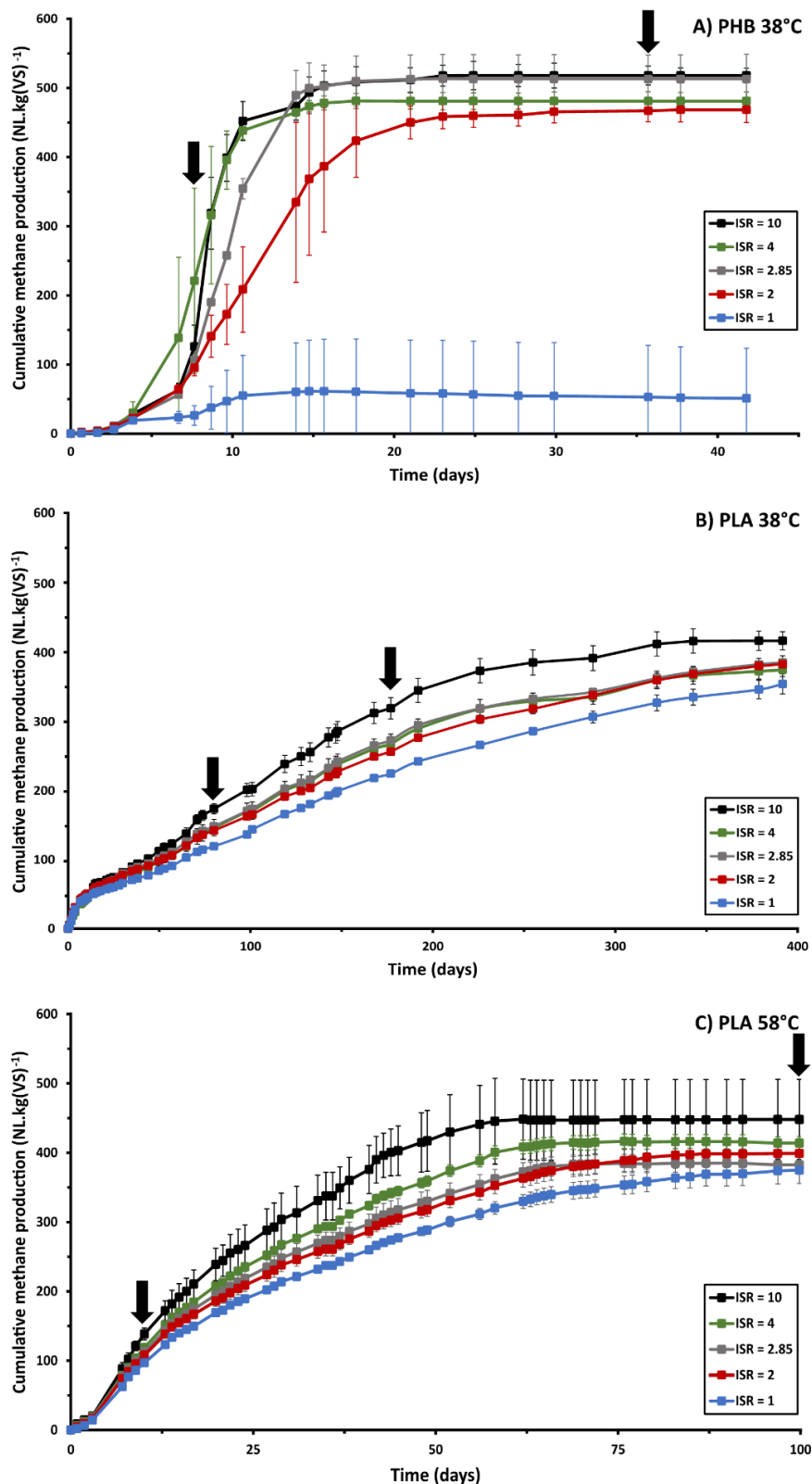


Figure II.1. The mean cumulative methane production ($L\ CH_4\ kg^{-1}\ VS$) from the various ISRs tested for PHB under mesophilic conditions (A), PLA under mesophilic conditions (B), and PLA under thermophilic conditions (C). The error bars represent the standard deviation. The arrows indicate the times at which the samples for the microbial community analyses were collected.

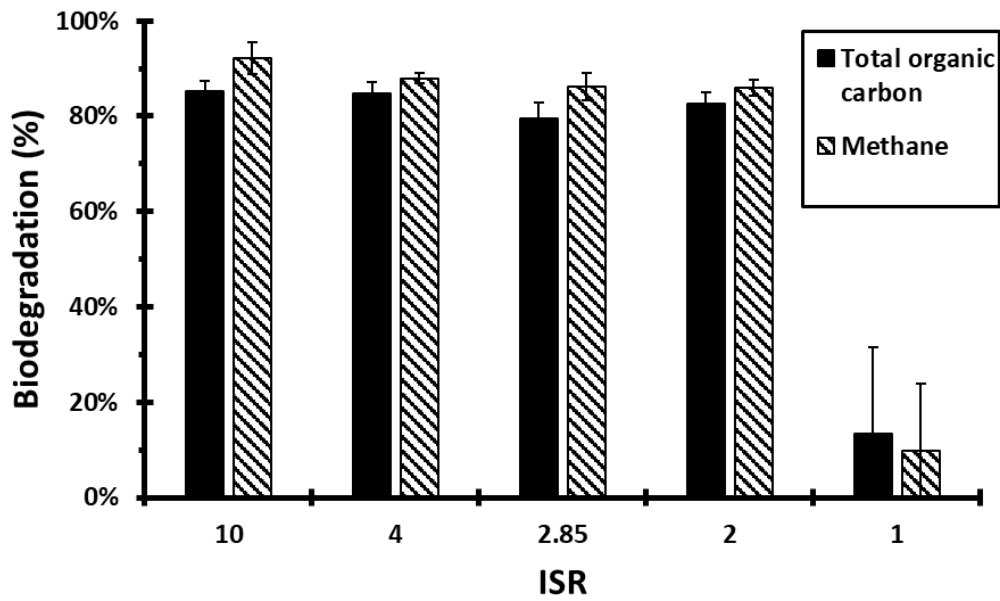


Figure II.2. The ultimate biodegradation level measured by the total organic carbon or methane-based method for PHB with different ISRs under mesophilic conditions.

Table II.2. Parameters of the Gompertz modeling

Substrate	Temperature (°C)	ISR	G(0) Methane potential (L CH ₄ kg VS ⁻¹)	λ Lag phase (days)	Rmax Methane production rate (L CH ₄ kg VS ⁻¹ d ⁻¹)	R ²
PHB	38	10	504 ± 17	6.62 ± 0.13	149.55 ± 15.19	0.997
		4	485 ± 5	6.37 ± 0.05	120.59 ± 0.26	0.998
		2.85	515 ± 36	6.53 ± 0.15	90.13 ± 6.75	0.999
		2	488 ± 4	6.07 ± 0.51	49.93 ± 27.43	0.994
		1	60 ± 71	27.82 ± 31.27	10.79 ± 15.59	0.625
PLA	38	10	426 ± 15	0	1.95 ± 0.22	0.997
		4	385 ± 2	0	1.51 ± 0.04	0.996
		2.85	401 ± 4	0	1.54 ± 0.01	0.994
		2	417 ± 12	0	1.42 ± 0.03	0.994
		1	404 ± 4	0	1.24 ± 0.02	0.994
PLA	58	10	456 ± 60	0	11.29 ± 0.84	0.992
		4	423 ± 10	0	9.17 ± 0.26	0.990
		2.85	390 ± 12	0	8.37 ± 0.52	0.989
		2	404 ± 19	0	7.49 ± 0.16	0.987
		1	374 ± 19	0	6.68 ± 0.01	0.986

3.1.1) PHB biodegradation under mesophilic conditions:

With most of the ISRs used, poly(hydroxyl butyrate) underwent almost complete and rapid conversion into methane under mesophilic AD, as was the case for cellulose (**Figure II.1A**). Interestingly, the ISR of 1 resulted in almost no methane production (*i.e.*, 51 ± 72 L CH₄ kg⁻¹ VS). Acidification of the reactors using an ISR of 1 was noted at the end of the test (pH = 5.5 ± 0.1). This acidification of the reactors was the result of substrate overload. The rate of PHB hydrolysis and VFAs generation was too high compared with the VFAs consumption rate, thereby leading to a drop in the pH (to 5.5 ± 0.1) and inhibition of the methanogenic archaea. For ISRs between 2 and 10, and after a lag phase of approximately 6 days (**Table II.2**), PHB was degraded between 84.8 and 93.8% in 10 to 25 days. This calculation was based on a theoretical methane production of 552 L CH₄ kg VS⁻¹ of PHB. Higher ISRs were associated with an increase in the methane production rate. However, the methane production rate did not increase linearly with the ISR ($R^2 = 0.75$, data not shown). The methane production rates of ISRs between 2.85 and 10 were not statistically different. There was a high standard deviation for the methane production rates of reactors using an ISR of 2 (49.9 ± 27.4 L CH₄ kg⁻¹ VS d⁻¹), which was probably due to a temporary accumulation of VFAs and consequent reduction of the activity of the methanogens. The ultimate methane production predicted by the Monod-Gompertz model corresponded to $91.3 \pm 3.1\%$, $87.9 \pm 0.9\%$, $93.3 \pm 6.5\%$, and $84.4 \pm 0.7\%$ of the theoretical methane production for ISRs of 10, 4, 2.85, and 2, respectively. According to the statistical analyses performed on the parameters of the Monod-Gompertz model (**Table II.2**), the ultimate methane productions did not differ according to the ISRs used, except for in case of an ISR of 1. The ISRs used did not affect the observed methane production when they were between 2 and 10. The ultimate biodegradation levels observed are consistent with the literature (Abou-Zeid et al., 2004, 2001; Benn and Zitomer, 2018; Budwill et al., 1992; Majone et al., 1995; Puechner et al., 1995). For example, Yagi et al. (2014) reported a biodegradation of 90% in 9 days for a PHB plastic film.

3.1.2) PLA biodegradation under mesophilic conditions

The anaerobic digestion of PLA under the mesophilic conditions stood out for its quite slow biodegradation kinetics (**Figure II.1B**). At the ISRs used, more than 340 days were required to reach the ultimate biodegradation level. At this stage, between $68.0 \pm 3.6\%$ and $80.0 \pm 2.1\%$ of the PLA

was converted into methane, depending on the ISR used. This calculation was based on a theoretical methane production of 522 L CH₄ kg⁻¹ VS for PLA (**Table II.1**). A similar biodegradation behavior has been reported for mesophilic anaerobic digestion (Bernat et al., 2021; Yagi et al., 2014b). Bernat et al. (2021) and Yagi et al. (2014) observed a biodegradation level between 29 and 66% after 280 days of digestion. Interestingly, in our study, there was not a lag phase during the AD of PLA, whereas a lag phase was observed for cellulose and PHB under the exact same conditions (**Figure II.1** and **Table II.2**).

It is worth noting that the ultimate methane production (G(0)) estimated by non-linear regression was higher than the observed values for ISRs between 1 and 2.85. This suggests that the methane production did not reach a steady state at 400 days under these conditions (**Table II.2**). The ultimate methane productions (estimated by the model) corresponded to 81.6 ± 2.9%, 73.8 ± 0.4%, 76.8 ± 0.8%, 79.9 ± 2.3%, and 77.4 ± 0.8% of the theoretical methane production for ISRs of 10, 4, 2.85, 2, and 1, respectively. Only the ultimate methane productions from PLA at ISRs of 4 and 10 were different, while the remainder were not statistically different. During the first few days of PLA digestion, the methane production rate was higher than that observed later on, irrespective of the ISRs used. This unexpectedly high level of methane production in the early days of the digestion could potentially correspond with the degradation of other compounds present in the polymer. Such a compound could be an additive present in the polymer that is degraded faster than the PLA itself (Chen et al., 2021). This hypothesis is supported by the fact that after a certain storage time, the surface of the PLA granules became coated with a fatty compound, presumably due to the loss (by exudation) of an additive. Contrary to the ultimate methane production, the methane production rates (R_{max}) increased linearly with the ISR (R² = 0.95, data not shown), thus suggesting that the kinetics of biodegradation were influenced by the ISR used (**Table II.2**). Consequently, the highest ISRs resulted in the best biodegradation performances, and the ultimate methane production was reached after 340 days. More than 60 additional days were needed to reach the steady state of methane production at the lowest ISR.

3.1.3) PLA biodegradation under thermophilic AD conditions

Biodegradation of PLA was more performant under thermophilic (58 °C) than mesophilic (38 °C) conditions. The time required to reach the steady state of biodegradation was reduced

approximately four-fold (**Figure II.1C**). Under the mesophilic conditions, more than 340 days were required, whereas only 60 to 100 days were needed under the thermophilic conditions. The faster biodegradation kinetics at the higher temperature could be partly attributed to the proximity of the PLA glass transition temperature (around 60 °C, Iannace et al., (2014)), which triggers chemical hydrolysis and facilitates access to microorganisms and enzymes (Itävaara et al., 2002; Shi and Palfery, 2010). Irrespective of the ISRs used, the ultimate biodegradation under thermophilic conditions was high, between $71.8 \pm 3.7\%$ and $85.85 \pm 11\%$. A similar biodegradation behavior has been observed elsewhere for PLA under thermophilic AD conditions (Hegde et al., 2018; Narancic et al., 2018; Yagi et al., 2009). PLA underwent a near-complete conversion into methane (79-90%) in these experiments, with timeframes varying from 36 to 100 days (Hegde et al., 2018; Narancic et al., 2018; Yagi et al., 2009). Similar to the mesophilic conditions, no lag phase was observed in the thermophilic reactors (**Table II.2**). Interestingly, the phase of higher methane production in the early days of PLA digestion observed under mesophilic conditions was not observed with the thermophilic conditions (**Figure II.1C**). This phase was hypothetically attributed to the degradation of a second compound. The degradation of this second compound was probably masked under the thermophilic conditions by the overall higher rate of methane production from PLA at this temperature.

A higher ISR also enhanced the biodegradation kinetics of the PLA under the thermophilic conditions. The methane production rate was significantly enhanced as the ISR increased, and the correlation was linear ($R^2 = 0.94$, data not shown). The highest ISR resulted in the highest methane production rate and thus also the shortest time to reach the maximum methane production (60 days). At the lowest ISR, this period extended to 100 days. On the other hand, the ultimate methane production (**Table II.2**) was not significantly different according to the ISR used. The ultimate methane production predicted by the Monod-Gompertz model corresponded to $87.4 \pm 11.5\%$, $81.0 \pm 1.6\%$, $74.7 \pm 2.3\%$, $77.4 \pm 3.6\%$, and $71.6 \pm 3.6\%$ of the theoretical methane production for ISRs of 10, 4, 2.85, 2, and 1, respectively. It is worth noting that the methane production rates from the reactors with the highest ISR, *i.e.*, corresponding to a low amount of sample added (approximately 0.5 g) to the reactors, were highly variable. This highlights the risk of non-reproducible experiments when using high ISRs.

3.1.4) Appropriate ISR for estimating the biodegradation of a plastic

As seen before, the use of an ISR of 1 during the BMP test of PHB led to inhibition of the AD process. Inhibition is often observed due to substrate overload with readily biodegradable substrates at a low ISR (Basak et al., 2021; Li et al., 2018). We demonstrated here that such a phenomenon could be observed for a readily biodegradable plastic and that the use of an ISR of 1 should be avoided for estimation of the BMP of an unknown biodegradable plastic. Similarly, an ISR of 2 applied to reactors fed with PHB led to temporary inhibition of the AD process, resulting in a highly variable methane production rate among replicates. This condition represents a risk of a degree of inhibition if an inoculum with a lower buffer capacity is used. Higher ISRs, between 2.85 and 10, resulted in similar ultimate methane productions for both plastics. Based on the recommendations provided by Holliger et al. (2016), ISRs leading to similar ultimate methane productions could be considered to be the most appropriate and could be used to determine the biochemical methane potential of a substrate. However, even though the use of different ISRs was not associated with a change in the ultimate methane production, the methane production rates (*i.e.*, the biodegradation kinetics) were significantly different based on the ISR used. A similar observation has been reported previously in other studies (González-Fernández and García-Encina, 2009; Raposo et al., 2006). The change in the biodegradation rate based on the ISR is problematic as the digestion time is a key parameter for classifying a plastic as biodegradable. This is especially so as the range of ISRs recommended by the standards is very broad, between 1.75 and 50 for ISO 14853 and between 2.4 and 14 for ISO 13975. Therefore, the ISRs provided in these standards should be more precise in order to accurately determine the biodegradation of a polymer under anaerobic digestion conditions. On the other hand, using high ISRs, such as ISR = 10, resulted in an increase in the variability between replicates, as a low amount of sample is introduced into the reactors (Raposo et al., 2008).

In the light of these results, we recommend the use of ISRs between 2.85 and 4 for biodegradable plastics. In this range, the ultimate methane productions and the methane production rates (biodegradation kinetics) were not statistically different. Moreover, the amount of substrate introduced into the reactor is sufficiently high, allowing reproducible observations to be made while avoiding inhibitions due to substrate overload.

3.2) Microbial community analyses

Anaerobic digestion of a substrate usually consists of three phases: a lag phase lasting a few days with no or a low level of methane production; an intensive methane production phase lasting several days; and finally a stationary phase with low or zero methane production (Koch et al., 2019). The microorganisms involved in the biodegradation of organic substrates are thought to be active during the intensive methane production phase and to have a low activity during the stationary phase. This is why the objective was to perform the sampling during these two phases. Nonetheless, the methane production from PLA under the mesophilic conditions was very slow and the stationary phase was reached after 350 days. Samples were, therefore, collected at two time points during the methane production phase, as shown in **Figure II.1**. The sampling during the last phase was not technically feasible due to the very long duration of the test and the sequencing platform schedule. The sampling days are indicated in **Figure II.1** and, as described before, it was only carried out with extreme ISRs (*i.e.*, 10 and 1) and with the ISR typically used in our laboratory (*i.e.*, 2.85).

A total of 892,377 sequences were collected after normalization to 12,933 sequences per sample. Almost 91.4% of the total sequences were assigned to the bacterial domain; while the remaining 9.6% were from the archaeal domain. The mesophilic reactors exhibited higher values of alpha diversity indices than the thermophilic reactors (data not shown). For example, there were an average of 270 ± 26 OTUs in the mesophilic communities and 154 ± 21 OTUs in the thermophilic communities. These findings are in accordance with the previous description of microbial communities of mesophilic and thermophilic AD reactors (Azizi et al., 2016; Levén et al., 2007; Moset et al., 2015; Yuji Sekiguchi et al., 1998). Under both conditions, the distribution of the populations in the different samples were highly uneven, thus indicating the presence of few dominant OTUs (representing the majority of the sequences) and a large number of rare OTUs

3.2.1) Distribution of the archaea:

In the mesophilic reactors, archaea represented an average of $6.52 \pm 6.28\%$ of the sample sequences. Only a small number of archaea were found to be active in the inoculum ($0.94 \pm 0.1\%$ of the sample sequences). A greater proportion of sequences related to archaea was found during the anaerobic digestion of PLA and PHB in the mesophilic digesters (at the first and second

sampling time). In the reactors fed with PLA under mesophilic conditions, archaea represented $3.03 \pm 0.61\%$ and $16.81 \pm 3.34\%$ at the first and second sampling time, respectively. In parallel, in the reactors fed with PHB, archaea represented $1.43 \pm 0.32\%$ and $6.66 \pm 1.45\%$ at the first and second sampling time, respectively. Interestingly, a high proportion of sequences attributed to methanogenic archaea was observed in samples collected during the steady state of methane production (*e.g.*, from the PHB-fed reactors at the second sampling time). The data in **Figure II.1** suggests that the methane production at these times and in these reactors was close to zero. However, this figure represents the methane production corrected for both the amount of substrate added and for the production in the blank reactor. Based on the raw production (**Figure II.3A & B**), it can be seen that methane was produced in these reactors at these times. Consequently, it is not surprising that there were active methanogens during the steady state. It was, however, surprising to find evidence of active methanogenic archaea ($6.53 \pm 0.68\%$ of the sample sequences) in reactors in which the apparent methane production was inhibited (*i.e.*, reactors fed with PHB at an ISR of 1). Some active methanogenic consortiums have previously been reported to be active after exposure to extremely acidic conditions under thermophilic AD (Han et al., 2019). Despite changes in abundance during the digestion process, the profile of the archaeal population remained unaltered irrespective of the plastic and ISR used. Three genera were dominant, namely, *Methanosaeta*, *Methanoculleus*, and *Methanospirillum*, representing $63.1 \pm 9.5\%$, $18.5 \pm 7.4\%$, and $13.8 \pm 5.7\%$ of the sample sequences, respectively. *Methanosaeta* produce methane by the acetoclastic pathway, while *Methanoculleus* and *Methanospirillum* are hydrogenotrophic methanogens (Amin et al., 2021; Sundberg et al., 2013). The predominance of acetoclastic methanogens ($63.1 \pm 9.5\%$ of the sample sequences) suggests that the methane was mainly generated by the acetoclastic pathway and that hydrogenotrophic methanogenesis plays a secondary role ($36.9 \pm 9.5\%$ of the sample sequences). Kirkegaard et al. (2017) also found a dominance of *Methanosaeta* when they investigated the microbial community of 32 full-scale anaerobic digesters in Denmark. Indeed, in their study, *Methanosaeta* represented between 60 and 80% of the sequences assigned to archaea from mesophilic reactors (Kirkegaard et al., 2017). Additionally, *Methanosaeta* and *Methanoculleus* have previously been found to represent a large part of the methanogenic archaea during mesophilic AD of PLA blends (Peng et al., 2022b).

In thermophilic reactors treating PLA, methanogenic archaea represented a greater proportion of the microbial community than under mesophilic conditions. Indeed, sequences assigned to the archaeal domain corresponded to an average of $11.33 \pm 6.02\%$ of the sample sequences, while the bacterial domain represented $88.67 \pm 6.02\%$. The proportion of archaea did not increase over time, amounting to $14.07 \pm 2.71\%$ and $14.66 \pm 3.29\%$ of the sample sequences at the first and second sampling time, respectively. By contrast, the apparent amount of methanogens decreased when the methane production was close to zero (**Figure II.3C**). At the last sampling time, with ISRs of 10 and 2.85, the methane production rates were between 0.8 and 1 mL.day⁻¹ and the relative abundance of archaea only amounted to between $1.04 \pm 0.1\%$ and $2.46 \pm 0.6\%$, respectively, of the sample sequences. *Methanosarcina* was the most abundant methanogen in the different reactors, as it represented 96.3% of the total archaea sequences. Hydrogenotrophic methanogens (mainly represented by *Methanoculleus* and *Methanothermobacter*) have previously been found to be dominant in thermophilic reactors fed with different PLA-based biodegradable plastics (Cazaudehore et al., 2021a). Similarly, Peng et al. (2022) found that there was an enrichment of *Methanothermobacter* during the thermophilic AD of a PLA blend. In their study, *Methanoculleus* and *Methanosarcina* were the other most abundant methanogenic archaea in thermophilic reactors fed with biodegradable plastics. Hydrogenotrophic methanogens (from the *Methanobacteriaceae* family) were also found to be predominant in thermophilic reactors fed with PLA (Bandini et al., 2020). *Methanosarcina* is able to carry out both the hydrogenotrophic and the

acetoclastic pathway, so it is not possible to determine which methane production pathway was favored in this experiment (De Vrieze et al., 2012).

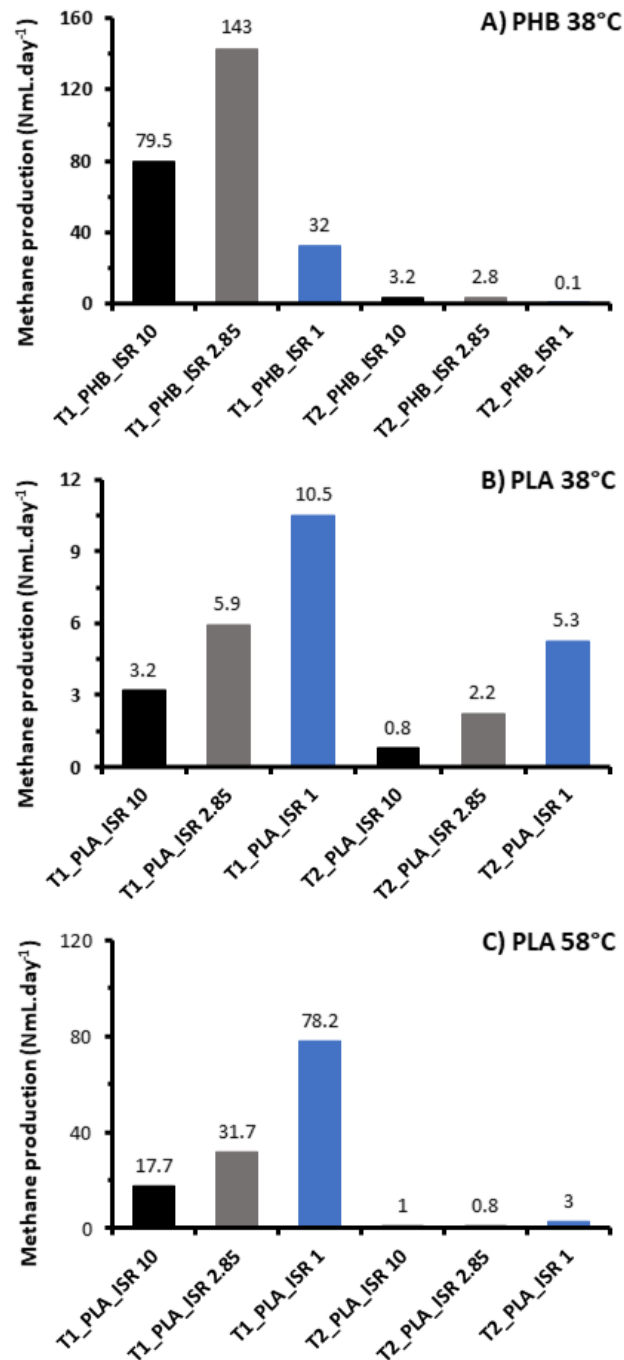


Figure II.3. Raw methane production (uncorrected for the mass of the sample and the production of the blank reactors) at the first (T1) and second (T2) sampling times for microbial analysis in reactors fed with PLA and incubated at 38 °C (A), at 58 °C (B), or fed with PHB and incubated at 38 °C (C).

3.2.2) Comparison of the microbial communities:

The composition of the microbial communities in the various samples was explored using redundancy analysis (RDA) (**Figure II.4**). Multivariate analysis was performed to determine correlations between the OTUs' relative change in abundance and the environmental variables. Only OTUs with high relative changes in abundance and representing more than 1% of the sequences in at least one sample were taken into account (**Figure II.5**). The environmental variables selected were the following: methane production under mesophilic conditions from PLA and from PHB, methane production under thermophilic conditions from PLA, the presence or absence of inhibition due to PHB overload, and the nature of the samples (inoculum or digestate from BMP reactors).

The RDA (**Figure 4A**) revealed that thermophilic and mesophilic BMP reactors formed very distinct active microbial communities. *Bacteroidota* (33%), *Desulfobacterota* (13.6%), *Chloroflexi* (12.8%), and *Firmicutes* (12%) were the most abundant phyla in the mesophilic reactors; while *Firmicutes* (44%), *Coprothermobacter* (11.7%), and *Proteobacteria* (9.5%) were dominant in the thermophilic reactors. These phyla have previously been observed to be dominant phyla in thermophilic and mesophilic AD reactors (Guo et al., 2015; Li et al., 2015; Nelson et al., 2011; Sundberg et al., 2013). The temperature has also previously been shown to be a determining factor affecting the microbial community composition (Azizi et al., 2016; Levén et al., 2007; Sundberg et al., 2013). To gain a better appreciation of the changes in the microbial populations induced by the ISR, individual RDAs were performed on mesophilic samples on the one hand and thermophilic samples on the other hand (**Figure II.4B & C**).

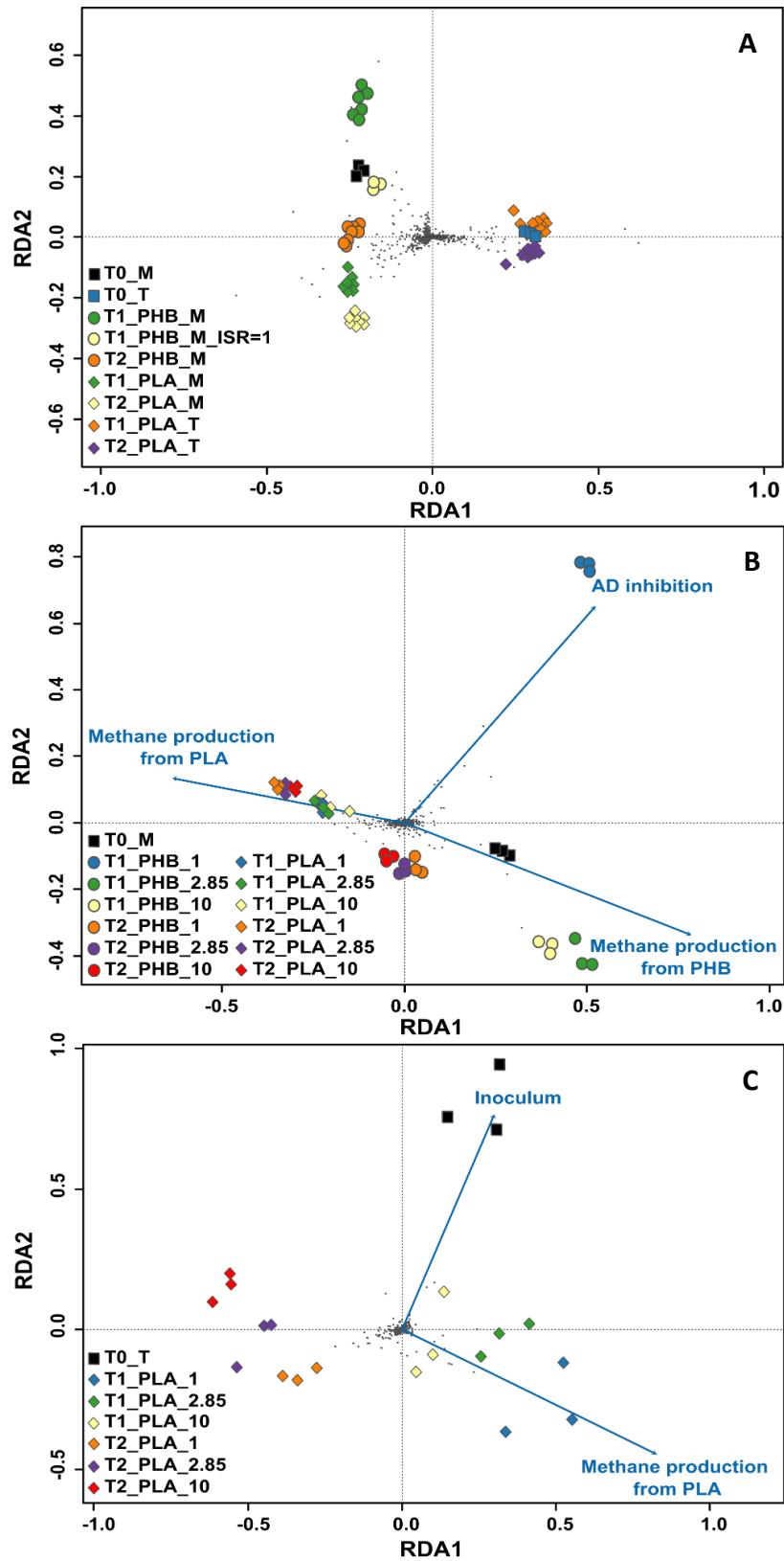


Figure II.4. Redundancy analysis (RDA) representing the environmental variables (arrows), OTUs (dots), and samples (colored rectangles, circles, and diamonds) on the entire dataset (A), the mesophilic samples (B), and the thermophilic samples (C).

Depending on the ISR used, the populations from reactors fed with PHB differed at the first sampling (**Figure II.4B**). Under non-inhibited conditions (ISRs of 2.85 and 10), the populations were very similar at the first sampling time. On the other hand, the populations from reactors with inhibited methane production were very different from the remainder at the first sampling time. Quite surprisingly, the apparent active microbial populations at the second sampling time of the reactors with PHB at the different ISRs were very similar. In a sense, this observation may seem trivial because each sample was obtained from a reactor in the post-digestion phase, and even though the conversion into methane was inhibited in one case (ISR = 1), the PHB was still biodegraded. However, as the pH was very different in the reactors at ISR = 1 (*i.e.*, pH = 5.5) and the other ISRs (*i.e.*, pH = 7.5), it is surprising that the active microbial populations appeared to be similar. The main OTU correlated with inhibition of the methane production was *Ilyobacter delafieldii*. It represented 13.6% of the sequences in the inhibited reactors, whereas it represented no more than 0.1% of the sequences in the other mesophilic reactors. The ability of this bacterial species to ferment PHB has been observed previously and it has a particular capacity to produce extracellular PHB depolymerase (Janssen and Harfoot, 1990a). The other major OTUs correlated with inhibition (ISR = 1) were *Ruminofilibacter* (8.3%), *Hungateiclostridium UCG-012* (5.8%), and *Lachnospiraceae* (2.3%), which have been reported to be involved in the hydrolysis and fermentation of organic AD substrates but not specifically PHB (Vacca et al., 2020; Wang et al., 2020; X. Zhang et al., 2018). The observation of a large number of bacteria capable of hydrolysis and fermentation corroborates the notion that an imbalance between the rate of hydrolysis, VFAs generation, and VFAs consumption by the methanogens are the reasons behind the inhibition of the methane production. On other hand, the OTUs correlated with the production of methane from PHB were mainly *Enterobacter*, *Lentimicrobium*, and *Desulfobulbus*, representing $13.5 \pm 4.5\%$, $10.5 \pm 0.4\%$, and $7.8 \pm 0.1\%$, respectively, of the sequences in the samples exhibiting high levels of methane production. Species from the genus *Enterobacter* have previously been isolated from tropical marine environments and identified as PHB-degrading microorganisms (Volova et al., 2010a). *Lentimicrobium* is another hydrolyzing bacterium (Wirth et al., 2019a). Unlike the correlation with the inhibition, where all the positively correlated OTUs were related to the hydrolysis step, some OTUs positively correlated with methane production from PHB were related to other steps of the anaerobic digestion. These OTUs, namely *Desulfobulbus*, *Treponema*, and

Syntrophales, have been related to sulfate reduction, propionate-oxidation, and homoacetogenesis (El Houari et al., 2017; Nobu et al., 2020; Wang et al., 2013). Correlation of the sulfate-reducing *Desulfobulbus* with methane production from the PHB was explained by the presence of sulfur in the composition of the PHB (0.1% TS). A certain amount of hydrogen sulfide was, therefore, produced during the anaerobic digestion of PHB. On the other hand, the PLA did not contain sulfur, and no hydrogen sulfide was produced during its digestion.

Under mesophilic conditions, the active populations on PLA-fed reactors were very similar at the same sampling time, irrespective of the ISR used (**Figure II.4B**). On the other hand, the populations changed over time, as samples from the first and the second sampling time were clustered separately (**Figure II.4B**). The methane productions at these two periods were, however, not very different (**Figure II.3B**). Multivariate analysis highlighted the correlation of some OTUs with the production of methane from PLA. However, these OTUs represented a relatively low proportion of the active populations of PLA-fed reactors, *i.e.*, *Smithella* ($2.2 \pm 1.9\%$), *Dehalobacter* ($1.9 \pm 1.3\%$), and *Izomoplasmatales* ($1.6 \pm 0.9\%$). The only exception was *Anaerolineaceae ADurb.120*, which represented between 12.5 and 17.5% of the sequences in the PLA-fed reactors, while it represented an average of $7.6 \pm 1.7\%$ of the sequences of the other mesophilic samples. *Anaerolineaceae ADurb.120* was identified as a member of the core microbiome of the mesophilic reactors irrespective of the substrate or variation, as determined by Prem et al. (2020). Cultivated strains of *Anaerolineaceae* can ferment numerous carbohydrates but they are not able to use lactate (the product of PLA hydrolysis), and they are often found to be part of a syntrophic relationship with hydrogenotrophic methanogens (Yamada et al., 2007, 2006). Even though some members of the *Anaerolineaceae* family have been reported to play a key role in the activation of long-chain alkane biodegradation, they have not been shown to be implicated in the biodegradation of polyesters (such as PLA) (Liang et al., 2016).

Under thermophilic conditions, the microbial communities were distributed according to their methane production (**Figure II.4C**). Multivariate analysis highlighted the correlation of members of the genus *Tepidanaerobacter* with the increasing methane production from PLA in the thermophilic reactors. Members of this genus have been reported elsewhere to be lactate-utilizing bacteria in thermophilic AD systems (Sekiguchi, 2006). Previous studies have observed that lactate-utilizing bacteria were key bacteria during the thermophilic AD process of compounds based on

PLA (Cazaudehore et al., 2021a; Tseng et al., 2019). Another study determined that lactate accumulation had a negative effect on the physicochemical depolymerization of PLA and that these bacteria allowed the physicochemical depolymerization rate of PLA to be enhanced by the use of lactate (Tseng et al., 2020b). Moreover, an OTU related to the genus *Thermogutta* was found to be highly correlated with the methane production from PLA. A strain of *Thermogutta* was previously described for its ability to produce esterase (Sayer et al., 2015; Slobodkina et al., 2015), and it may be involved in the hydrolysis of long-chain PLA under thermophilic conditions.

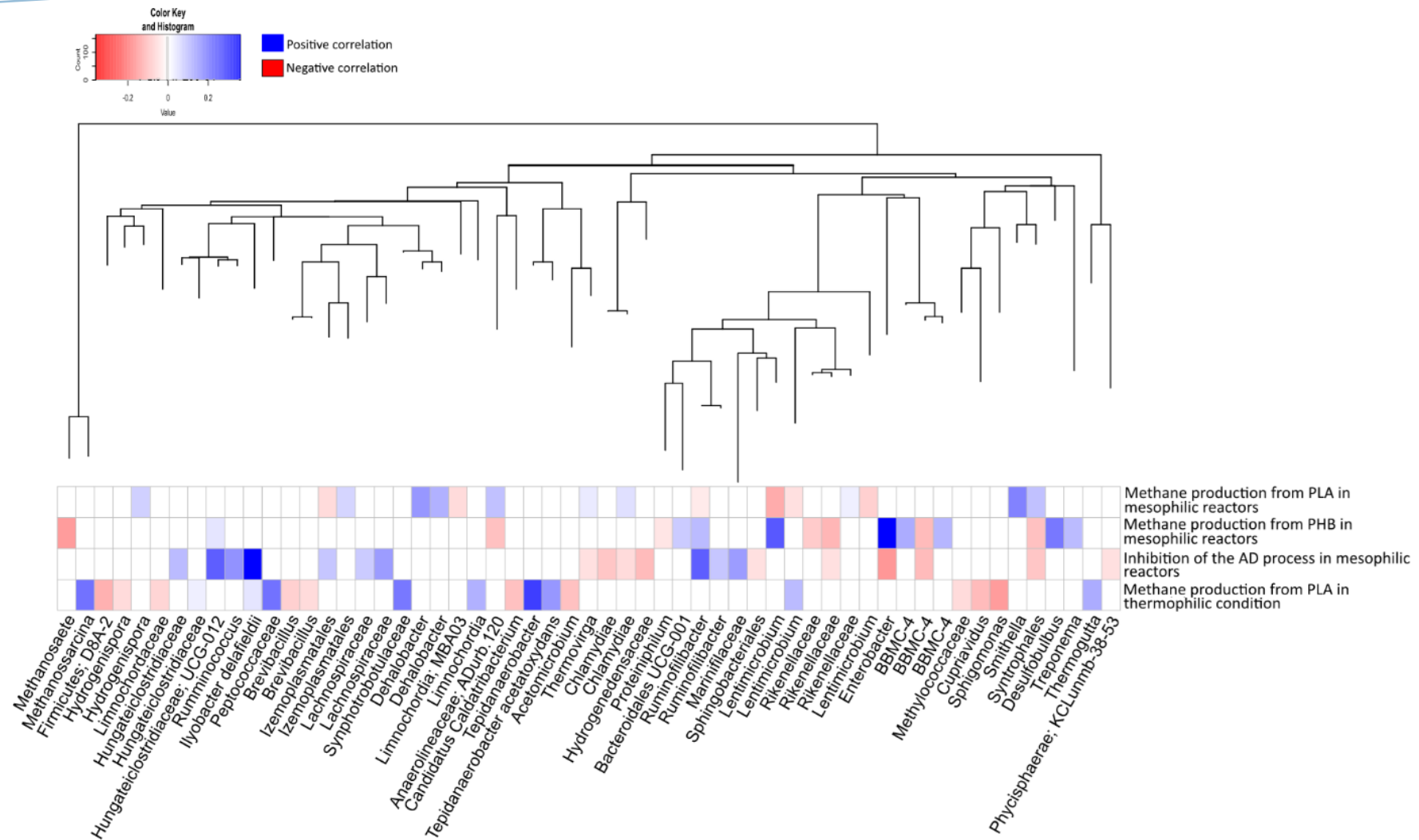


Figure II.5. Heatmap of the multivariate regression analysis. Significant correlation (P -value < 0.05) and the major OTUs (representing more than 1% of the sequences in at least one sample) are depicted. Blue rectangles represent a positive change in the relative abundance whereas red rectangles represent a negative correlation. The intensity of the colors correlates with the magnitude of the Hellinger transformed abundance change.

4) Conclusion

The results of this study suggest that the ISR is a key parameter for estimating the biodegradation behavior of biodegradable plastics in BMP tests. The ultimate methane production was not significantly altered by the ISR used, except when there was inhibition of the methane production. However, an increase in the ISR was associated with an increase in the methane production rate. Based on these observations, we recommend avoiding low ISRs (≤ 2) to prevent overloading. Similarly, we do not recommend using high ISRs in order to avoid an excessive dilution of the substrate in the reactor, which reduces the accuracy of the test. ISRs between 2.85 and 4 are recommended for BMP testing of biodegradable plastics. In parallel, the analysis of the correlation between the change in the relative abundance of the OTUs and the methane production from PLA under mesophilic conditions revealed the importance of a fermentative bacterium related to *Anaerolineaceae ADurb.120*. This same analysis for PHB highlighted the importance of two PHB degraders: *Ilyobacter delafieldii* and *Enterobacter*. Under thermophilic conditions, *Tepidanaerobacter* stood out as a probably lactate-utilizing bacteria and *Thermogutta* due to its hydrolyzing ability. Their involvement has, however, not been ascertained and needs to be confirmed by further studies.

CHAPTER III

Batch anaerobic digestion experiments

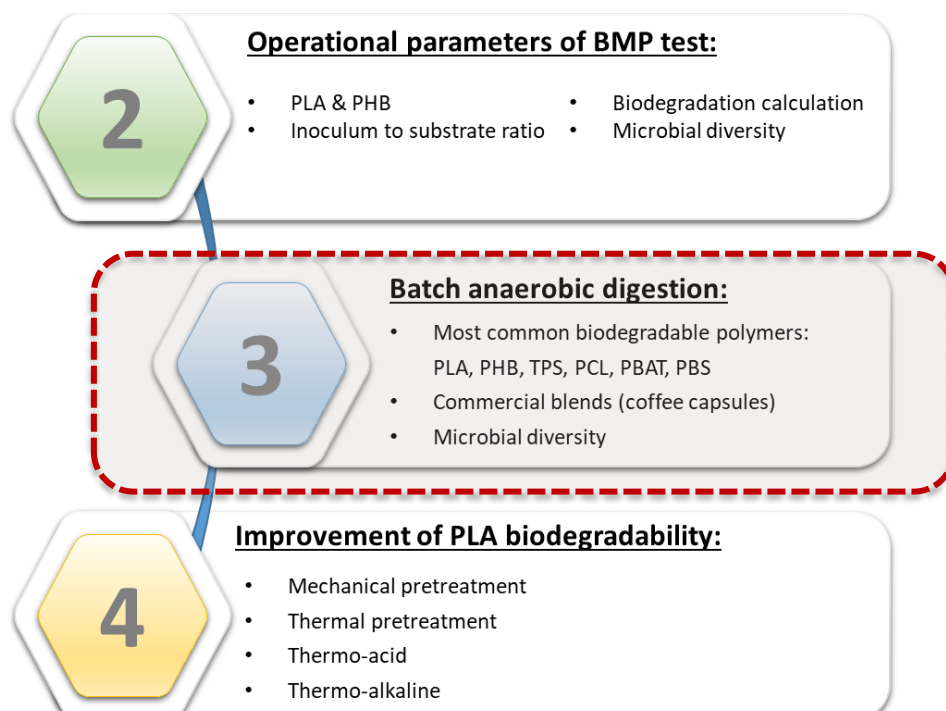
This chapter is based on a modified version of two scientific papers:

Part A in preparation:

Cazaudehore, G., Monlau, F., Gassie, C., Lallement, A., Guyoneaud, R., 2022. Active microbial communities during biodegradation of biodegradable plastics through mesophilic and thermophilic anaerobic digestion (in preparation).

Part B published in Science of The Total Environment:

Cazaudehore, G., Monlau, F., Gassie, C., Lallement, A., Guyoneaud, R., 2021. Methane production and active microbial communities during anaerobic digestion of three commercial biodegradable coffee capsules under mesophilic and thermophilic conditions. Science of The Total Environment 784, 146972. <https://doi.org/10.1016/j.scitotenv.2021.146972>



Foreword

The capacity to estimate the biodegradation behavior of biodegradable plastics in anaerobic digestion systems is crucial. The introduction of biodegradable plastics, unable to undergo complete biodegradation in the classical Hydraulic Retention Time (HRT) of industrial anaerobic digesters, will result in biodegradable plastic persistence in digestate and ultimately in the environment.

As highlighted in the previous chapter, the inoculum to substrate ratio (ISR) used in biochemical methane potential (BMP) test has an influence on the measured biodegradation kinetics. An increase of the ISR is associated with an improved biodegradation kinetic and to an increase of the variability. On the contrary, low ISRs are associated with a risk of observing an inhibition of the methane production. Therefore, the BMP tests performed in the rest of this work uses an ISR of 2.85. Furthermore, as the two methods of calculating biodegradation resulted in similar ultimate biodegradation, the method based on methane production is preferred in the rest of the manuscript, as it requires less experiments and thus limits the associated experimental errors.

During the ISR tests, BMPs have been performed on two biodegradable plastics that are two of the most used (PLA) or promising (PHB) polymers for biodegradable plastics. Nonetheless, a wide variety of polymers can be used for biodegradable plastic production (Narancic et al., 2018). According to European Bioplastics (2020), the main biodegradable polymers are starch blends (38.4% of the biodegradable plastic production in 2019), poly(lactic acid) (PLA, 25.0%), poly(butylene adipate/terephthalate) (PBAT, 24.1%), poly(butylene succinate) (PBS, 7.7%) and polyhydroxyalkanoates (PHAs, 2.2%) (European Bioplastics, 2019). PHAs are a family of polyester, Poly(3-hydroxybutyrate) (PHB) is the most widespread members of this family (Bugnicourt et al., 2014). Additionally, Poly(ϵ -caprolactone) (PCL) could be added to the list as it is generally found blended with other biodegradable polymers notably with thermoplastic starch (TPS) (Arakawa and DeForest, 2017). Up to date, few scientific papers had investigated the mesophilic and thermophilic biodegradation of the main biodegradable plastics using a same experimental protocol (Narancic et al., 2018; Yagi et al., 2014b, 2013b).

In the next chapter, the biodegradation of eight of the most common biodegradable polymers in both mesophilic and thermophilic condition was measured and the microbial communities involved in the process were examined. The test duration has been expanded in comparison with standard test (ISO 14853 and ISO 13975) and with classical retention time in order to estimate the ultimate biodegradation. However, these polymers are usually blended and combined with additives to generate industrial grades. Therefore, in a second time, the biodegradation and the microbial communities involved in anaerobic digestion of commercial blends of biodegradable plastics used in coffee capsules has been investigated. The commercial blends were purchased in the form of three types of coffee capsules, certified biodegradable under industrial composting conditions.

PART A: Biodegradation of the main biodegradable polymers

Abstract

The biodegradation of the main biodegradable plastics (PLA, PCL, PHB, TPS, PBS and PBAT) was measured under thermophilic and mesophilic anaerobic digestion. Active microbial communities involved in the process were observed by amplicon sequencing of the 16S rRNA. Under mesophilic conditions (38 °C), TPS and PHB achieved high biodegradation (82.6 and 64.3-80.3%, respectively) within a relatively short time period (25-50 days). By contrast, PCL and PLA exhibited very low biodegradation rates at 38 °C and 500 days were required to reach the ultimate methane production (corresponding to 49.4% of biodegradation for PCL and 74.7-80.3% for PLA). Little or no degradation was measured for PBAT (13.4%) and PBS (0%) at 38°C in 500 days. Under thermophilic condition (58°C), TPS, PHB and PLA reached high level of biodegradation (80.2%, 57-72.3% and 74.6%, respectively). PBS, PBAT and PCL were not converted in methane under thermophilic conditions. The methane production rate from PLA was greatly enhanced at 58°C since only 60 to 100 days were required to reach the ultimate methane production. Previously identified PHB degraders (*Enterobacter* and *Cupriavidus*) were found to be correlated with methane production from PHB-fed reactors at 38°C and 58°C, respectively. Similarly, the importance of lactate utilizing bacteria (*Moorella* and *Tepidimicrobium*) was highlighted during the thermophilic anaerobic digestion of PLA. Finally, OTUs affiliated to Clostridium genus were evidenced during mesophilic and thermophilic AD of TPS, respectively.

1) Introduction

Biodegradable plastics have been developed to represent an environmentally friendly alternative to conventional plastics. The biodegradable plastics include a wide range of different polymers, each with their own properties and biodegradation behavior. According to the European bioplastic association, the main biodegradable polymers are starch blends (representing 38.4% of the biodegradable plastic production in 2019), poly(lactic acid) (PLA, 25.0%), poly(butylene

adipate/terephthalate) (PBAT, 24.1%), poly(butylene succinate) (PBS, 7.7%) and polyhydroxyalkanoates (PHAs, 2.2%) (European Bioplastics, 2019). PHAs are a family of polyester, Poly(3-hydroxybutyrate) (PHB), poly(3-hydroxyvalerate) (PHV) and their copolymer poly(3-hydroxybutyrate-cohydroxyvalerate) (PHBV) are the most widespread members of this family (Bugnicourt et al., 2014). Poly(ϵ -caprolactone) (PCL) could be added to the list, PCL is generally found blended with other biodegradable polymers such as thermoplastic starch (TPS) (Arakawa and DeForest, 2017).

Their biodegradable nature provides new end-of-life opportunities such as composting and anaerobic digestion. Currently, industrial composting is the privileged end-of-life option for biodegradable plastics. Therefore, polymers commonly referred to biodegradable are, at least, biodegradable under industrial composting condition according to the EN 13432 standard (Abraham et al., 2021; European Bioplastics, 2020). Their biodegradability is however strongly linked to the environmental conditions and thus, their biodegradation under other conditions is not assured (Narancic et al., 2018).

Anaerobic digestion represent a promising valorization route, allowing the recovery of a part of the energy through methane generation (Abraham et al., 2021). The variability of the operating protocols and the plastics used (*i.e.*, origin and physico-chemical properties) in anaerobic digestion makes the results difficult to compare across studies and sometimes contradictory results are reported. For example, Vasmara and Marchetti, (2016) observed no biodegradation for ground PLA cups (<1mm) in 90 days under mesophilic anaerobic digestion. In a similar digestion period Itävaara et al. (2002) reported a biodegradation of 60% for PLA granules under mesophilic anaerobic digestion in 100 days.. In general rules, it has been demonstrated that polymers degrade better under thermophilic than mesophilic conditions (Batori et al., 2016; Abraham et al., 2021). Cazaudehore et al. (2022) have defined three main categories of biodegradables plastics in anaerobic digestion: polymers that are readily biodegradable in both mesophilic and thermophilic conditions like TPS and PHB; polymers that are biodegraded very slowly under mesophilic conditions and much faster under thermophilic conditions such as PCL and PLA and finally the polymers that are biodegradable under industrial compositing conditions according to the EN 13432 standard and poorly or not at all biodegraded by either mesophilic or thermophilic AD such as PBAT and PBS. Nonetheless, not all the main biodegradable polymers have received the same

attention, as the majority of studies have focused on mesophilic degradation of PHB, PHBV, PCL, and PLA and PLA, PCL, PBS on thermophilic conditions (Bátori et al., 2018a; G. Cazaudehore et al., 2022).

Furthermore, the ultimate methane production from biodegradable plastics is often underestimated due to the use of unsuitable digestion period during the AD tests. Indeed, most of the biodegradable plastics have low methane production rate; therefore the time required to reach the ultimate methane production is much higher than the typical retention time observed in industrial biogas plants (around 30-50 days for biowastes, 50-120 days for agricultural wastes). Narancic et al. (2018) and García-Depraect et al. (2021) had described the biodegradation behavior of the main biodegradable plastics in several anaerobic digestion process, according to ISO 15985 (simulating high solid and thermophilic AD) and ISO 14853 (simulating semi-liquid and mesophilic AD). In these two studies, the duration of the test was between 56 (mesophilic AD) and 127 days (thermophilic AD). The digestion period was insufficient for observing the ultimate methane production from most of the biodegradable plastics, especially under mesophilic condition where biodegradation rates are lower.

As anaerobic digestion relies on complex microbial populations, the structure of these populations is a fundamental parameter for AD process (Castellano-Hinojosa et al., 2018; De Vrieze et al., 2018; Peng et al., 2022b). However, little is known about the microbial populations involved in the AD of biodegradable plastics (F. Bandini et al., 2020; Cazaudehore et al., 2021a; Emadian et al., 2017a; Tseng et al., 2019; Venkiteshwaran et al., 2019; Yagi et al., 2014b, 2013a). Venkiteshwaran et al. (2019) explored the microbial ecology during continuous co-digestion of PHB and biowaste but the analysis did not bring to light previously known PHB degraders. Similarly, Peng et al. (2022) highlighted *Clostridium* (sensu stricto), *Streptococcus* and *Caldicoprobacter* as the genera being responsible for the difference between thermophilic reactors fed with a blend of PLA/PBAT and reactors not fed with plastics. Recently, Tseng et al. (2020, 2019) and Cazaudehore et al. (2021) highlighted the importance of lactate utilizing bacteria from the genus *Tepidimicrobium* during the thermophilic AD of PLA and of PLA blends, respectively.

For these reasons, this study intended to examine the AD of the main biodegradable polymers (TPS, PLA, PBAT, PCL, PBS and PHB) under both mesophilic and thermophilic semi-liquid AD. The

test duration was increased in comparison with standard testing methods and retention times of industrial anaerobic digesters in order to measure the ultimate methane production from the main biodegradable plastics. The identification of the microorganisms involved in the anaerobic digestion of the different plastics was examined through MiSEQ analyses of the 16S rRNA (active microbial populations).

2) Material and methods

2.1) Origin and characterization of samples and inocula

Polyhydroxybutyrate (PHB) and Poly(lactic acid) (PLA) are among the most studied polymer to replace the petroleum derived plastics (Boey et al., 2021). That is why two examples of these polymers were selected for this study. PLA was purchased from Natureplast® (SF 141, Natureplast®, Iffs, France) and Total Corbion® (Luminy LX 175, Total Corbion®, Gorinchem, Netherlands). PHB was purchased from KD Feddersen (Paris, France) and from Biomer® (P209, Biomer®, Schwalbach, Germany). The following plastics were purchased from specified sources: PCL (Capa™ 6250, Perstorp, Malmö, Sweden), PBAT (Ecoworld 009, Jinhui Zhaolong, Fuxi St. Taiyuan City, China), PBS (PBE 003, Natureplast®, Iffs, France). In addition, a TPS was obtained from the laboratory CATAR CRITT Agro ressources (Toulouse, France). It was processed on a Clextral Evolum (Clextral, Firminy, France) from 70% starch obtained from wheat flour (type 55, Gers farine, France) and 30% glycerol (>99%, Gaches Chimie, Escalquens, France). Cellulose (positive control) was purchased from Tembec® (Témiscaming, Canada) in the form of plates and were ground using a plant shredder (GHE 355, Stihl®, Waiblingen, Germany).

Two inocula, from a mesophilic anaerobic digestion plant (Méthelayou, Préchacq-Navarrenx, France), were acclimated to either the mesophilic (38 °C) or the thermophilic (58 °C) anaerobic condition. They were put in condition of anaerobic digestion and were fed twice a week with a mix of sludge from wastewater treatment and green grass. Control parameters (pH, oxydo-reduction potential, FOS/TAC, concentration of ammonia and volatile fatty acids) and the ability to convert the positive control (cellulose) in methane during biochemical methane potential test (BMP) were measured regularly. The inocula were sieved to a 2mm mesh size prior to use in the BMP test to reduce the biogas production from the residual undegraded organic fraction.

The total solids (TS) and the volatile solids (VS) of the different plastics, the cellulose and the inocula were determined using APHA standard methods (American Public Health Association., 2005). Analysis of the elemental composition (determination of the carbon, hydrogen, nitrogen and sulphur content) was performed using an Elemental Vario Macro Cube analyser (Elementar, Langensfeld, Germany). Oxygen content was calculated by difference between the VS content and the sum of the carbon, hydrogen nitrogen, and sulphur content. Ammonia titration was performed using kits (Spectroquant® Ammonium Cell Test, Merck, Darmstadt, Germany). VFAs content was determined by gas chromatography (7890B, Agilent, USA) and alkalinity by titration using sulfuric acid (0.1N) (Lallement et al., 2021). **Table III.1** summarize the characterization of the inocula and samples.

2.2) Biodegradation test using biochemical methane potential test:

Experimental set up of the biochemical methane potential (BMP) test was used in order to determine the biodegradation behavior of the main biodegradable plastics and of cellulose (positive control). This protocol was adapted from the recommendations made during the European inter-laboratory study on BMP test (Hafner et al., 2020; Holliger et al., 2021, 2016; Ribeiro et al., 2020). Test were carried out under thermophilic (58 °C) or mesophilic (38°C) condition in 600 mL bottles. 300 mL of a mixture of water, inoculum and test material were used. The amount of inoculum introduced was the same in the different bottles/tests; on the contrary, the amount of water and test material was adapted to satisfy an inoculum substrate ratio of 2.85 g VS g⁻¹ VS and to have the same working volume in the different reactors. Each condition was run in triplicate. Before the airtight closing of the bottles, pH and redox of the mixture was measured, then anaerobic condition was reached by nitrogen flushing (Alphagaz™ Smartop, Air Liquide, Paris, France) of the headspace. Biogas production was monitored over time using a manometric method (Digitron 2023P, Digital Instrumentation Ltd, Worthing, United Kingdom). Then, the biogas composition was determined using a gas chromatography (Micro GC 490, Agilent, Santa Clara, USA), as described elsewhere (Cazaudehore et al., 2021a). A triplicate of blanc control, consisting of only water and inoculum, was run in parallel. The mean biogas production from the blanc controls was subtracted to the production of the other bottles. It corresponded to the biogas production from the residual organic material included in the inocula. pH was measured at the end of the test to observe a potential acidification of the reactors. All of the results are presented for

normalized conditions of temperature and pressure (P_{atm} , 0 °C). The biodegradation yield was estimated using **Equation 1**. Cumulative methane production curves of the different substrates were modeled according to a modified Monod-Gompertz model (Rakmak et al., 2019), **eq.2**. The parameters of the model were determined for each condition using R software (version 3.6.2) and Nonlinear Least Squares method (nlsLM function from minpack.lm package).

$$\text{Eq.1: Biodegradation (\%)} = \frac{\text{Observed methane production}}{\text{Theoretical methane production}}$$

$$\text{Eq. 2: } G(t) = G(0) * \exp \left[- \exp \left(\frac{R_{max} * \exp(1)}{G(0)} * (\lambda - t) + 1 \right) \right]$$

Where:

- $G(t)$ is the cumulative methane production at the t time in $L \text{ CH}_4 \text{ kg}^{-1} \text{ VS}$
- $G(0)$ is ultimate methane produced in $L \text{ CH}_4 \text{ kg}^{-1} \text{ VS}$
- λ is the time lag in days
- R_{max} the methane production rate in $L \text{ CH}_4 \text{ kg}^{-1} \text{ VS days}^{-1}$

The theoretical methane production was calculated from the elemental composition using the **Eq.3** (Achinas and Euverink, 2016a; Boyle, 1977; Buswell and Mueller, 1952).

$$\text{Eq.3: Theoretical methane production (NL(CH}_4\text{).g}^{-1}\text{(C}_x\text{H}_y\text{O}_z\text{N}_n\text{S}_s\text{))} = \frac{22.4 \times \left(\frac{x}{2} + \frac{y}{8} - \frac{z}{4} - \frac{3n}{8} - \frac{s}{4} \right)}{12x + y + 16z + 14n + 32s}$$

2.3) Analysis of the microbial diversity

2.3.1) Sampling

Samples of digestate were taken, in triplicate, at the beginning of the BMP test (T_0) and during the anaerobic digestion of the different plastics. When possible, the samplings were conducted during both a high methane production phase and a low methane production phase. Therefore, the days of the sampling were adapted to the biogas production of each substrate. The specific days are indicated in the **figure III.1**. Two volumes of RNAprotect Reagent® (Qiagen, USA) were mixed with one volume of digestate to ensure a stabilization of the RNA. After an incubation of 5 minutes, the mix was centrifuged (5000 rcf, 5 min), the supernatant discarded and the pellets stored at -20°C during a maximum of two weeks before being transferred to -80 °C.

2.3.2) Sequencing of the 16S rRNA transcripts

Fast RNA Pro Soil Direct kit (MP Biomedicals™, Irvine, USA) were used to extract the RNA according to manufacturer's instructions. Absence of DNA in the extracts was verified by polymerase chain reactions and gel electrophoresis. Quantification of the RNA was measured using Quant-iT™ RNA HS Reagent (Invitrogen™, Waltham, USA). 5 ng of extracted RNA were retro-transcribed using the M-MLV reverse transcriptase (Invitrogen™, Waltham, USA) and amplified using AmpliTaq Gold™ 360 Master Mix (Applied Biosystems, Waltham, USA) with the V4 V5 primers (515F and 928R, Wang and Qian, 2009) as described in Cazaudehore et al. (2021). Amplicons were sequenced by the PGTB service (Bordeaux, France) using Illumina MiSeq 250 bp paired-end technology.

2.3.3) Data processing

Treatment of the sequencing data was performed on Galaxy FROGS pipeline (Afgan et al., 2018) according to the method described by Escudié et al. (2018). Sequences were merged, denoised and dereplicated using the pre-processing tools of the system. Operational Taxonomic Units (OTU) were produced based on a three base aggregation distance. Minor OTUs, consisting of less than 0.0005% of the total sequences, and chimera OTUs were withdrawn. Finally, the taxonomic assignments was produced using the Silva database v128.1 (Pruesse et al., 2007). The number of sequences per samples were normalized to 14508 sequences. The data have been deposited in GenBank under the accession number PRJNA783887.

Statistical analysis were carried out on R version 3.6.2. Alpha diversity metrics (Richness, Chao1, Exp. Shannon and Inv. Simpson) were calculated for each samples using the phyloseq package. Alpha diversity indices were compared using Kruskal-Wallis and Dunn test (package dunn.test). After an Hellinger transformation of the OTU's abundances, the relationship between environmental variables and the microbial composition of the different samples was explored using redundancy analysis (RDA) (Paliy and Shankar, 2016; Ramette, 2007; X. Wang et al., 2012). Significant environmental variables were selected using ANOVA on RDA (p value < 0.05). Graphic representation of the samples and environmental variables was produced using vegan package. Multivariate regression analysis (function lm and ANOVA) was realized to select the OTUs that are significantly correlated with the variation of the environmental variables (p-value < 0.05) and to determine direction (positively or negatively) and magnitude of the correlation (X. Wang et al.,

2012). Finally, heatmaps representing the top 10 positively correlated OTUs for each environmental variable were generated using heatmap.2 package. OTUs were reordered on the heatmaps to match with the phylogenetic tree made using ggtree package.

3) Results and discussions

3.1) Anaerobic biodegradation test

Biodegradations observed under both mesophilic and thermophilic AD condition for the different biodegradable plastics and cellulose (positive control) are represented in **Figure III.1**. Biodegradations were calculated by comparing observed methane production with theoretical methane production (**Table III.1 and Eq.3**). Then, methane production curves were modeled using the Gompertz–Monod model, which allows to describe the data by three parameters: the duration of the lag phase (λ , days), the methane production rate (R_{max} , L CH₄ kg⁻¹ VS d⁻¹) and the ultimate methane production ($G(0)$, L CH₄ kg⁻¹ VS). The parameters of the model are listed in the **table III.2**. The Gompertz-Monod model was not suitable for all the conditions. In some cases, the biodegradation was relatively close to zero and so the model predicted aberrant parameters values. For other conditions, good correlation coefficients ($R^2 \geq 0.991$) were measured indicating that the Monod-Gompertz model accurately described the cumulative methane production.

The quality of the inocula used was validated using BMP test on cellulose (positive control) in both mesophilic and thermophilic conditions. Under both conditions, the positive control met the requirements (ultimate biodegradation $\geq 85\%$) fixed by the interlaboratory study on BMP test (Holliger et al., 2021, 2016), showing the inocula was efficient and the test valid. Indeed, an ultimate biodegradation of $90.2 \pm 3.4 \%$ in 25 days was reached under mesophilic condition and $93.7 \pm 3.2 \%$ in 20 days under thermophilic condition. In parallel, the final measurement of the pH in the different BMP test vessels (pH between 7.5 and 8.07) revealed the absence of acidification and thus failure of the AD system. Therefore, the low biodegradation observed for some conditions could not be attributed to an acidification of the reactors.

Table III.1: Properties of the inocula, the cellulose and the various biodegradable plastics used in this study. Values are expressed as average (\pm standard deviation)

Parameters	pH	Redox	N-NH ₄ ⁺	FOS/TAC	VFA	TS	VS	C	H	N	S	O*	Theoretical methane production
Units	-	mV	g L ⁻¹	-	g L ⁻¹	% raw mass	% raw mass	% TS	% TS	% TS	% TS	% TS	L CH ₄ .kg VS ⁻¹
Mesophilic inoculum	7.73	-365	1.5 (\pm 0.2)	0.31 (\pm 0.02)	0.1 (\pm 0.0)	4.0 (\pm 0.1)	2.5 (\pm 0)	-	-	-	-	-	-
Thermophilic inoculum	7.94	-403	1.7 (\pm 0.1)	0.25 (\pm 0.04)	0.1 (\pm 0.0)	2.7 (\pm 1.4)	1.9 (\pm 0.4)	-	-	-	-	-	-
Cellulose	-	-	-	-	-	90.0 (\pm 0)	100.0 (\pm 0)	40.7 (\pm 0.1)	6.5 (\pm 0)	0 (\pm 0)	0.2 (\pm 0)	52.6	378
TPS	-	-	-	-	-	92.1 (\pm 0)	92 (\pm 0)	38.8 (\pm 0.3)	7.4 (\pm 0.3)	0 (\pm 0)	0.1 (\pm 0)	53.8	379
PLA Natureplast	-	-	-	-	-	99.6 (\pm 0)	97.7 (\pm 0)	51.8 (\pm 0.2)	6.1 (\pm 0)	0 (\pm 0)	0 (\pm 0)	40.2	522
PLA Corbion	-	-	-	-	-	99.7 (\pm 0)	99.7 (\pm 0)	49.2 (\pm 0.2)	5.3 (\pm 0.1)	0 (\pm 0)	0 (\pm 0)	45.5	449
PBAT Jinhui	-	-	-	-	-	98.9 (\pm 1.1)	98.9 (\pm 0)	62.5 (\pm 0.3)	6.3 (\pm 0.1)	0 (\pm 0)	0 (\pm 0)	31.2	650
PCL Capa	-	-	-	-	-	99.9 (\pm 0)	99.9 (\pm 0)	63.1 (\pm 0)	10 (\pm 0.2)	0 (\pm 0)	0 (\pm 0)	27	773
PBS Natureplast	-	-	-	-	-	99.9 (\pm 0)	99.9 (\pm 0)	55.7 (\pm 0.2)	6.6 (\pm 0.1)	0 (\pm 0)	0 (\pm 0)	4.7	574
PHB K.D. Feddersen	-	-	-	-	-	98.8 (\pm 0.1)	98.7 (\pm 0)	53.2 (\pm 0.2)	7 (\pm 0)	0.2 (\pm 0)	0.1 (\pm 0)	39.5	552
PHB Biomer	-	-	-	-	-	99.3 (\pm 0.1)	97.3 (\pm 0)	55.6 (\pm 0.3)	7.2 (\pm 0.3)	0.1 (\pm 0.1)	0 (\pm 0)	35	609

* Calculated

3.1.1) Mesophilic condition

Anaerobic digestion experiments under the mesophilic condition were marked by particularly unfavorable kinetics for most of the polymers (**Fig 1A**). The duration of the test was therefore extended until 500 days. Biodegradation of TPS and PHB represented an exception; they were quickly converted in methane. Thermoplastic starch was biodegraded to $82.6 \pm 7.8\%$ in 25-30 days. A relatively large standard deviation was however measured for TPS, probably due to plastic heterogeneity linked to its laboratory production. Narancic et al. (2018) also found a near complete mineralization of TPS in 56 days at 35°C .

The two biodegradable plastics made up of PHB were digested during this experiment, however, although both plastics were made up with the same polymer, their ultimate biodegradation and the time required to achieve it were very different. While PHB from Biomer[®] was biodegraded to $64.3 \pm 0.6\%$ in around 50 days, PHB from K.D. Feddersen was biodegraded to $80.1 \pm 1.8\%$ in 25 days. It is worth noticing that both biodegradable plastics have different theoretical methane potential (**Table III.1**). These observations highlight that the nature of the polymer is not the only factor affecting the biodegradation, others factors associated with the properties of the plastic can have a significant impact on biodegradation such as crystallinity, molecular weight, accessible surface area etc. (Ahmed et al., 2018; Artham and Doble, 2008; Tokiwa et al., 2009). Similar observation was made by Benn and Zitomer, (2018) that reported biodegradation around $67 \pm 19\%$ under mesophilic AD condition for four PHB from different producers (*i.e.*, TianAn Biologic, Metabolix, Mango Materials).

Other biodegradable plastics exhibited low or zero biodegradation rate under mesophilic condition. PBS was not converted at all in methane even with the very long digestion period applied here. Similarly, PBAT was only converted to $13.4\% \pm 0.4\%$ in 500 days. These results are in accordance with the data from literature (Cho et al., 2011b; Massardier-Nageotte et al., 2006; Narancic et al., 2018; Shin et al., 1997; Svoboda et al., 2018; Yagi et al., 2014b). On the other side, PCL and PLA had slow biodegradation rate but their ultimate biodegradation was important after 500 days. PCL was biodegraded to 49.4 ± 0.9 in 500 days while PLA from Natureplast[®] and Total Corbion[®] reached $80.3 \pm 6.1\%$ and $74.7 \pm 2.0\%$ of biodegradation, respectively. Similarly, Bernat et al. (2021) and Yagi et al. (2014) found a low biodegradation rate for PCL and PLA. The anaerobic

digestion of these polymers did not result in a steady state of methane production after 277 to 280 days. Overall, both PLA from Total Corbion® and Natureplast® had close biodegradation kinetics. However, while the digestion of the PLA from Total Corbion® started with a relatively long lag phase (around 30 days, **table III.2**), the digestion of the one from Natureplast® started with a shorter lag phase (around 10 days) followed by an intense methane production phase. After few days, the methane production rate from Natureplast® PLA decreased. That surprising methane production in the early days of the digestion could correspond to the degradation of another compound present in the polymer (*i.e.*, probably an additive). Indeed, after a certain storage time, the surface of the Natureplast® PLA granules became coated with a fatty compound, presumably due to the loss (by exudation) of an additive.

3.1.2) Thermophilic condition

The duration of the thermophilic test was reduced in comparison with mesophilic as PLA, PHB, TPS and cellulose samples had reached their ultimate methane production within 100 days (**Figure III.1**).

TPS digestion resulted in similar biodegradation under mesophilic ($82.6 \pm 7.8\%$) and thermophilic ($80.2 \pm 4.5\%$) conditions. However, the biodegradation kinetic (R_{max} , **table III.2**) was higher in thermophilic condition. Similarly, the ultimate biodegradation of PLA did not change depending on the process temperature or the PLA producer. Natureplast® PLA was biodegraded to $74.6 \pm 2.1\%$ and Total Corbion® PLA to $74.6 \pm 2.4\%$ in thermophilic reactors. Nonetheless, the difference in methane production rate of PLA samples between mesophilic and thermophilic process was very important (**Table III.2**). Indeed, the steady state of biodegradation was reached between 60 and 100 days in thermophilic reactors while 500 days were required for mesophilic reactors. Interestingly, the phase of high methane production in the early days of Natureplast® PLA digestion observed in mesophilic condition could not be evidenced in thermophilic conditions. The degradation of the second hypothetical compound should occur in parallel with the degradation of PLA and thus be masked by the higher methane production rate from PLA under thermophilic conditions.

On the contrary, worse performances (in terms of kinetics and ultimate biodegradation) could be observed for PHBs, PCL and PBAT in thermophilic condition than in mesophilic condition (**Figure**

III.1). K.D. Feddersen and Biomer® PHBs were biodegraded to $72.3 \pm 5.0\%$ and $57.6 \pm 3.7\%$ respectively in approximately 40-50 days. The slower biodegradation of PHB was mainly due to an increased duration of the lag phase (**Table III.2**). In the case of K.D. Feddersen's PHB, this lag phase (25 days) was special. The methane production actually started (from the 3rd day) and stopped some days later (8th day) for approximately 15 days (**Figure III.1**). This type of lag phase is characteristic of a temporary inhibition of the methane production due to an accumulation of volatile fatty acids (Koch et al., 2019). It is likely that with a lower organic load, better biodegradation kinetics would have been observed, at least for K.D. Feddersen's PHB.

PCL was not converted to biogas under thermophilic conditions and these results do not match data described in literature. Narancic et al. (2018) and Yagi et al. (2013, 2009) found a near complete mineralization for PCL (87-92%) in a period between 45 and 127 days. Similarly, Šmejkalová et al. (2016) reported a conversion in biogas between 54 and 60% for several PCLs with different molecular mass in period between 70 and 140 days. BMP test were replicated using a new inoculum and adding an additional PCL (Mn= 45,000; Sigma Aldrich®, Saint Louis, USA), in that test plastic samples were introduced in the form of granule. Both PCL from Sigma Aldrich® and Perstorp® resulted in no significant biodegradation after 100 days (data not shown). After 100 days, the granules were still in solid form and appeared unchanged except for a yellowing coloration. The granules were however more brittle, they could be broken by hand. The temperature used in our test (*i.e.*, 58 °C) is higher than that of the tests described in the literature (*i.e.*, 55 °C) (Narancic et al., 2018; Šmejkalová et al., 2016; Yagi et al., 2013a, 2009) and is close or equal to the melting temperature of PCL (*i.e.*, 58-60°C). The lack of biodegradation at 58°C could be related to the proximity of the process temperature and the melting temperature of PCL. However such a phenomenon is not in line with thermal degradation (occurring at \geq melting temperature) resulting in chain scission and depolymerisation reaction (Niaounakis, 2015).

PBAT and PBS were weakly or not biodegraded under thermophilic condition, $1.7 \pm 1.8\%$ and $0.0 \pm 1.8\%$ respectively in 100 days. Low or no biodegradation was reported elsewhere for PBAT (Svoboda et al., 2018) and PBS (Dvorackova et al., 2015; Narancic et al., 2018; Yagi et al., 2013a).

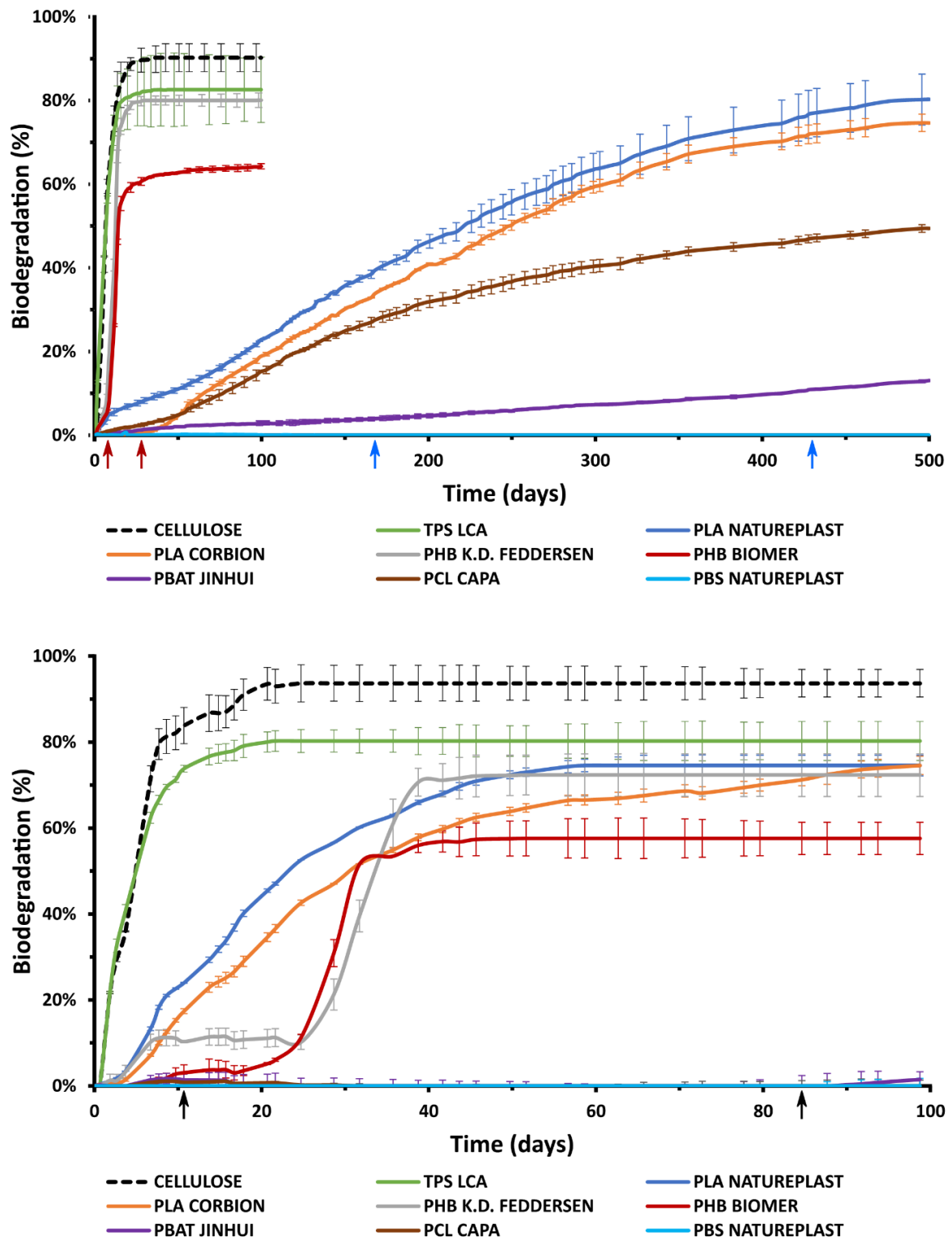


Figure III.1. The mean biodegradation (based on theoretical methane production) from the different biodegradable plastics under mesophilic condition (A) and thermophilic condition (B). The error bars represent the standard deviation. Arrows indicate the sampling period for microbial analysis and the color indicate in which reactor the digestate was sampled (black: all reactors, red: PHB and TPS fed reactors; Blue: PLA, PBS, PBAT and PCL fed reactors).

Table III.2. Parameters of the Monod-Gompertz modeling for the various experiments and conditions tested

Substrates	Temperature (°C)	λ Lag phase (days)	Rmax Methane production rate (L CH ₄ kg ⁻¹ VS d ⁻¹)	G(0) Methane potential (L CH ₄ kg VS ⁻¹)	R ²
Cellulose	38	1.67 ± 0.19	38.22 ± 3.03	345.1 ± 10.9	0.997
TPS LCA		0.89 ± 0.02	33.99 ± 0.60	309.5 ± 31.1	0.997
PLA Natureplast		10.9 ± 1.28	1.29 ± 0.05	438 ± 43.70	0.999
PLA Corbion		37.6 ± 0.39	1.16 ± 0.01	344.4 ± 13.58	0.995
PBAT Jinhui		40.58 ± 41.4	0.18 ± 0.02	159.7 ± 47.26	0.987
PCL Capa		25.40 ± 8.89	1.49 ± 0.15	366.9 ± 4.75	0.996
PBS Natureplast		N/A	N/A	N/A	N/A
PHB K.D. Feddersen		5.99 ± 1.39	46.18 ± 19.03	491.5 ± 82.72	0.991
PHB Biomer		7.58 ± 0.28	46.26 ± 3.48	383.4 ± 1.96	0.993
Cellulose		58	0.63 ± 0.08	50.64 ± 2.84	354.48 ± 15.34
TPS	0.02 ± 0.17		38.68 ± 1.78	311.52 ± 13.13	0.990
PLA Natureplast	2.08 ± 0.06		13.85 ± 0.37	419.09 ± 11.57	0.996
PLA Corbion	2.89 ± 0.46		8.83 ± 0.09	352.66 ± 8.81	0.995
PBAT Jinhui	N/A		N/A	N/A	N/A
PCL Capa	N/A		N/A	N/A	N/A
PBS Natureplast	N/A		N/A	N/A	N/A
PHB K.D. Feddersen	25.04 ± 0.45		36.70 ± 1.47	418.18 ± 28.11	0.986
PHB Biomer	22.89 ± 0.45		38.68 ± 6.72	360.44 ± 25.04	0.995

N/A non applicable

3.2) Microbial analysis

A total of 1027 OTUs were observed during the digestion of the different plastics with an average of 286 ± 77 OTUs per sample in mesophilic reactors and 124 ± 20 in thermophilic reactors. Most of them (95 %) could be affiliated at the genus level. Samples from mesophilic reactors had higher level of alpha diversity than samples from thermophilic reactors (data not shown). However, at both temperatures the microbial communities were highly uneven, suggesting the presence of few dominant OTUs and a large number of rare OTUs. *Bacteroidota* (29.8%), *Chloroflexi* (14.9%), *Desulfobacterota* (9.4%), *Firmicutes* (8.9%) and *Euryarchaeota* (7%) dominated microbial communities from mesophilic reactors at the phylum level, representing together more than 70% of the sequences. Samples from thermophilic reactors were dominated by sequences from the *Firmicutes* (47.6%), *Proteobacteria* (13.6%) and *Coprothermobacter* (4.4%), representing together 65.6% of the sequences found in thermophilic condition whereas *Euryarchaeota* observed in mesophilic condition only represented 1% of the sequences in thermophilic reactors. The composition and the variation of the active microbial communities from the different samples was explored using redundancy analysis (RDA) (**Figure III.2**). To have a better appreciation of the active microbial populations' changes induced by the digestion of the different plastics, individual RDAs were performed on mesophilic samples on one side and on thermophilic samples on another side (**Figure III.2A and 2B**).

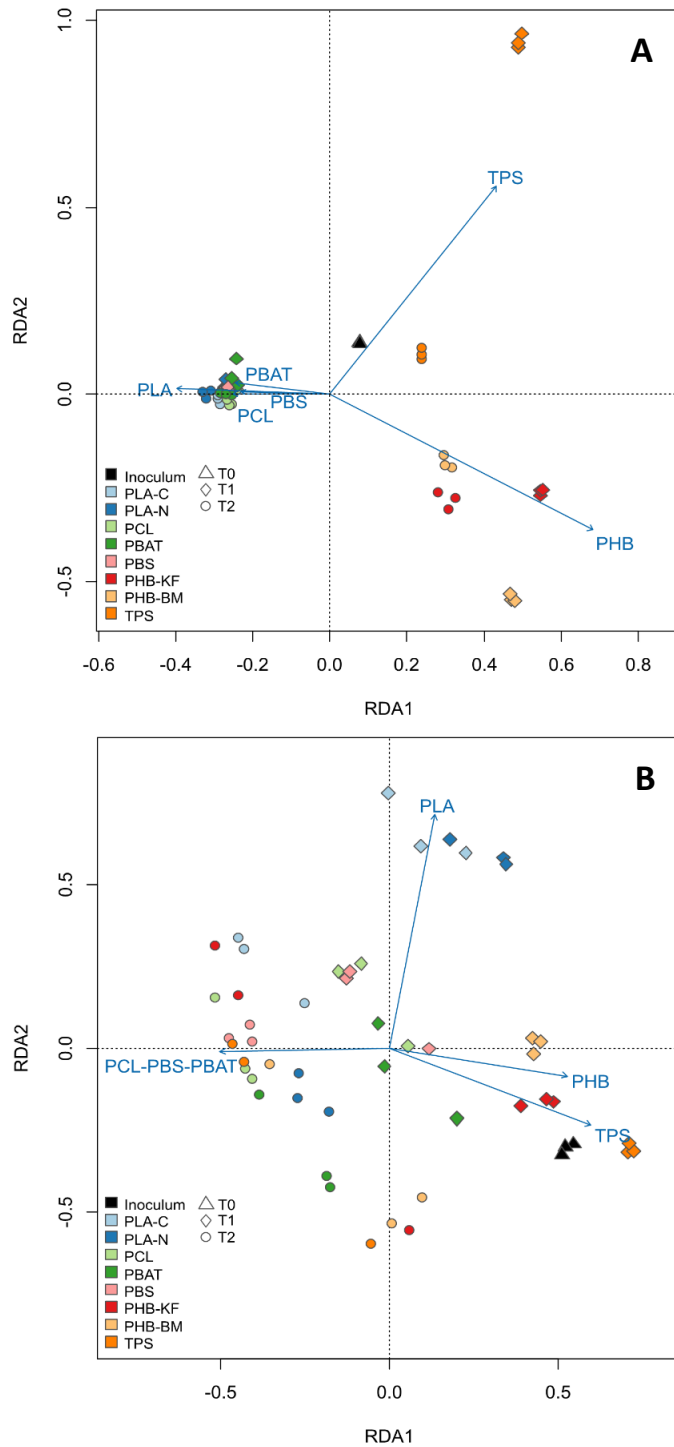


Figure III.2. Redundancy analysis (RDA) representing the environmental variables (arrows, i.e., the methane production from the different biodegradable plastics) and microbial communities from the different samples (colored circles, diamonds and triangle) under mesophilic condition (A) and thermophilic condition (B). PLA-C and PLA-N are referring to PLA Corbion and Natureplast, respectively. PHB-KF and PHB-BM are referring to PHB from KD Feddersen and PHB from Biomer respectively.

3.2.1) Changes in mesophilic microbial communities

The RDA (**Figure III.2A**) revealed that active populations from reactors fed with PLA, PCL, PBS and PBAT were similar. Indeed, in these reactors the methane production rate was low or zero all over the test as indicated in **Figure III.1A** and by the R_{max} in **Table III.2**. It could be assumed that the low polymers biodegradation rate in these reactors avoided observing significant differences in active microbial communities during the digestion of the different biodegradable plastics. By contrast, active microbial populations from reactors fed with PHB (from K.D. Feddersen and Biomer®) and TPS were clearly different from the rest. For the reactors fed with these three plastics, the active microbial communities were also different between the sampling times. In other words, the active microorganisms were different when the samples were performed during the high methane production phase (noted T1 in **Figure III.2**) and when samples were taken in a steady state of methane production (note T2). Therefore, the microorganisms involved in the AD of the biodegradable plastics are supposed to be active when the methane production was high.

Multivariate analysis was performed to display correlations between the methane production from the biodegradable plastics and the change in OTUs relative abundance. Since the digestion of PLA, PBS, PBAT and PBS resulted in low methane production and very similar active microbial populations, the multivariate analysis only highlighted OTUs representing a very low proportion of the reactors active populations. Therefore, the correlations with these variables were not displayed on **Figure III.3** and not further discussed. The digestion of the two PHB samples resulted in close active microbial populations; the correlation with the methane production from these two biodegradable plastics was therefore investigated together.

The methane production from TPS under mesophilic condition was correlated with an increasing abundance of OTUs affiliated to *Clostridium* genus, *Treponema* genus and to the *Paludibacteraceae* family (**Figure 3 III.**). *Clostridium*, *Treponema* and *Paludibacteraceae* represented, respectively, 7.1, 6.1 and 5.7 % of the OTUs of the reactors fed with TPS at the first sampling time. Moreover, their apparent abundance decreased to less than 0.1% at the second sampling time, when the methane production from TPS was close to zero. Interestingly, OTU associated to *Clostridium* were previously highlighted during the mesophilic anaerobic digestion of a mix of biodegradable plastic (PLA/PBAT blend) and biowaste (Peng et al., 2022b). It was one

of the main OTUs responsible for the difference between communities from plastic-fed and non-plastic-fed reactors. Some members of the family *Paludibacteraceae* were found to be fermentative bacteria able to produce acetate or propionate (Tonanzi et al., 2020; Ueki, 2006). *Treponema* species, such as *Treponema amylovorum*, were reported to be hydrolytic bacteria able to degrade starch (Wyss et al., 1997) which is one of the main components of TPS.

Similarly, OTU from the *Enterobacter* genus was correlated to the methane production from the two PHBs (**Figure III.3**). This genus represented a large part of the relative abundance of the reactors fed with PHB at the first sampling time (between 13.8 and 25%) while it represented only a small part of the populations of other reactors (between 0 and 1.8% of the sequences). Some *Enterobacter* bacteria were previously isolated from a tropical marine environment and were identified as PHB degraders (Volova et al., 2010b). The *Enterobacter sp.* could be key bacteria in the anaerobic biodegradation of PHB. Additionally, the methane production from PHB was also correlated with other OTUs, but unlike *Enterobacter*, these OTU represented a relatively small proportion of the abundance (< 3 %, except for *Sphingobacteriales ST-12K33*) and their relative abundance had a relatively low variation when the methane production from PHB decreased (between the first and second sampling time).

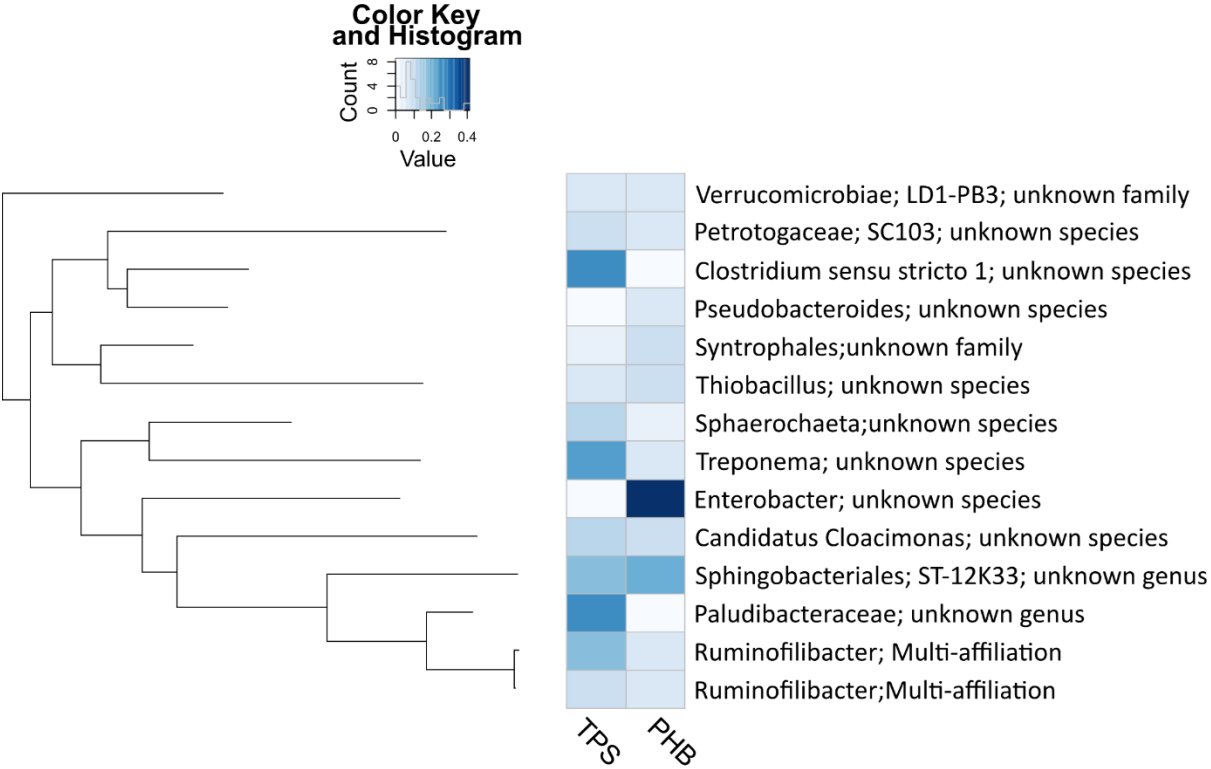


Figure III.3. Heatmap of the multivariate regression analysis from mesophilic samples. Only the top 10 OTUs having the highest positive correlation with the methane production from PHB and TPS were displayed. The intensity of the colors correlates with the magnitude of the Hellinger transformed abundance change.

3.2.2) Change in thermophilic microbial communities

Under thermophilic condition, as seen on the RDA (**Figure III.2B**) microbial populations sampled during a low methane production phase were scattered on the center and on the left side of the RDA. Indeed, reactors for which biodegradation was zero throughout the test (reactors fed with PCL, PBAT, PBS) had microbial populations located in the center of the RDA at the first sampling time and in the left side at the second sampling time. On the other hand, reactors fed with PLA, PHB and TPS had microbial populations located in the top and right side when the methane production was very active (first sampling time) and in the left side when methane production was over (second sampling time). Interestingly, the replicates were not clustered together in the left side of the figure, suggesting that when the methane production was close to zero the microbial communities were highly heterogeneous. Additionally, at the first sampling time for PHB, PLA and TPS, the microbial communities were clustered separately based on the polymer digested. Moreover, active microbial populations during the AD of PLA from Natureplast® and Total Corbion® were close at the first sampling time. A similar observation was made for PHB from K.D. Feddersen and Biomer®.

Multivariate analysis was performed to display correlations between the methane production from the different biodegradable plastics and the change in OTUs relative abundance. As discussed before, the conditions resulting in very low methane production (PCL, PBS, PBAT) were excluded from the multivariate analysis. Moreover, since active microbial populations from the digestion of the two PLAs were very close, they were treated together in this analysis. It was also the case for PHBs.

Methane production from TPS under thermophilic condition was mainly correlated to OTUs affiliated to *Clostridium* genus and to *Limnochordia* family. The OTU associated to *Clostridium* was closely related to *Clostridium thermarum* that was isolated from a hot springs and exhibited the ability to degrade starch (Liu et al., 2020). Moreover, that OTU was not found active in all reactors (abundance $\leq 0.1\%$) except in the TPS-fed reactors at first sampling (when methane production was high) suggesting an implication in TPS degradation. By contrast, *Limnochordia*-affiliated OTUs accounted for a high proportion of the active population of not only TPS-fed reactors but also of reactors fed with different others biodegradable plastics.

Methane production from PLA under thermophilic conditions showed high positive correlation with *Moorella* and *Tepidimicrobium*. *Moorella* and *Tepidimicrobium* represented around 9 to 13% of the sequences in PLA-fed reactors when methane production was active and only 0.4 to 1.9% when the methane production was near to zero, suggesting an implication in the conversion of PLA in methane. Both *Tepidimicrobium sp.* and *Moorella sp.* have been isolated from thermophilic anaerobic digester treating PLA and demonstrated the capacity of utilizing lactate as carbon sources (Cazaudehore et al., 2021a; Giri et al., 2019; Harada et al., 2018; Tseng et al., 2020a, 2019). Tseng et al. (2020) isolated a strain of *Tepidimicrobium xylanilyticum* from a thermophilic anaerobic reactor fed with PLA and found that *T. xylanilyticum* was preventing the inhibition of the PLA physicochemical depolymerisation by scavenging the lactate produced.

Among positively correlated OTUs with the methane production from PHB, only *Cupriavidus* was previously highlighted as a PHB degrader. Indeed, several members of the *Cupriavidus* genus had extracellular and intracellular PHB depolymerase (Emadian et al., 2017a; Knoll et al., 2009). However, *Cupriavidus* represented only a relatively low proportion of the active populations of the PHB-fed reactors during the high methane production phase (*i.e.*, between 1.0 and 1.3% of the sequences). On the contrary, other OTUs positively correlated to the methane production from PHB had a high abundance in PHB-fed reactors, such as *Coprothermobacter*, *Tepidimicrobium* and *Candidatus Caldatribacterium*. On one hand, *Coprothermobacter* and *Candidatus Caldatribacterium* were found to be active in almost all the reactors at the first sampling time. Moreover, *Coprothermobacter* also represented a large part of the active bacteria from the inoculum (19.2% of the sequences). It is highly probable that *Coprothermobacter* and *Candidatus Caldatribacterium* were not specific to the PHB degradation in thermophilic AD system. *Coprothermobacter spp.* has been demonstrated to be fermentative bacteria able to provide hydrogen to hydrogenotrophic archaea (Tandishabo et al., 2012). *Candidatus Caldatribacterium* has not been yet isolated and cultivated in laboratory, its implication in fermentation and syntrophic acetate oxidation was however supposed (Dyksma et al., 2020). On other hand, *Tepidimicrobium sp.* had only a relative high abundancy in reactors fed with PHB from Biomer® (9.3% of the sequences) but not in reactors fed with K.D. Feddersen's PHB (0.5% of the sequences). As discussed before, *Tepidimicrobium* was previously identified as a lactate utilizing bacteria in thermophilic anaerobic digesters treating PLA or PLA products (Cazaudehore et al., 2021a; Tseng

et al., 2019). It was therefore surprising to found that OTU among the OTUs correlated with the methane production from PHB.

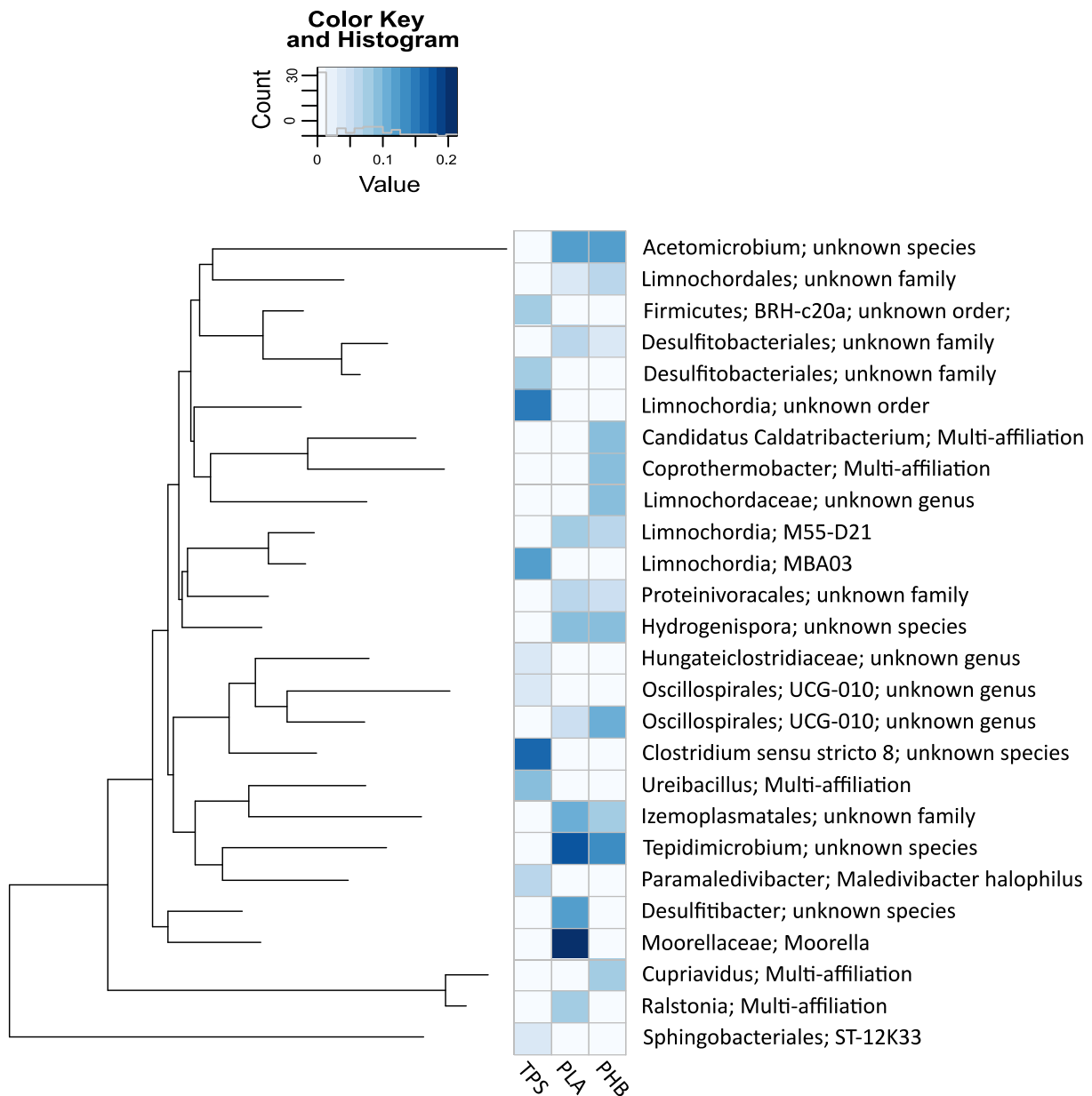


Figure III.4. Heatmap of the multivariate regression analysis from thermophilic samples. Only the top 10 OTUs having the highest positive correlation with the methane production from TPS, PLA and PHB were displayed. The intensity of the colors correlates with the magnitude of the Hellinger transformed abundance change.

3.2.3) Archaea diversity in mesophilic and thermophilic conditions

In mesophilic reactors, archaea represented 8.1% of the total sequences. The combined relative abundance of the archaeal OTUs ranged from 2.4 to 13.7 % of the different reactors sequences. *Methanosaeta* was predominant in mesophilic reactors accounting for 65.7 % of the total archaeal abundance, followed by *Methanospirillum* and *Methanoculleus* representing 15.9 and 11.8 % of the total archaeal abundance, respectively. *Methanosaeta* performs methanogenesis by the acetoclastic pathway, while *Methanoculleus* and *Methanospirillum* are hydrogenotrophic methanogens (Amin et al., 2021; Sundberg et al., 2013). The predominance of acetoclastic methanogens (65.7% of the archaeal sequences) suggests the methane was mainly produced by the acetoclastic pathway and that hydrogenotrophic methanogenesis played a secondary role (33.5% of the archaeal sequences). The dominance of *Methanosaeta* in mesophilic digesters is supported by other studies exploring the microbial diversity on full-scale digesters (Kirkegaard et al., 2017; Sundberg et al., 2013).

In thermophilic reactors, methanogenic archaea represented a lower proportion of the microbial community than under mesophilic conditions. Indeed, archaeal sequences represented only 1% of the total sequences, the combined relative abundance of the archaeal OTUs ranged from 0.3 to 2.1 % of the different reactors sequences. A low proportion of archaea was previously reported for functioning anaerobic digesters in co-digestion of wastes from slaughterhouses, restaurants, households (Sundberg et al., 2013). Indeed, Sundberg et al., (2013) observed an average relative proportion of archaea of 1.8% (range 0-4.4%), when studying the microbial communities from 14 co-digestion anaerobic digesters including both thermophilic and mesophilic processes. Sequences affiliated to *Methanothermobacter* genus were predominant in thermophilic reactors accounting for 91.5 % of the total archaeal abundance. *Methanosarcina* was the second most abundant archaeal genus representing 7.9 % of the total archaeal abundance. Based on these observations, hydrogenotrophic pathway was the predominant methane production pathway in thermophilic reactors (De Vrieze et al., 2018; Wasserfallen and de Macario, 2000). Hydrogenotrophic archaea dominance is common in many thermophilic anaerobic reactors and was confirmed by the observed high abundance of syntrophic bacteria (e.g., *Tepidimicrobium*, *acetomicrobium*) (Goberna et al., 2009; Krakat et al., 2010; Sasaki et al., 2011; Sundberg et al., 2013).

Conclusion:

Only PHB and TPS showed a high biodegradation in a relative short period of time (25-50 days) under both mesophilic and thermophilic AD. On the contrary, methane production rates from PLA and PCL under mesophilic conditions were very low and 500 days were required to reach the ultimate methane production from these polymers. Thermophilic AD of PLA resulted in enhanced biodegradation kinetics whereas PCL was not degraded in thermophilic condition. PBAT and PBS showed minor or zero biodegradation at both temperatures even with 500 days period used in mesophilic condition. These results have to be confirmed in continuous co-digestion experiment in order to properly appreciate the biodegradation kinetics and the long-term effect of plastic addition in anaerobic digesters. Previously known PHB degraders (*i.e.*, *Enterobacter* and *Cupriavidus*) were observed during mesophilic and thermophilic AD of PHB. Similarly, starch-degrading bacteria (from *Clostridium* genus) were highlighted during TPS digestion at 38 °C and 58°C. Strong correlation was found between the relative abundancy of lactate utilizing bacteria (*Tepidimicrobium* and *Moorella*) and the methane production from PLA under thermophilic conditions, confirming the importance of these bacteria during thermophilic AD of PLA.

PART B: Biodegradation of commercial blends of biodegradable polymers

Abstract

Biodegradable plastics market is increasing these last decades, including for coffee capsules. Anaerobic digestion, as a potential end-of-life scenario for plastic waste, has to be investigated. For this purpose, mesophilic (38 °C) and thermophilic (58 °C) anaerobic digestion tests on three coffee capsules made up with biodegradable plastic (Beanarella®, Launay® or Tintoretto®) and spent coffee (control) were compared by their methane production and the microbial communities active during the process. Mesophilic biodegradation of the capsules was slow and did not reach completion after 100 days, methane production ranged between 67 and 127 L CH₄ kg⁻¹ VS. Thermophilic anaerobic digestion resulted in a better biodegradation and reached completion around 100 days, methane productions were between 257 and 294 L CH₄ kg⁻¹ VS. The microbial populations from the reactors fed with plastics versus spent coffee grounds were significantly different, under both the mesophilic and the thermophilic conditions. However, the different biodegradable plastics only had a small impact on the main microbial community composition at a similar operational temperature and sampling time. Interestingly, the genus *Tepidimicrobium* was identified as a potential key microorganisms involved in the thermophilic conversion of biodegradable plastic in methane.

1) Introduction:

Due to inadequate end-of-life management, conventional plastics have become one of the most abundant sources of environmental pollution. Geyer et al. (2017) estimated that almost 60% of all plastics ever produced, corresponding to 5 billion tons, have been discarded in the environment (natural or landfills). These plastics have accumulated in the world's oceans; where large quantities of debris represent a threat to marine species by entanglement, suffocation, or ingestion (Compa et al., 2019; Gregory, 2009). Microplastics are a threat to both marine life and human beings (Ajith et al., 2020; Sarker et al., 2020). The use of biodegradable plastics, will be promoted by the European directive 2018/581, which plans for the generalization of separation at source and treatment of organic wastes. A biodegradable plastic can be defined as a plastic that undergoes a significant change in its chemical structure under specific environmental conditions, resulting in the loss in some of its properties by the action of naturally occurring microorganisms in a given period of time. This is measured by standard test methods appropriate to the plastic and to the application (ISO 472, 2013). The biodegradation of a biodegradable plastic depends greatly on the environment in which it takes place. This is why biodegradable plastics should not be discarded directly in the environment but should be preferentially treated in a controlled recycling environment (composting or anaerobic digestion).

Anaerobic digestion (AD) is a biological process by which organic matter is converted, in an oxygen-free environment, into biogas (mainly composed of carbon dioxide and methane). The process of AD has proven to be a promising method for the valorisation of organic materials such as agricultural wastes (manure, crop residues, and winery waste products), food wastes, and sewage sludge (Da Ros et al., 2017; Hanum et al., 2019; Monlau et al., 2013b; Moretti et al., 2020). The literature recommends balancing the carbon to nitrogen (C/N) ratio of feedstocks to between 20 and 30 to prevent both nutrient limitation and ammonia toxicity (Esposito et al., 2012; Hawkes, 1980b). Some feedstocks that are usually processed in anaerobic digesters, such as food waste or sewage sludge, have a low C/N ratio (Esposito et al., 2012). Plastics, on the other hand, contain very low levels of nitrogen. Co-digestion of biodegradable plastics with other types of waste can help adjust the carbon-nitrogen ratio to the recommended values (Benn and Zitomer, 2018). Anaerobic digesters are typically operated at mesophilic (30-40 °C) or thermophilic (around 55 °C) temperatures in wet or dry processes (Brown et al., 2012). The process of AD can be divided into

four main steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis through acetotrophic, hydrogenotrophic or methylotrophic pathway (Demirel and Scherer, 2008; Evans et al., 2019).

The microbiome involved in the process of AD has undergone intensive study in recent years (Azizi et al., 2016; Castellano-Hinojosa et al., 2018; De Vrieze et al., 2018; Guo et al., 2015; Levén et al., 2007; Li et al., 2015; Liu et al., 2018; Sundberg et al., 2013). However, little is known about the microorganisms involved in the AD of biodegradable plastics. In the reviews by Shah et al. (2014, 2008) and Emadian et al. (2017), several microorganisms were reported to decompose biodegradable plastics, but most of them did not come from anaerobic media. Yagi and co-workers have published several studies aimed at the detection of microorganisms that participate in anaerobic digestion of biodegradable plastic. They performed denaturing gradient gel electrophoresis of the 16S rRNA amplicons (RT-PCR-DGGE). In their first two papers they were not successful at identifying the microorganisms responsible for the thermophilic digestion of poly(lactic acid) (PLA) (Yagi et al., 2011b, 2010). However, they highlighted that some of the microorganisms participating in the anaerobic biodegradation of cellulose and PLA at 55°C differ. Subsequently, Yagi and co-workers succeeded in identifying some microorganisms that participated in the degradation of PLA, poly(ϵ -caprolactone) (PCL), and poly(3-hydroxybutyrate) (PHB) under mesophilic and thermophilic conditions (Yagi et al., 2014b, 2013a). Venkiteshwaran et al. (2019) have examined the microbial community shift during anaerobic co-digestion of PHB and synthetic primary sludge (dog food and basal nutrients) by Illumina sequencing. No previously known PHB degraders were observed in the co-digesters. Finally, Tseng et al. (2019) examined the microbial populations involved in anaerobic digestion of PLA under thermophilic conditions by PCR-DGGE. They pointed out the importance of the genus *Tepidimicrobium*, which is thought to comprise key bacteria that decompose lactic acid and that supply CO₂ and H₂ to hydrogenotrophic methanogens.

The aim of this study was to evaluate the methane potential of three biodegradable plastics under mesophilic and thermophilic conditions using Biochemical Methane Potential (BMP) testing and to analyze the active microbial communities (16S rRNA) during the process. These plastics were commercially available in the form of coffee capsules and were certified as being biodegradable under industrial composting conditions according to the EN 13432 standard. The EN 13432

standard defines the requirements for packaging recoverable through composting and biodegradation, with test scheme and evaluation criteria for the final acceptance of packaging.

2) Materials and methods

2.1) Biodegradable plastic supports and inocula

Biodegradable plastics were purchased in the form of coffee capsules (Launay[®], Beanarella[®], and Tintoretto[®]) certified as being biodegradable under industrial composting conditions (EN 13432). They were made of Vegemat[®] from Vegeplast (France), Ecovio[®] from BASF (Germany), or Mater-Bi[®] from Novamont (Italy). Mater-Bi[®] is a class of compounds based on thermoplastic starch (TPS) and other polymers such as cellulose acetate, poly(vinyl alcohol), PCL, and PBAT (Aldas et al., 2020). Ecovio[®] (BASF) is a mixture of PLA and a fossil-based and biodegradable polymer commercialised as Ecoflex[®] (BASF) (Ecovio[®] BASF website, 2020). Vegemat[®] (Vegeplast) is a mixture of biobased and biodegradable polymers obtained through the processing of agropolymers and polyesters (Pluquet et al., 2016). The capsules were separated from the coffee, and the opercula and were ground using a cutting mill (SM 100 Retsch, Haan, Germany) and a centrifugal mill (ZM 100, Retsch, Haan, Germany) to a particle size of 1 mm. In order to produce the spent coffee and to simulate a passage through a coffee maker, the ground coffee (Grand'Mère[®], France) was mixed with water using a magnetic stirrer (200 rpm, 15 min). After a centrifugation step (5 000 x g, 5 min), the solid fraction was collected and dried at 60 °C until a constant mass was obtained.

The total solids (TS) and the volatile solids (VS) were determined using APHA standard methods (APHA, 2005). Elemental analysis (Carbon, Hydrogen, Nitrogen, and Sulphur) was performed on the different samples using an Elemental Vario Macro Cube analyser (Elementar, Germany). The oxygen content was estimated by the difference between the VS, carbon, hydrogen, nitrogen, and sulphur content. The theoretical methane production was estimated from the elemental composition using the following equation (Achinas and Euverink, 2016a; Boyle, 1977; Buswell and Mueller, 1952):

$$\text{Theoretical methane production (NL(CH}_4\text{)}.g^{-1}\text{(C}_x\text{H}_y\text{O}_z\text{N}_n\text{S}_s\text{))} = \frac{22,4 \times \left(\frac{x}{2} + \frac{y}{8} - \frac{z}{4} - \frac{3n}{8} - \frac{s}{4}\right)}{12x + y + 16z + 14n + 32s}$$

Two different inocula were used in this study. They were prepared from wastewater treatment plant (WWTP) sludge and acclimated for two months for anaerobic digestion at 38 °C (mesophilic inoculum) or 58 °C (thermophilic inoculum). The mesophilic inoculum was fed with grass and WWTP sludge; the thermophilic inoculum with grass, wheat waste, and WWTP sludge. The chemico-physical characteristics of the substrates and inocula used here are presented in **Table III.3**.

Table III.3. The main characteristics of the samples (inocula and substrates) used in this study.

Parameters	pH	TS	VS	Ash	C	H	N	S	O*	Theoretical methane production
Units	-	% raw mass	% raw mass	% raw mass	% TS	% TS	% TS	% TS	% TS	L CH ₄ kg VS ⁻¹
Beanarella®	-	99.8 (± 0)	70.6 (± 0.1)	29.2 (± 0.1)	37.7 (± 0)	4.4 (± 0.3)	0	0	28.6	531
Launay®	-	99.7 (± 0)	94.1 (± 0)	5.6 (± 0)	49.7 (± 0)	5.8 (± 0)	0	0.4 (± 0.2)	38.5	518
Tintoretto®	-	99.8 (± 0)	98.4 (± 0)	1.4 (± 0)	54 (± 0)	6.1 (± 0)	0	0.2 (± 0)	38.4	546
Spent coffee		95.3 (± 0.1)	90.9 (± 0.1)	4.4 (± 0.1)	48.9 (± 0.3)	6.7 (± 0.1)	2.8 (± 0.1)	0.3 (± 0)	36.5	524
Mesophilic inoculum	7.59	3.9 (± 0)	2.6 (± 0.2)	1.3 (± 0.2)	-	-	-	-	-	-
Thermophilic inoculum	8.02	3.7 (± 0.1)	2.5 (± 0.3)	1.3 (± 0.3)	-	-	-	-	-	-

2.2) Biochemical Methane Potential (BMP) tests

Ground biodegradable plastic samples, spent coffee, and cellulose (positive control purchased from Tembec, France) were digested in batch bottles under mesophilic (38 °C) and thermophilic (58 °C) conditions according to an experimental protocol adapted from the recommendations of the European Inter-Laboratory studies in which APESA is involved (Hafner et al., 2020). The BMP bottles were filled with 300 mL of inoculum, water, and test material mixture at an inoculum to substrate ratio (ISR) of 2.7 g VS g⁻¹ VS. The organic loading of the test corresponded to 9 g VS L⁻¹. Previous research work has suggested an ISR ≥ 2 in order to obtain reproducible constant kinetics (Chynoweth et al., 1993; Raposo et al., 2011, 2008). A blank control without test material was also

carried out. Before placing an airtight seal on the bottle, the gas phase was flushed out with nitrogen (Alphagaz™ Smartop, Air Liquide, France). The daily biogas production was estimated by the increase in pressure using a manometer (2023P, Digitron, Croatia). The biogas composition was determined using a gas chromatograph (Micro GC 490, Agilent, USA) equipped with two columns. The first column (M5SA 10m, Agilent, USA) was used at 80 °C and 200 kPa to separate O₂, N₂, and CH₄ using Argon as the carrier phase; the second column (PPU 10m BF) was used at 80 °C and 150 kPa to separate the CO₂ from the other gases using Helium as the carrier phase. The injector temperature was 110 °C. The detection of gaseous compounds was achieved using a thermal conductivity detector. The biogas production of the negative control, endogenous to the inoculum, was subtracted from the production of the other bottles. The BMP tests were performed in triplicate. The calibration was carried out with two standard gases composed of 9.5% CO₂, 0.5% O₂, 81% N₂ and 10% CH₄ and 35% CO₂, 5% O₂, 20% N₂, and 40% CH₄ (special gas, Air Liquide®, France). All of the results are presented for normalized conditions of temperature and pressure (P_{atm}, 0 °C). The degradation yield was estimated by comparing the observed methane production to the theoretical methane production (**Table III.3**).

2.3) Microbial analysis

2.3.1) Sampling

Digestate samples were collected, in triplicate, at the start of the BMP tests (T₀) in order to characterise the initial state of the microbial populations, as well as at two different times (T₁ and T₂) during the anaerobic digestion process. When feasible, the sampling dates were adjusted to occur during either a phase of high methane production in the case of the first sampling (T₁) or later during a phase of lower methane production in the case of the second sampling time (T₂). The specific days of the sampling are indicated in **Figure III.5**. The digestate samples were mixed with two volumes of RNAprotect Bacteria Reagent® (Qiagen, USA), incubated for 5 min at room temperature, and then centrifuged (5 000 x g, 5 min). The supernatants were discarded and the pellets were stored at -20 °C for less than two weeks before their transfer to -80 °C.

2.3.2) Nucleic acid extraction, reverse transcription of the RNA, and PCR amplification

Nucleic acid extractions were performed using a Fast RNA® Pro Soil Direct kit (Qiagen, USA), according to the manufacturer's instructions to collect a mixture of RNA and DNA. The DNA and

the RNA were then separated using an AllPrep DNA/RNA kit (Qiagen, USA). The absence of DNA in the RNA extracts was verified by PCR followed by 1% agarose gel electrophoresis. RNA reverse transcription was carried out using the instructions provided with the M-MLV reverse transcriptase (Invitrogen™, USA).

PCR of the V4-V5 region (nucleotides 515-928) of the 16S rRNA gene (from cDNA and DNA) was performed using AmpliTaq Gold™ 360 Master Mix (Applied Biosystems, USA) with the following reaction mix: AmpliTaq 1X, 515F primer 0.6 μM (GTGYCAGCMGCCGCGGTA, Wang and Qian, 2009), 928R primer 0.6 μM (ACTYAAAKGAATTGRCGGGG, Wang and Qian, 2009), and cDNA 10 μL or DNA 1 μL. The amplification was performed with an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 65 °C, and 40 s at 72 °C. The amplification reaction ended with a 7-min extension step at 72 °C. The amplification was confirmed by analysis of the reaction mix by 1% agarose gel electrophoresis.

2.3.2) Sequencing and bioinformatics analyses

Amplicons were sequenced by the Get-PlaGe sequencing service (INRA, Toulouse, France) using Illumina MiSeq 250 bp paired-end technology. Bioinformatics processing of the data was performed using the method described by Escudié et al., 2018 on the Galaxy FROGS pipeline (Afgan et al., 2018). After a pre-processing step (merging, denoising, and dereplications of the reads), the sequences were clustered into Operational Taxonomic Units with an aggregation distance of three bases. OTUs containing less than 0.0005% of the total sequences were deleted, as were chimeric OTUs. Taxonomic assignments were performed using the Silva database v.128 (Pruesse et al., 2007). The number of sequences per sample was normalized based on the minimum number of sequences per sample found (7 744 sequences). The sequence data have been deposited in GenBank under accession number PRJNA648017.

The statistical analyses were performed using R Studio version 3.2.3 software (<http://www.r-project.org>). Diversity indices (Richness, Chao, Exponential of Shannon, and Inverse of Simpson) were calculated using Vegan and Bat packages. The differences in the diversity index between the different samples and conditions were tested using the Kruskal-Wallis test. Non-metric distance scaling (NMDS) plots of the community data were generated using the Bray-Curtis distance

measures with the Phyloseq and ggplots2 packages. Heatmaps and Venn diagrams were produced using gplots package.

3) Results and discussion

3.1) Performances of the anaerobic digestion processes

The methane productions during the anaerobic digestion of the three coffee capsules (Launay®, Beanarella®, and Tintoretto®), spent coffee and cellulose, under the mesophilic (38 °C) and the thermophilic (58 °C) conditions, are represented in **Figure III.5**.

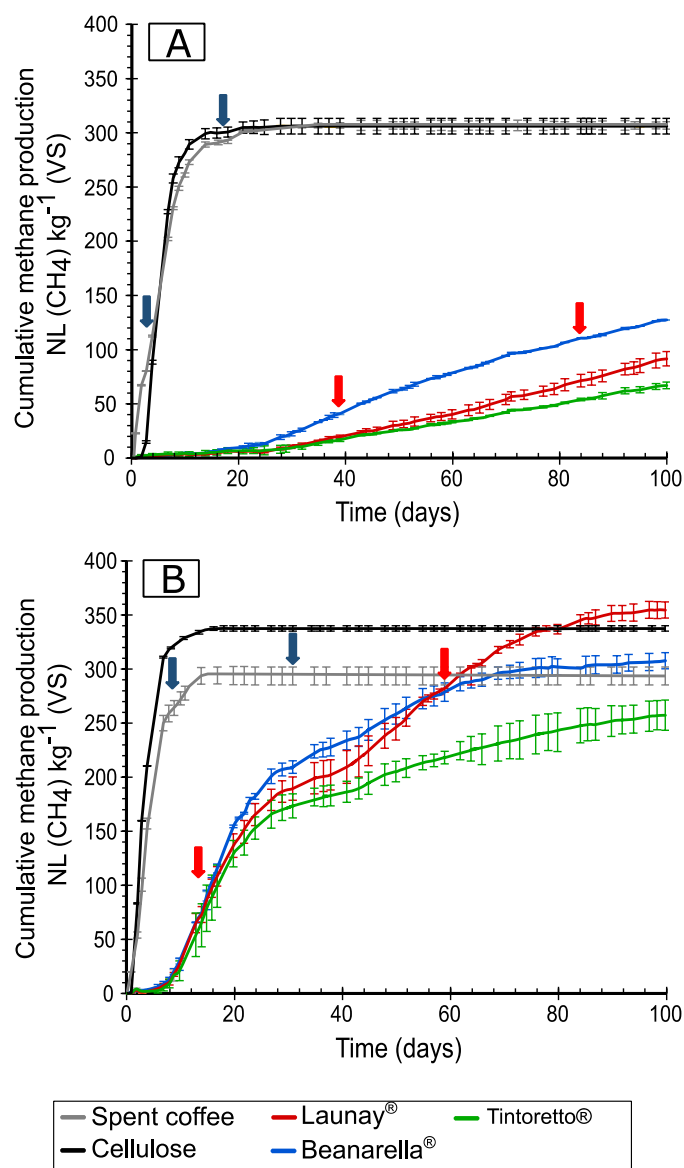


Figure III.5. The mean cumulative methane production (NL CH₄ kg⁻¹ VS) in the mesophilic digesters (38 °C, A) and the thermophilic digesters (58 °C, B). The error bars represent the standard deviations of the biological replicates. The arrows indicate the times at which the samplings for

the microbial community analyses were conducted. The colour of the arrows indicates whether the sampling was done on plastic-fed digesters (red arrows) or on digesters fed with spent coffee grounds (blue arrows).

The methane potential of cellulose (positive control) under mesophilic and thermophilic conditions was 315 ± 5 L CH₄ kg⁻¹ VS and 337 ± 3 L CH₄ kg⁻¹ VS, respectively. Similar values and kinetics have been reported elsewhere for cellulose controls (Hansen et al., 2004; Raposo et al., 2011), thus indicating that the inocula were well-suited to anaerobic digestion under both the thermophilic and the mesophilic conditions.

The spent coffee was readily biodegradable, under both the mesophilic and the thermophilic conditions, with almost no lag phase (< 1 day) and a high level of methane production over a period of 15 to 20 days. A similar degradation behaviour was noted under the mesophilic and the thermophilic conditions; the methane potentials were 294 ± 8 L CH₄ kg⁻¹ VS and 301 ± 3 L CH₄ kg⁻¹ VS, respectively, corresponding to a conversion into methane of between 56 and 57% of the spent coffee. The methane production and kinetics from spent coffee were comparable to those of cellulose. The methane potential of spent coffee has been previously described, ranging from 240 to 340 L CH₄ kg⁻¹ VS (Lane, 1983; Neves et al., 2006).

The digestion of the biodegradable plastics exhibited very different behaviors under the mesophilic versus the thermophilic conditions. Under the mesophilic conditions, the AD of the three plastics exhibited a long lag phase (almost 25 days) and slow kinetics (**Figure III.5A**), suggesting that the hydrolysis step is limiting and underperforming. Finally, the conversion of the plastics into biogas did not reach completion within the 100-day test period. The methane potential of the Beanarella[®], Launay[®], and Tintoretto[®] samples at the end of the test was 127 ± 1 L CH₄ kg⁻¹ VS, 92 ± 0 L CH₄ kg⁻¹ VS, and 67 ± 3 L CH₄ kg⁻¹ VS, respectively. The biodegradability corresponded to $24 \pm 0\%$, $18 \pm 0\%$, and $12 \pm 1\%$ for the Beanarella[®], Launay[®], and Tintoretto[®] samples, respectively. The thermophilic anaerobic digestion seems to be more suitable to the treatment of the three biodegradable plastics selected (**Figure III.5B**). The biodegradation kinetics were better, with a shorter lag phase (almost 10 days) and an greater rate of methane production. The methane potentials were reached during the 100-day test period for the three plastics samples. The methane potential of the Beanarella[®], Launay[®], and Tintoretto[®] samples was 308 ± 7 L CH₄ kg⁻¹ VS, 355 ± 7 L CH₄ kg⁻¹ VS, and 257 ± 14 L CH₄ kg⁻¹ VS, respectively. Digestion of the

Beanarella[®], Launay[®], and Tintoretto[®] samples exhibited $58 \pm 1\%$, $69 \pm 1\%$, and $47 \pm 1\%$ conversion of plastic to methane, respectively. Mater-Bi[®], the main constituent of the Tintoretto[®] capsule, has been digested previously in other studies (Calabro et al., 2019; Puechner et al., 1995; Vasmara and Marchetti, 2016). In a similar period (90 days), the degradation of Mater-Bi[®] resulted in 33 L (CH₄) kg⁻¹ (VS) at 35 °C, while it resulted in 267 L (CH₄) kg⁻¹ (VS) at 55 °C in the study by Vasmara and Marchetti (2016); similar methane productions were observed for Tintoretto[®] in our study. No data regarding anaerobic digestion of the two other materials (Vegemat[®] and Ecovio[®]) are available in the literature. Most polymers, such as PLA, PCL, poly(butylene adipate-co-terephthalate) (PBAT), and poly(butylene succinate) (PBS), are biodegraded slowly by AD under mesophilic conditions (Abou-Zeid et al., 2004; Day et al., 1994; Nunziato et al., 2018; Vargas et al., 2009; Vasmara and Marchetti, 2016; Yagi et al., 2014b). For example, in the study by Yagi et al., (2014), only between 29 and 49% of the PLA was degraded within 277 days in mesophilic digesters, and the degradation did not reach completion. However, some polymers are biodegraded rapidly even under mesophilic condition, such as PHB, poly(3-hydroxybutyrate) (PHBV), and TPS (Abou-Zeid et al., 2001; Benn and Zitomer, 2018; Greene, 2018a; Ryan et al., 2017b; Soda et al., 2016). PHB was reported to degrade between 50 and 80% within 40 days depending on the grade used (Benn and Zitomer, 2018). A difference in efficiency between mesophilic and thermophilic digestion of plastics has already been shown elsewhere (Nunziato et al., 2018; Vargas et al., 2009; Vasmara and Marchetti, 2016; Yagi et al., 2014b, 2013a).

Higher level of plastic conversion in methane were observed in thermophilic condition (between 47 and 69%) than in mesophilic condition (between 12 and 24%) in 100 days. There are two possible explanation for this. Firstly, the thermophilic condition (58°C) provides more favorable environmental conditions for the degradation of plastics than the mesophilic condition (38 °C). When the temperature rises and approaches the glass transition temperature of a polymer, this polymer becomes more accessible to microorganisms and makes it easier to degrade. (Shi and Palfery, 2010). Secondly, the microbial composition of thermophilic and mesophilic inoculum is different. The microorganisms present in thermophilic reactors could be more efficient to degrade biodegradable plastics than mesophilic ones.

During the mesophilic and the thermophilic anaerobic digestion of spent coffee and coffee capsules, the digestates were sampled at two different times for microbial analysis, as described

in **Figure III.5**. For the thermophilic condition, the sampling was carried out in two distinct phases. The first sampling took place during a high methane production phase; the methane production rate was between 10.8 and 12.5 L CH₄ kg⁻¹ VS d⁻¹ for the coffee capsules. At the second sampling time, the methane production rate was much lower (between 1.2 and 3.3 L CH₄ kg⁻¹ VS d⁻¹). Due to the low efficacy of mesophilic anaerobic digestion of plastics, both of the samplings took place during a low methane production phase. The methane production rate was between 0.8 and 2.1 L CH₄ kg⁻¹ VS d⁻¹ for the various plastics.

3.2) Sequence data analysis, community diversity indices, and beta diversity

The microbial community composition was evaluated by amplicon sequencing of the 16S rRNA transcripts and the 16S rRNA gene in order to compare the active and the total microbial community. Samples collected in triplicate at the different sampling times (T0, T1, and T2) and under both the mesophilic and the thermophilic conditions were analyzed by amplicon sequencing of the 16S rRNA transcripts. The digestates sampled at the initial and the final sampling time (T0 and T2) were analyzed by amplicon sequencing of the 16S rRNA gene only. A total of 650 496 sequences were collected after normalization (7 744 sequences per sample). Almost 96.3% of the total sequences were assigned to the bacterial domain, while the remaining 3.7% could be assigned to the archaeal domain. Similar Bacterial/Archaea ratios have been reported previously in the literature (Guo et al., 2015; Moseet et al., 2015; Sundberg et al., 2013; Yang et al., 2014). For instance, Sundberg et al., 2013 reported an average bacterial abundance of 95% of the total sequences during the examination of the microbial community composition of 21 full-scale anaerobic digesters.

A comparison of the alpha diversity indices from the analysis of the 16S rRNA gene and transcripts is shown in **Table III.4**. The first analysis focused on the total microbial population (DNA-based analysis) while the second one shows the active microbial community (RNA-based analysis). Lower diversity was observed in the RNA-based analysis compared to the DNA-based analysis, as expected. Similarly, lower diversity indices were observed for the thermophilic samples than for the mesophilic ones, as reported previously (Azizi et al., 2016; Levén et al., 2007; Li et al., 2015; Y. Sekiguchi et al., 1998; Sundberg et al., 2013). The low values of the composite indices (exponential of Shannon and inverse of Simpson) compared to the richness indices (number of OTU and Chao1)

indicate a highly uneven distribution of the populations in the different samples. This highlights a paucity of dominant OTUs (with a high number of sequences) and a high number of rare OTUs (with a low number of sequences).

	Mesophilic conditions				Thermophilic conditions			
	Richness	Chao1	Exp. Shannon	Inv. Simpson	Richness	Chao1	Exp. Shannon	Inv. Simpson
Amplicon sequencing of 16S rRNA gene (DNA based) analysis								
T0	372 (± 12)	487 (± 44)	51 (± 7)	17 (± 3)	271 (± 20)	388 (± 47)	18 (± 3)	8 (± 1)
T2 Coffee	395 (± 4)	482 (± 27)	83 (± 10)	31 (± 5)	264 (± 10)	350 (± 47)	27 (± 2)	10 (± 1)
T2 Beanarella®	385 (± 4)	459 (± 28)	81 (± 2)	31 (± 2)	284 (± 2)	340 (± 13)	33 (± 5)	9 (± 1)
T2 Launay®	390 (± 5)	450 (± 6)	75 (± 2)	25 (± 1)	270 (± 12)	333 (± 18)	29 (± 7)	8 (± 3)
T2 Tintoretto®	380 (± 11)	468 (± 8)	67 (± 11)	21 (± 6)	290 (± 14)	356 (± 12)	39 (± 7)	11 (± 3)
Amplicon sequencing of 16S rRNA gene transcripts (RNA based) analysis								
T0	289 (± 70)	373 (± 68)	27 (± 10)	7 (± 1)	192 (± 37)	365 (± 61)	8 (± 2)	4 (± 0)
T1 Coffee	208 (± 12)	295 (± 31)	20 (± 5)	8 (± 1)	134 (± 24)	192 (± 66)	12 (± 1)	5 (± 0)
T1 Beanarella®	215 (± 16)	273 (± 23)	40 (± 11)	17 (± 6)	125 (± 13)	168 (± 31)	19 (± 5)	10 (± 4)
T1 Launay®	224 (± 23)	336 (± 86)	32 (± 1)	13 (± 0)	113 (± 5)	176 (± 31)	14 (± 1)	7 (± 1)
T1 Tintoretto®	210 (± 5)	270 (± 23)	36 (± 5)	15 (± 3)	119 (± 3)	178 (± 23)	14 (± 2)	6 (± 1)
T2 Coffee	230 (± 8)	284 (± 28)	37 (± 4)	17 (± 2)	171 (± 28)	221 (± 24)	25 (± 5)	12 (± 2)
T2 Beanarella®	209 (± 27)	259 (± 37)	42 (± 1)	20 (± 2)	119 (± 14)	166 (± 34)	22 (± 2)	13 (± 1)
T2 Launay®	195 (± 5)	252 (± 22)	42 (± 2)	20 (± 2)	136 (± 9)	174 (± 13)	25 (± 0)	14 (± 0)
T2 Tintoretto®	196 (± 10)	267 (± 23)	42 (± 4)	20 (± 3)	262 (± 47)	326 (± 63)	40 (± 7)	14 (± 2)

Table III.4. Alpha diversity indices of the mesophilic and the thermophilic samples from amplicon sequencing of the 16S rRNA gene (DNA-based) and transcripts (RNA-based) analyses (Hill numbers).

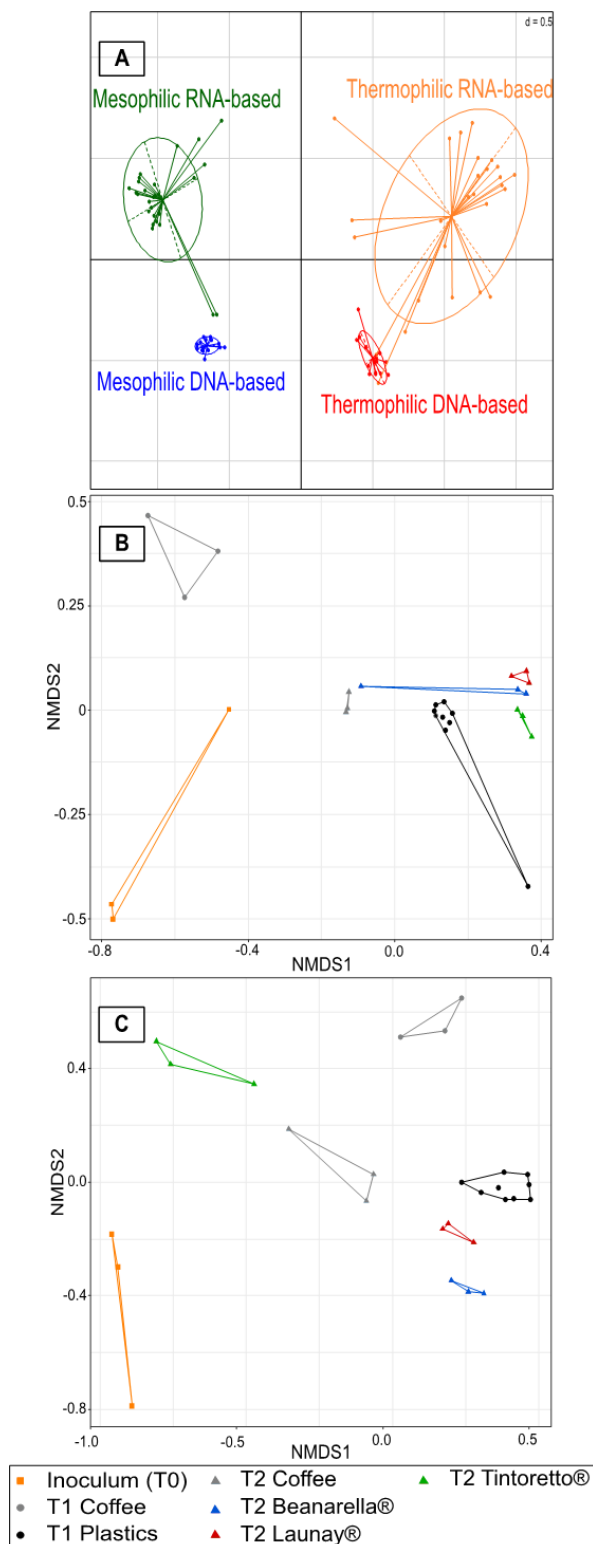


Figure III.6. Non-metric distance scaling (NMDS) analysis of the Bray-Curtis distance indices for the microbial communities at the OTU level of the entire data set (A), of RNA from the mesophilic digesters (B) and the RNA from the thermophilic digesters (C). The inocula (T0) are represented as squares, the first session of sampling (T1) as dots, and the second session (T2) as triangles. The different plastics at the first session of sampling are grouped as T1 plastics because their microbial populations were very close.

Non-metric distance scaling based on the Bray-Curtis index was performed to examine the beta diversity of all of the samples collected from both the mesophilic and the thermophilic digesters over time, and for the digestion of the different substrates (**Figure III.6**). The first NMDS (**Figure III.6A**), on the entire data set, distinguished microbial communities from the mesophilic and the thermophilic reactors. The temperature was been shown to be one of the most important parameters driving the microbial community composition of anaerobic digesters (Azizi et al., 2016; Sundberg et al., 2013). In the same way, the microbial communities from the DNA-based and the RNA-based analysis were clearly separated. The analysis of the 16S rRNA transcripts revealed a different microbial community structure than that of the 16S rRNA gene, thus showing the relevance of studying RNA (De Vrieze et al., 2018). For this reason, the RNA-based analysis will be discussed in more detail below. The other two NMDS (**Figure III.6 B and C**) focused on the thermophilic and the mesophilic samples based on RNA analysis. With the thermophilic condition, the microbial communities of the reactors digesting plastics (Beanarella[®], Launay[®], and Tintoretto[®]) were clustered together at the first sampling time, and they shared a similar microbial composition structure. On the other hand, at the second sampling time, a number of big differences were noted between the communities from the reactors digesting the different plastic samples. In particular, the communities of the Beanarella[®] reactors were very different from those of the Launay[®] and the Tintoretto[®] reactors. The difference in activity between T1 and T2 could explain this shift. At the first sampling time, the measured methane production was high, while it was quite low at the second sampling time (see 3.1). Thus, one could expect to find more bacteria involved in the hydrolysis and fermentation of the substrate being active at the first sampling time than at the second. Similarly, different community compositions were observed for reactors with spent coffee grounds at the first and the second sampling time, under either the mesophilic or the thermophilic condition. However, the digestion of the plastic samples under mesophilic conditions was inefficient and no differences in methane production were observed between the first and the second sampling time. This led to very similar active microbial populations at the two sampling times from the mesophilic reactors digesting the different plastic samples.

3.3) Distribution of the main phylum

Twelve major phyla (represented by more than 1% of the total sequences each in at least one sample) were observed in the mesophilic reactors (**Figure III.7**). As described above, the thermophilic reactors were less diverse; sequences could only be assigned to eight major phyla (**Figure III. 9**).

3.3.1) Archaea

Methanogenesis, the final step of the anaerobic digestion resulting in the production of methane, is exclusively due to the *Archaea*. *Euryarchaeota* was the major phylum of the archaeal domain, representing 99.5 and 100% of the total archaea sequences in the mesophilic and the thermophilic digesters, respectively. *Bathyarchaeota* represented 0.5% of the archaeal sequences found in the mesophilic digesters. The abundance of the *Euryarchaeota* phylum in the inocula was quite low: 0.4% and 0.6% of the total sequences, in the mesophilic and thermophilic inocula, respectively. The low abundance of active archaea at the beginning of the BMP test (T0) could be explained by the fact that the inocula were exhausted before their use (they were not fed) in order to lower the endogenous methane production (**Figures III.7 and 9**). The active *Euryarchaeota* increased during the digestion of the compounds (spent coffee or plastics) to between 1% and 11% of the total sequences (**Figures III.7 and 9**). At the genus level, *Methanothermobacter* and *Methanoculleus* dominated the thermophilic digesters, representing 67.5 and 27.6% of the total archaeal sequences, respectively. *Methanothermobacter* was the most abundant archaea in the plastic-fed digesters at 58 °C, while it was *Methanoculleus* in the coffee-fed reactors. Similarly, *Methanoculleus* and *Methanosaeta* were dominant in the mesophilic condition, at 55.7 and 29.4% of the total archaeal sequences, respectively. *Methanosaeta* were predominant in the digesters fed with spent coffee, and *Methanoculleus* in the plastic-fed reactors, at 38 °C.

Except for members of the *Methanosarcina* genus, which are able to perform all three pathways of methane production (De Vrieze et al., 2012); all the archaeal genera found here could be assigned to a specific methane production pathway. Most of the archaeal sequences could be attributed to hydrogenotrophic methanogens genera. Under the thermophilic conditions, hydrogenotrophic methanogens (*Methanoculleus*, *Methanothermobacter*, and *Methanobacterium*) represented 96.7% of the archaeal sequences. Acetoclastic methanogens

represented 0.1% (*Methanosaeta*) and methylotrophic methanogens 1.6% (*Methanosillicoccus*). *Methanosarcina* accounted for 1.6% of all of the archaeal sequences. In the mesophilic reactors, the dominance of hydrogenotrophic methanogens (*Methanoculleus*, *Methanospirillum*, *Methanobacterium*, and *Methanothermobacter*) was less pronounced, at 69.4% of all of the archaeal sequences. Acetoclastic methanogens (*Methanosaeta*) accounted for 29.4% of the archaeal sequences. Similarly, methylotrophic methanogens (*Methanomasillicoccus*) accounted for 1.1% of under the mesophilic conditions.

A dominance of hydrogenotrophic methanogens is common in many thermophilic anaerobic digesters (Goberna et al., 2009; Hori et al., 2006; Krakat et al., 2010; Sasaki et al., 2011; Sundberg et al., 2013). A number of recent studies have shown that an increase in temperature correlated with a growing abundance of hydrogenotrophic methanogens (Liu et al., 2018; Pap et al., 2015). Hydrogenotrophic dominance is less common in mesophilic digesters, although it has been reported in the literature (Sundberg et al., 2013; Wirth et al., 2012; Zhu et al., 2011). Several parameters such as temperature, a change in substrate composition, and a high concentration of ammonia or volatile fatty acids have been shown to favor the development of hydrogenotrophic microorganisms (De Vrieze et al., 2012; Sasaki et al., 2011; Schnürer and Nordberg, 2008). This situation was supported by the high abundance of acetate-oxidizing bacteria able to engage in a syntrophic partnership with hydrogenotrophic methanogens, such as *Tepidanaerobacter*, *Thermacetogenium*, and *Tepidanaerobacter* (Hattori et al., 2000; Westerholm et al., 2011).

3.3.2) Bacteria:

a) Mesophilic condition

The *Bacteroidetes* phylum was predominant in the initial inoculum, representing 61% of the sample sequences, with *Firmicutes* (16%) and *Chloroflexi* (13%) being the other two major phyla. The *Bacteroidetes* phylum decreased during the anaerobic digestion, although it remained the most abundant phylum in each reactor (between 25% and 58% of the sequences of the samples). The digestion of the compounds (spent coffee or plastics) also pointed to an increase in abundance of the *Proteobacteria* and *Synergistetes*. Interestingly, the *Spirochaetae* phylum was only abundant in the reactor with spent coffee grounds at the first sampling time (19%), while it was found in low abundance at the second sampling time and in the reactors with plastics (between 0.1 and 0.7%).

Most of these OTU (97%) were attributed to the genus *Treponema*. Some *Treponema* species have been reported to be homoacetogens (Graber et al., 2004; Zhang et al., 2009) while others are hydrolytic bacteria, such as *Treponema amylovorum*, which, for example, are able to degrade starch (Wyss et al., 1997).

As seen in the NMDS representation (**Figures III.6**), the microbial community composition of the plastic-fed reactors was very close for the same sampling time. At the phylum level, a similar profile was observed (**Figure III.7**), with a dominance of *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, and *Proteobacteria*. The methane production resulting from the digestion of the biodegradable plastic was not significantly different between the first and the second sampling time, which may explain the low variation of the phyla profile. As a result, the microbial community compositions of T1 and T2 were very similar, with the exception of a decrease in *Bacteroidetes* and a slight increase in *Proteobacteria* and other minor phyla (as *Fibrobacterales* and *Synergistetes*). Similarly, no notable differences were seen in the distribution of the 20 most abundant genera (**Figure III.8**). Thus, these analyses did not reveal OTUs specific for the degradation of plastic in anaerobic media, which may be due to the lack of efficacy of the mesophilic process. No previously known taxa participating in the anaerobic digestion of biodegradable plastic were found in the mesophilic reactors (Venkiteshwaran et al., 2019; Yagi et al., 2014b)

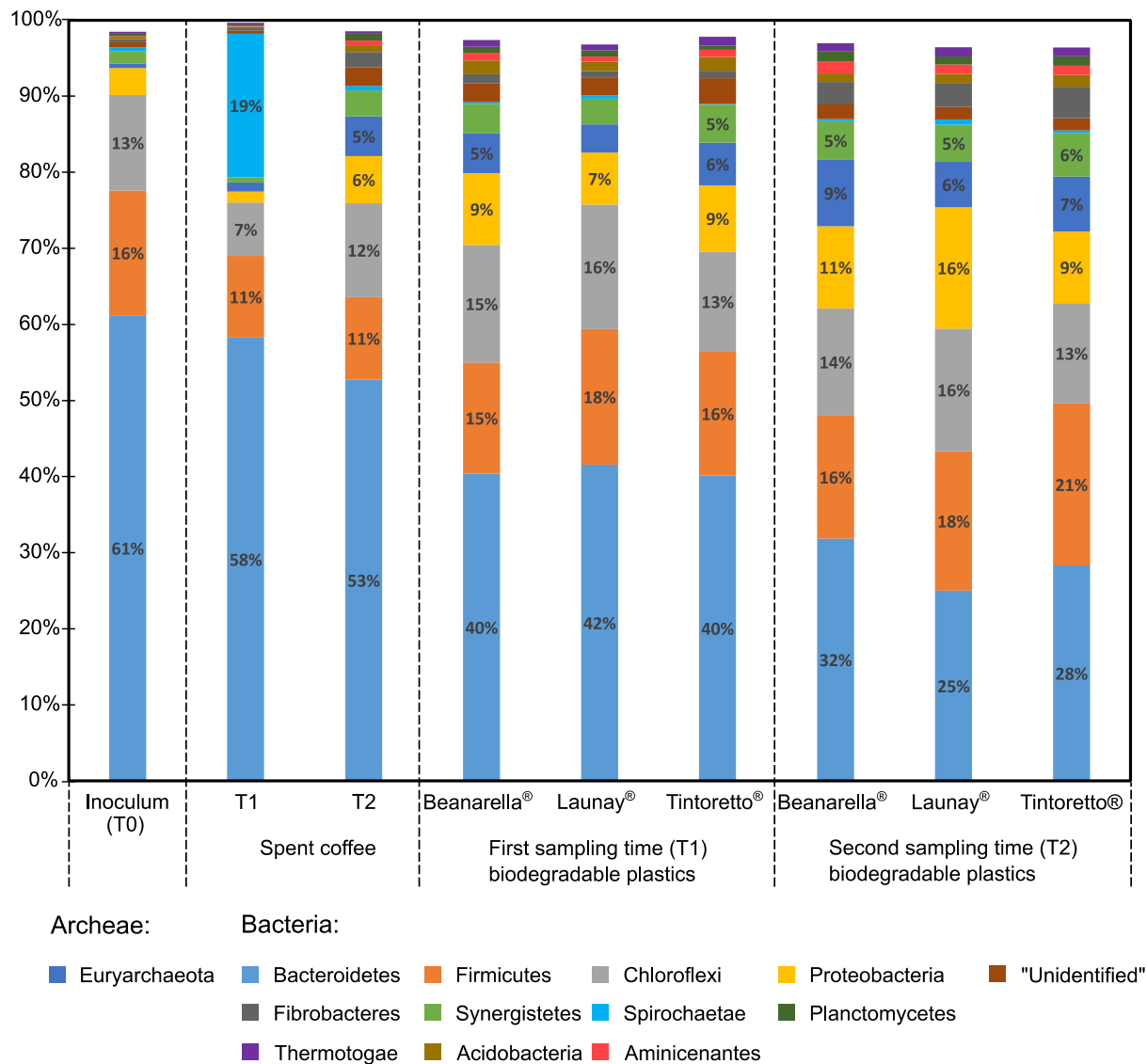


Figure III.7. The main bacterial and archaeal phyla (representing more than 1% of the total sequences in at least one sample) observed in the mesophilic digesters. Phyla with an abundance of less than 5% are not labelled.

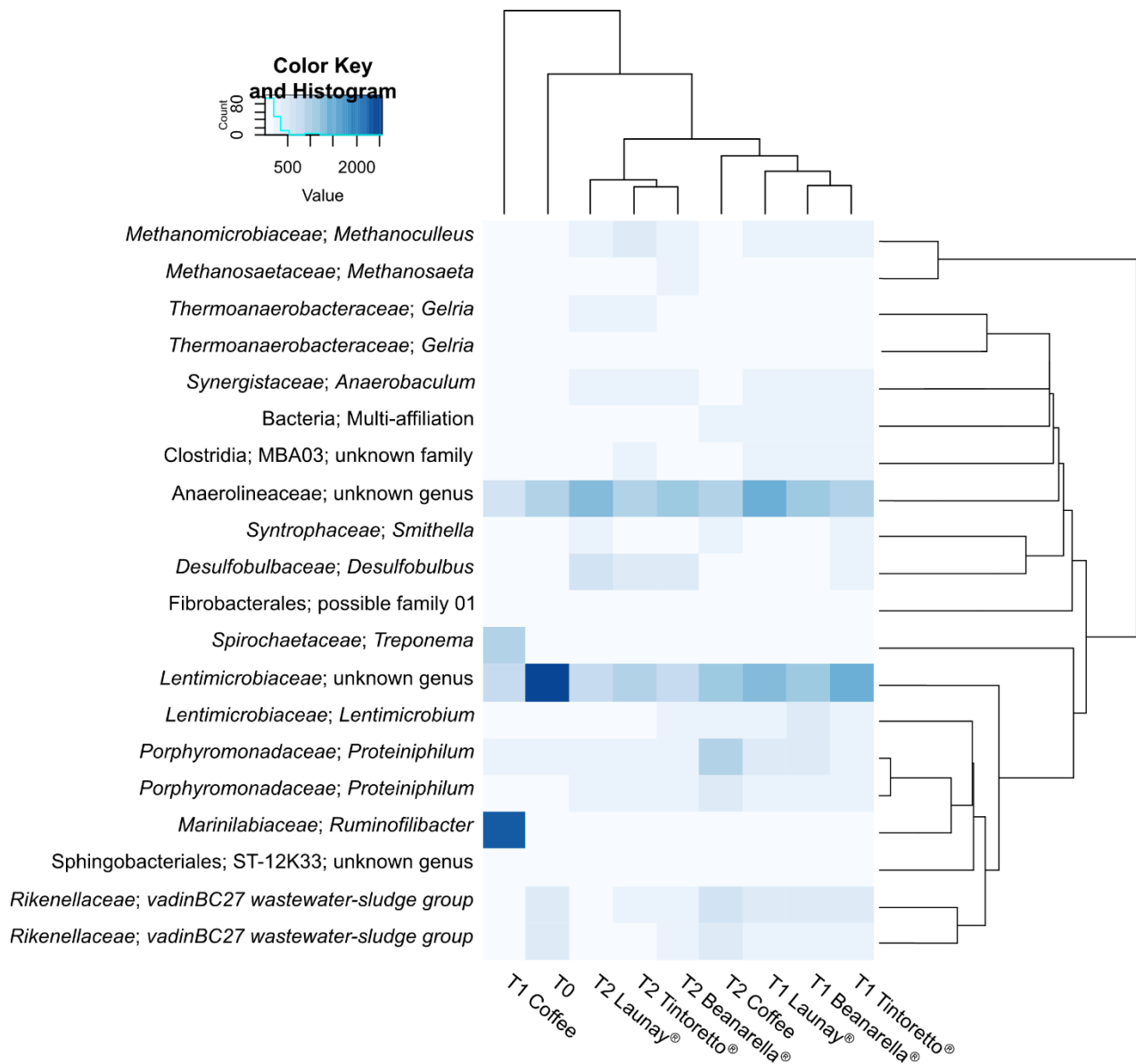


Figure III.8. Heatmap of the 20 most abundant genera observed in the mesophilic digesters. The legend shows the relative abundances. The sample clusterization is on the top of the heatmap and the evolutionary dendrogram of the OTU at the right of the heatmap.

b) Thermophilic condition

Bacteroidetes was the predominant phylum initially (accounting for 49% of the sample sequences) in the inoculum, followed by *Thermotogae* (27%) and *Firmicutes* (22%). However, during the digestion (of the various plastics and the spent coffee), *Firmicutes* became predominant, representing between 55% and 84% of the sequences. The digestion was also marked by a pronounced decrease in the abundance of *Bacteroidetes*, dropping from 49% of the sequences in the inoculum to less than 8% in the reactors treating the various substrates. By contrast,

Synergistetes increased from 1% in the inoculum to between 2 and 19% in the reactors. Interestingly, a high abundance of *Thermotogae* (37%) was found at the first sampling time for the reactor with spent coffee (**Figure III.9**), while they were found at low abundance (between 2 and 9%) elsewhere (in other reactors and at the second sampling time for the reactor with coffee). All of the sequences belonging to the phylum *Thermotogae* could be attributed to the genus *Defluviitoga* (**Figure III.10**). *Defluviitoga* are well-known bacteria involved in thermophilic hydrolysis and fermentation of a large diversity of monosaccharides, disaccharides, and polysaccharides including cellulose and xylan (Ben Hania et al., 2012; Li et al., 2019; Maus et al., 2016).

As described above, at the first sampling time, the microbial community composition of the thermophilic reactors treating the various plastic samples was very similar (**Figure III.9**). *Firmicutes* (77-84%) and *Synergistetes* (5-9%) dominated the microbial community of those reactors. However, a number of differences were observed at the second sampling time on the NMDS and on the repartition at the phylum level (**Figures III.9 and 10**). The digestion of Beanarella® and Tintoretto® resulted in a quite high amount of *Bacteroidetes* (8% and 5%, respectively), while digestion of Launay® capsules led to a low degree of development of this phylum (0.4%). Similarly, the digestion of Tintoretto® was marked by a high abundance of sequences attributed to *Proteobacteria* (6.5%), while they were very low for the other two plastics (0.3-0.7%). These *Proteobacteria* were mostly attributed to *Alpha-proteobacteria*, and more specifically to the *Phyllobacteriaceae* family. Most of the time, species of *Phyllobacteriaceae* are found in aerobic plant-associated environments, although they have also been identified in anaerobic digestion media (Guo et al., 2015; Willems, 2014).

At the first sampling time, the measured methane production was high, while it was quite low at the second sampling time (**see 3.1**). Thus, one could expect to find more bacteria involved in the hydrolysis of the substrate and its transformation to methane at the first sampling time than at the second sampling time. At this time point, the phylum *Firmicutes* dominated both reactors fed with plastics and spent coffee grounds. These *Firmicutes* corresponded to different genera depending on whether they came from reactors with spent coffee or biodegradable plastics (**Figure III.10**). For the digesters fed with spent coffee, most of the *Firmicutes* sequences belonged to the genus *Ruminiclostridium* (14% of sample sequences) and *Coprothermobacter* (15% of the

sample sequences). Species of the *Ruminiclostridium* genus are often found in rumen or other anaerobic media and they exhibit lignocellulolytic activity (Ravachol et al., 2015; X. Zhang et al., 2018). Members of the *Coprothermobacter* genus are known to be anaerobic and thermophilic microorganisms with a proteolytic activity (Pavan et al., 2018). For the digesters fed with plastics, most of the Firmicutes sequences belonged to the genus *Tepidimicrobium* (42% of the sample sequences). **Figure III.10**, representing the 20 most abundant OTU at the genus level observed in the thermophilic digesters fed with plastics, highlights the high abundance of *Tepidimicrobium* at the first sampling time (when the biodegradation of the plastic was active). By contrast, the *Tepidimicrobium* abundance was very low at the second sampling time (when the biodegradation of the plastic was low). This suggests that *Tepidimicrobium sp.* may have a significant role during the degradation of biodegradable plastics. Only two species of this genus have been described, *Tepidimicrobium ferriphilum* (Slobodkin, 2006) and *Tepidimicrobium xylanilyticum* (Niu et al., 2009). The first one was isolated from a freshwater hot spring and the second one from a thermophilic anaerobic digester treating municipal solid waste and sewage. The *Tepidimicrobium xylanilyticum* isolated from an anaerobic digester was able to grow on a variety of carbohydrates (xylan, xylose, glucose, cellobiose, etc.) and on a number of proteinaceous compounds. It has been reported that *Tepidimicrobium xylanilyticum* cannot use starch as a substrate (Niu et al., 2009), which we believe is one of the components of Launay® and Tintoretto® coffee capsules. Members of the *Tepidimicrobium* genus found in the reactors fed with plastic may be involved in the degradation of polyesters, such as PLA, PBAT, or PCL. Moreover, *Tepidimicrobium xylanilyticum* was identified by Tseng et al. (2019) in an anaerobic digester treating PLA or lactic acid under thermophilic conditions. Subsequently, Tseng et al. (2020) isolated and characterized one strain of *T. xylanilyticum* from this digester. Contrary to the type strain of *T. xylanilyticum*, that strain was able to consume lactate and could produce H₂, CO₂ and acetate. They also found out that the accumulation of lactate inhibited the physicochemical depolymerisation of PLA in lactate. More studies on *Tepidimicrobium* is crucial for gaining a better understanding of their involvement in the biodegradation of other biodegradable polymers.

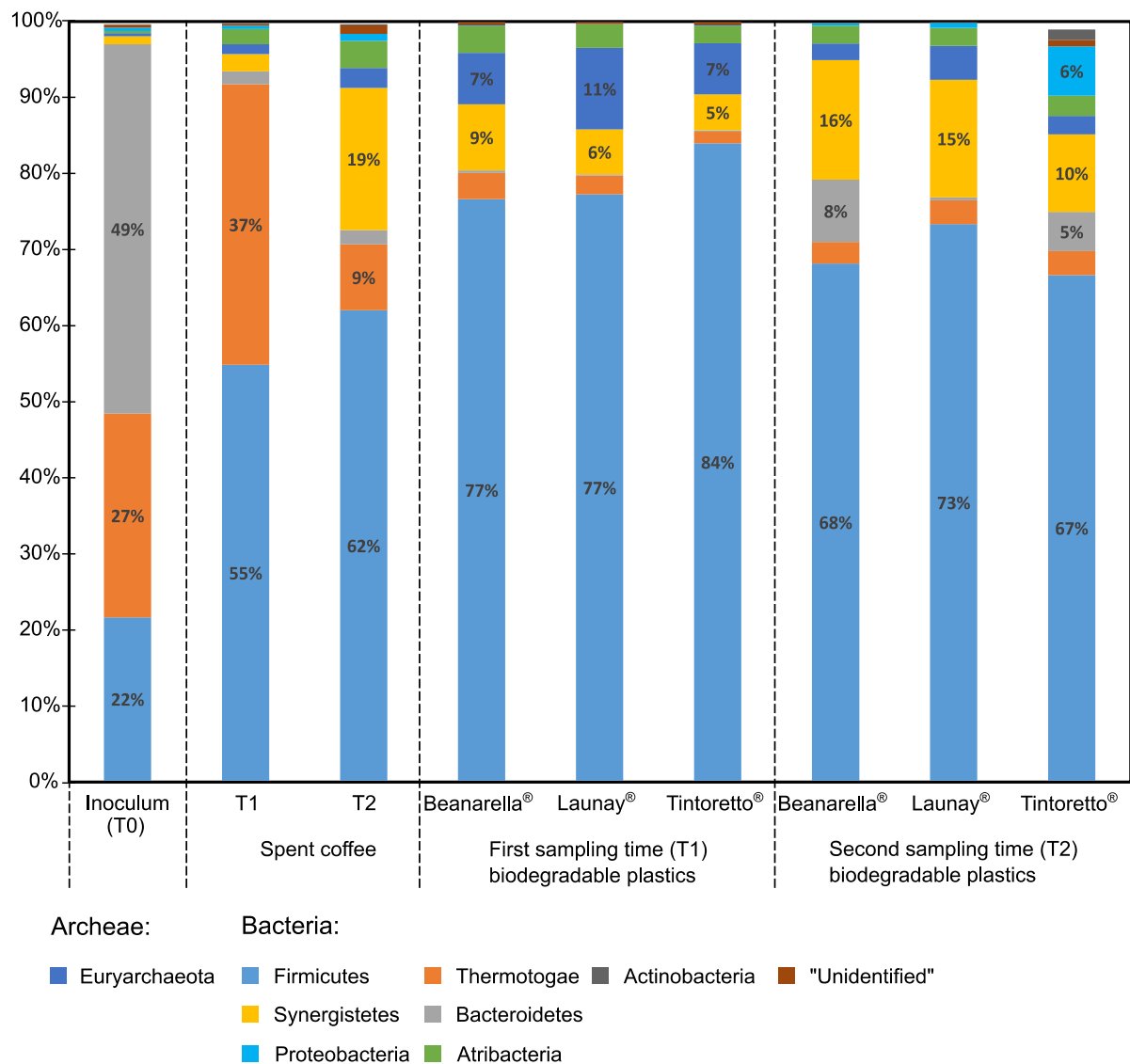


Figure III.9. The main bacterial and archaeal phyla (representing more than 1% of the total sequences in at least one sample) observed in the thermophilic digesters. Phyla with an abundance of less than 5% are not labelled.

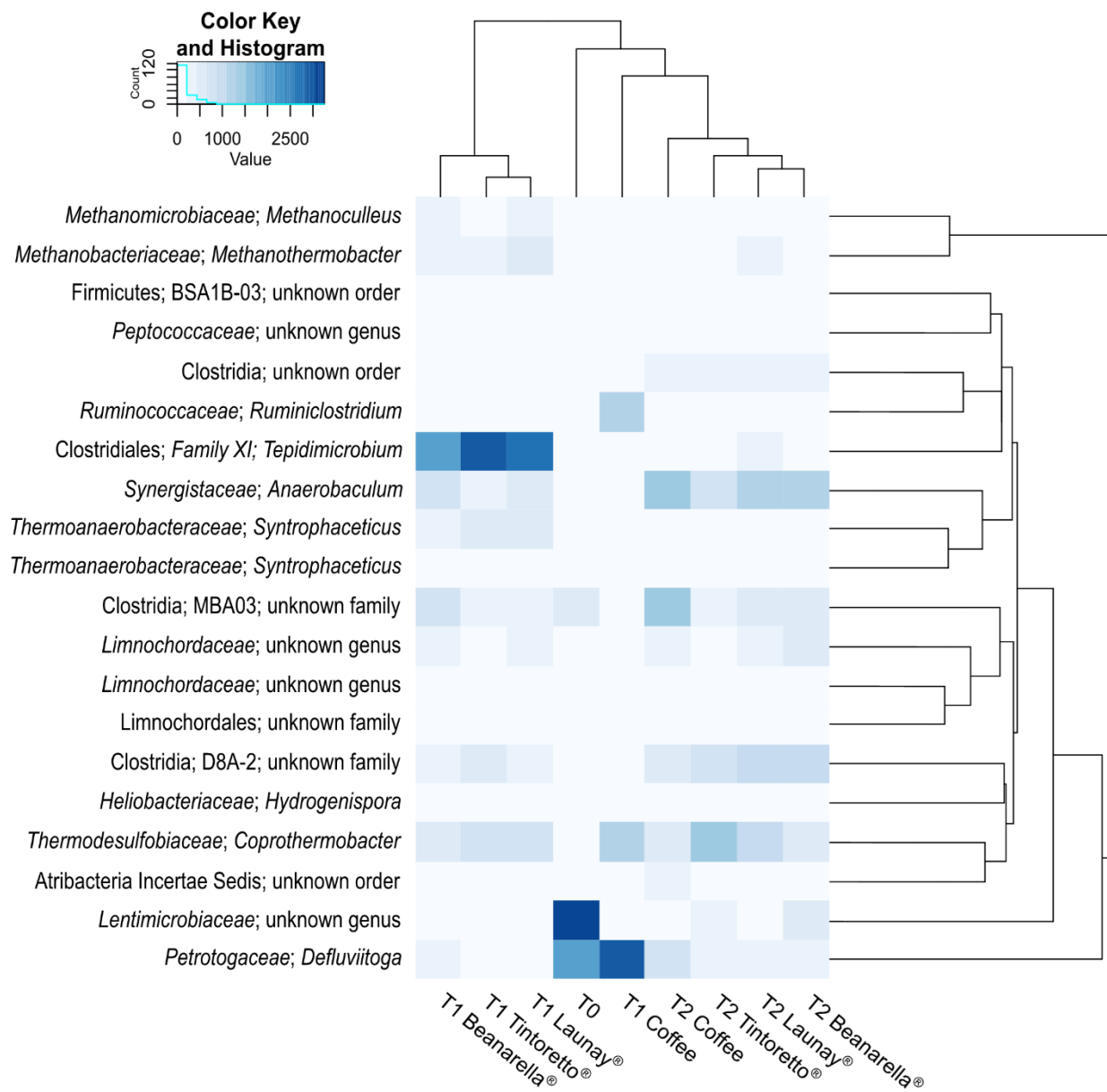


Figure III.10. Heatmap of the 20 most abundant genera observed in the thermophilic digesters. The legend shows the relative abundances. The sample clusterization is on the top of the heatmap and the evolutionary dendrogram of the OTU at the right of the heatmap.

4) Conclusion:

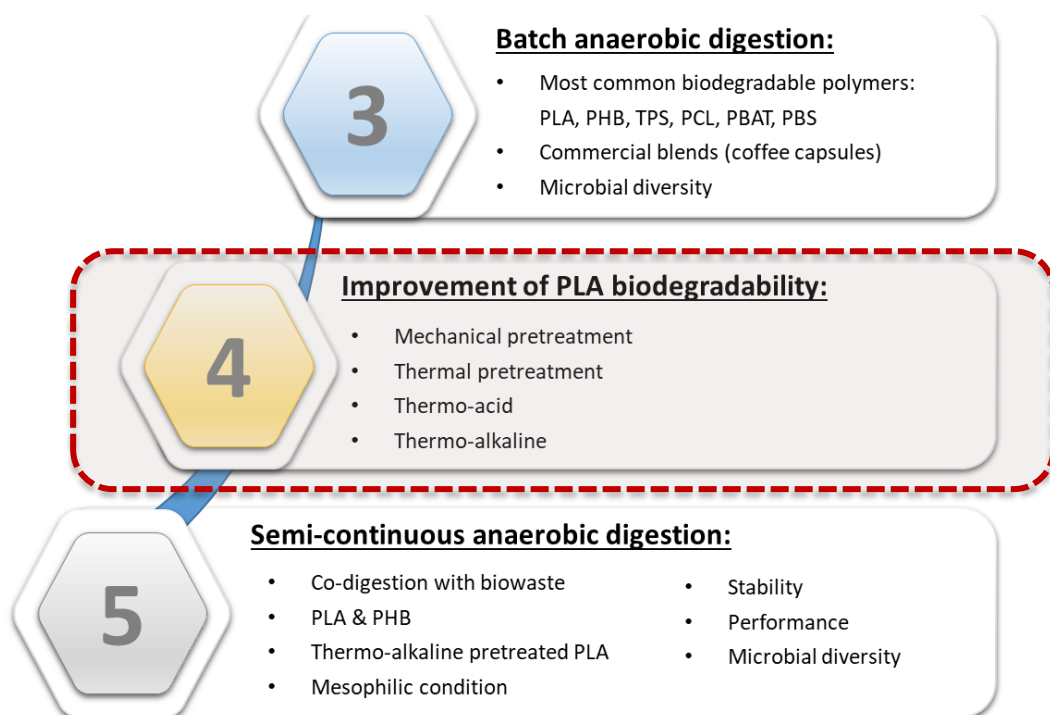
The digestion of the three biodegradable coffee capsules was much more efficient under the thermophilic than under the mesophilic conditions. The methane potential at 100 days, at the mesophilic temperature, ranged between 67 and 127 L CH₄ kg⁻¹ VS, while it ranged between 257 and 294 L CH₄ kg⁻¹ VS at the thermophilic temperature. Hydrogenotrophic methanogens were predominant in the plastic-fed reactors, at both operating temperatures. The microbial populations from the reactors fed with plastics versus spent coffee grounds were significantly different, under both the mesophilic and the thermophilic conditions. At the level of the main OTUs, at the same sampling time and at the same operational temperature, the populations coming from the reactors fed with the different plastics were only slightly different. Most of the differences between these populations were due to rare OTUs. The *Tepidimicrobium* genus was found to be dominant in the thermophilic digesters fed with biodegradable plastics during the high methane production phase. This genus may have a significant role during the biodegradation of the biodegradable plastics under thermophilic conditions. To extend the findings of this study, the microbial community composition and the dynamics of continuous anaerobic digestion pilot-fed with plastics and other organic wastes will be assessed in order to determine the performance and stability of the process.

CHAPTER IV

Improvement of PLA biodegradability

This chapter is based on a modified version of a scientific paper submitted to Chemosphere:

Cazaudehore, G., Guyoneaud, R., Vasmara, C., Greuet, P., Gastaldi, E., Marchetti, R., Leonardi, F., Turon, R., Monlau, F., 2022. Impact of mechanical and thermo-chemical pretreatments to enhance anaerobic digestion of poly(lactic acid) (under revision). Chemosphere.



Foreword

To consider possible the introduction of biodegradable plastics into anaerobic digesters, it is crucial that the biodegradation kinetics of biodegradable plastics are in phase with the Hydraulic Retention Time (HRT) of anaerobic digesters. These HRT are generally of about 30 days for biowastes and 80-120 days for agricultural wastes, (Shi et al., 2017; Van et al., 2019). Therefore, biodegradable plastics must ideally undergo significant biodegradation over a similar period (Bátori et al., 2018a). If it is not the case, persistent plastic debris in digestate could represent an obstacle to marketing and a source of environmental contamination. Contamination of the digestate can be overcome by using an additional step of composting on the solid digestate (Cucina et al., 2021a; Kern et al., 2018).

As demonstrated in the previous chapter, only TPS and PHB achieve high biodegradation (80.2-82.6% and 57-80.3%, respectively) within a relatively short period (25-50 days) under both mesophilic and thermophilic AD conditions. PBS and PBAT exhibit very poor biodegradation at both temperature, reaching a maximum biodegradation of 13.4% for PBAT in 500 days under mesophilic condition. The biodegradation rate of PCL and PLA is very low at 38 °C. Indeed, 500 days are required to reach the ultimate methane production, corresponding to 49.4% of biodegradation for PCL and 74.7-80.3% for PLA. Methane production rate from PLA was greatly enhanced at 58°C since only 60 to 100 days are required to reach the ultimate methane production, corresponding to 74.6% of PLA biodegradation. Ali Akbari Ghavimi et al. (2015) proposed an explanation for the low biodegradation rate of PLA. According to their theory, bacteria would not be able to handle high molecular weight PLA. They would be able to use this PLA only after a reduction of its molar mass caused by a hydrolysis. Hydrolysis rate of PLA is relatively low at 38 °C; by contrast, at 58 °C the hydrolysis rate is increased.

To overcome the limitations related on bad biodegradation kinetics, pretreatment technologies prior to anaerobic digestion can be applied, and are generally classified as physical, thermal, chemical, biological, or a combination of these (Atelge et al., 2020; Bordeleau and Droste, 2011; Millati et al., 2020). Among these technologies, mechanical and thermo-chemical pretreatments have been shown to enhance the anaerobic digestion of sludge (Elalami et al., 2019), biowastes

(Jin et al., 2016), manures (Passos et al., 2017) lignocellulosic biomasses (Barakat et al., 2014; Sambusiti et al., 2013b), algae biomasses (Passos et al., 2015), and, more recently, biodegradable plastics (Benn and Zitomer, 2018; Ryan et al., 2017b). In the case of biodegradable plastics, pretreatments technologies has mainly focus on thermal, thermo-chemical and mechanical (Battista et al., 2021; Benn and Zitomer, 2018; Hobbs et al., 2019; Vargas et al., 2009).

In this context, in the following chapter we focus on the application of pretreatment technologies (mechanical, thermal, thermochemical) to improve the biodegradation rate of PLA in batch system under mesophilic conditions. Its mesophilic conversion to methane is very slow, 500 days are needed to reach the ultimate methane production. Nevertheless, PLA has been chosen because it is one of the most studied biodegradable polymers to replace conventional plastics (Boey et al., 2021; Naser et al., 2021), and because it currently represents 25% of the biodegradable plastic production in 2019 (European Bioplastics, 2020).

Abstract:

To date, the introduction of biodegradable plastics such as PLA in anaerobic digestion systems has been limited by a very low rate of biodegradation. To overcome these limitations, pretreatment technologies can be applied. In this study, the impact of pretreatments (mechanical, thermal, thermo-acid, and thermo-alkaline) was investigated. Mechanical pretreatment of PLA improved its biodegradation rate but did not affect the ultimate methane potential (430-461 L CH₄ kg⁻¹ VS). In parallel, thermal and thermo-acid pretreatments exhibited a similar trend for PLA solubilization. Both of these pretreatments only achieved substantial solubilization (> 60%) at higher temperatures (120 and 150 °C). At lower temperatures (70 and 90 °C), negligible solubilization (between 1 and 6%). occurred after 48 h. By contrast, coupling of thermal and alkaline pretreatment significantly increased solubilization at the lower temperatures (70 and 90 °C). In terms of biodegradation, thermo-alkaline pretreatment (with 5% w/v Ca(OH)₂) of PLA resulted in a similar methane potential (from 325 to 390 L CH₄ kg⁻¹ VS) for 1 h at 150 °C, 6 h at 120 °C, 24 h at 90 °C, and 48 h at 70 °C. Reduction of the Ca(OH)₂ concentration (from 5% to 0.5% w/v) highlighted that a concentration of 2.5% w/v was sufficient to achieve a substantial level of biodegradation. Pretreatment at 70 and 90 °C using 2.5% w/v Ca(OH)₂ for 48 h resulted in biodegradation yields of

73% and 68%, respectively. Finally, a good correlation ($R^2=0.90$) was found between the PLA solubilization and its biodegradation.

1) Introduction

Every year, 335 million tons of plastics are produced globally (Abraham et al., 2021). Massive amounts of plastics are released into the terrestrial and marine ecosystems as industrial waste products, and, in 2014, over 250,000 tons of plastic were estimated to be afloat at sea (Eriksen et al., 2014). Indeed, due to insufficient recycling and poor end-of-life management, used plastics have become a major global problem, resulting in a clear risk to marine environments as well as to the safety of land animals and humans (Emadian et al., 2017a; Hegde et al., 2018; Karan et al., 2019). Growing concern regarding non-biodegradable plastics and the impact of these materials on the environment has led to increased interest in bioplastics and especially in biodegradable plastics as more environmentally friendly and sustainable alternatives to fossil-derived plastics (Cucina et al., 2021a; Filho et al., 2021). More specifically, biodegradable plastics are polymers that are mineralized into carbon dioxide, methane, water, inorganic compounds, or biomass through the enzymatic action of specific microorganisms (Folino et al., 2020). Poly(lactic acid) (PLA) is one of the main biodegradable plastics available on the market, accounting for approximately 25% of all biodegradable plastic production (European Bioplastics, 2019). Moreover, PLA is one of the most studied biopolymers as replacements for petroleum derived plastics (Boey et al., 2021). PLA is a linear aliphatic polyester generated from renewable resources. It is synthesized by direct polycondensation of lactic acid or by ring-opening polymerization of lactide (Long and Chen, 2009). Lactic acid is commonly produced by fermentation of various biomasses (*e.g.*, corn, wheat, sugar cane, and sugar beet) (Song et al., 2011). PLA is mainly used for the production of durable and disposable goods (cutlery, glasses, dishes, and packaging) but it is also used in building and construction, agricultural, medical applications, and fibers production (Cucina et al., 2021a; Muroga et al., 2018).

In parallel, the intensification of separate collection of general household wastes and biowastes in most European countries has also contributed to the development of biodegradable plastics, and their end-of-life is becoming a key issue. In 2019, it was reported that, in Italy, bioplastics represented 3-4% by weight of the Organic Fraction of Municipal Solid Wastes (OFMSW), and this

percentage is expected to increase (Cucina et al., 2021a). Among the various end-of-life scenarios, the potential of anaerobic digestion is increasingly being recognized for the valorization of organic wastes. At the end of 2018, there were 18 202 biogas plants in Europe: 11 084 in Germany, 1 655 in Italy, and 837 in France. AD is a biological process by which organic matter is converted into biogas (a mixture of CO₂ and CH₄). This process can be performed either at mesophilic (35-37 °C) or at thermophilic (52-55 °C) temperatures, although mesophilic reactors are more common due to the higher stability and lower requirement of investment and energy. In the past decade, PLA biodegradation has been investigated in both mesophilic and thermophilic AD. Faster biodegradation has been found to occur under thermophilic than under mesophilic conditions (Battista et al., 2021; Bernat et al., 2021; Mu et al., 2021; Narancic et al., 2018; Shrestha et al., 2020). For instance, Narancic et al. (2018) reported PLA biodegradation yields of 0% after 56 days and 88% after 80 days under mesophilic versus thermophilic conditions, respectively. Similarly, Mu et al. (2021) demonstrated very limited PLA biodegradation under mesophilic conditions (just 50.5 ± 0.5 L kg⁻¹ VS after 146 days) and, in contrast, it was highly biodegradable under thermophilic conditions (442.6 ± 1.1 L kg⁻¹ VS after 96 days). Bernat et al. (2021) and Yagi et al. (2014) found a very low biodegradation rate for PLA under mesophilic conditions, with the digestion not reaching a steady state of methane production after 277 to 280 days. Indeed, only approximately 50% of the PLA was biodegraded after this incubation time.

Generally, biogas plants treating OFMSW or biowastes operate with hydraulic retention times (HRTs) of 15–30 days. Consequently, biodegradable plastic supports (*e.g.*, collected bags, plates, cutlery) have to undergo significant degradation under these conditions (Bátori et al., 2018b). Unfortunately, the literature to date indicates that the biodegradation rates of PLA are not in keeping with the HRTs of industrial biogas plants. Therefore, subsequent industrial composting processes generally need to be applied to solid digestate fractions to reduce biodegradable plastic leaching into the soil ecosystem (Cucina et al., 2021b; Kern et al., 2018). The very limited biodegradability of PLA by mesophilic anaerobic digestion can be explained by its physicochemical properties (molecular weight, accessible surface area, porosity, and crystallinity), which limit microbial and enzymatic attack (Mu et al., 2021). To overcome these limitations, pretreatment technologies prior to anaerobic digestion can be applied, and these are generally classified as physical, thermal, chemical, biological, or a combination of these (Atelge et al., 2020; Bordeleau

and Droste, 2011; Millati et al., 2020). Among these technologies, mechanical and thermo-chemical pretreatments have been shown to enhance the anaerobic digestion of sludge (Elalami et al., 2019), biowastes (Jin et al., 2016), manures (Passos et al., 2017) lignocellulosic biomasses (Barakat et al., 2014; Sambusiti et al., 2013b), algae biomasses (Passos et al., 2015), and, more recently, biodegradable plastics (Benn and Zitomer, 2018; Ryan et al., 2016). Indeed, mechanical pretreatment and size reduction has been investigated for PCL (Yagi et al., 2009), PLA (Yagi et al., 2012b), and PHBV (Ryan et al., 2016). For instance, Ryan et al. (2016) demonstrated that mechanical size reduction improves the kinetics of biodegradation without any impact on the final methane potential for PHBV particles ranging from 10 to 3,900 μm . In parallel, the impact of thermal and thermo-chemical pretreatment has also been investigated for PLA (Battista et al., 2021; Benn and Zitomer, 2018; Hobbs et al., 2019; Mu et al., 2021; Vargas et al., 2009) and PHB (Benn and Zitomer, 2018). Contrasting effects were observed: acidic and alkaline pretreatment of starch-based plastic and PLA carried out at room temperature did not enhance the methane potential (Battista et al., 2021) whereas alkaline pretreatment enhanced the methane potential of starch-based bags, PLA, and PHA polymers (Benn and Zitomer, 2018; Calabro et al., 2019). Furthermore, as one of the main means for digestate valorization is their use for agronomic applications, it is particularly important to verify that the pretreatment will not affect the digestate's agronomic potential (Elalami et al., 2019). Indeed, thermo-chemical pretreatment can contribute to an increase in the content of certain elements in the digestate, directly by chemical addition (*i.e.*, Na, S, and Fe) or indirectly (through the formation of by-products such as furans and polyphenols). However, these elements or compounds can have detrimental impacts on the soil (Elalami et al., 2019; Monlau et al., 2014). To date, the knowledge regarding pretreatment technologies to enhance anaerobic biodegradability in anaerobic digestion remains very limited. Previous results have demonstrated that PLA biodegradation by mesophilic anaerobic digestion is very slow and not in keeping with the HRTs generally applied in biowaste biogas plants (HRTs of approximately 20-30 days) or agricultural biogas plants (HRTs of approximately 60-100 days) (Bátori et al., 2018b; Cucina et al., 2021b; Ruile et al., 2015). The main objective of the present study was, therefore, to further assess the effects of different pretreatment strategies on PLA solubilization and increased anaerobic digestion at mesophilic temperatures. For this purpose, the

effects of different pretreatments, namely mechanical (centrifugal milling), thermal (70 °C to 150 °C), and thermo-chemical (in the presence of $\text{Ca}(\text{OH})_2$ and H_3PO_4), were investigated.

2) Materials and Methods

2.1) PLA and inoculum used

The PLA used in this study was purchased from NaturePlast (Iffs, France). The grade was NP SF 141, which is a thermoplastic resin of PLA, biobased at more than 85% from annually renewable plant resources, with better flexibility and elongation properties compared to raw PLA. Total solids (TS) and volatile solids (VS) were determined by standard analytical methods (APHA, 2005). The main physicochemical properties of the PLA are presented in **Table IV.1**. The inoculum used for the anaerobic biodegradation testing was prepared in the APESA laboratory from digestate collected from a mesophilic anaerobic digestion plant. It was maintained at 38 ± 1 °C under agitation and it was fed twice a week with a mix of sludge from wastewater treatment as well as green grass. Control parameters (pH, redox, alkalinity, dry matter, volatile solids, concentration of ammonia, and volatile fatty acids) were measured at regular intervals. The inoculum was sieved to a 2-mm mesh size prior to use in the BMP tests to reduce the biogas production from the residual undegraded organic fraction contained in the inoculum. The main properties of the inoculum were: TS (% fresh mass): $3.8 \pm 0.3\%$; VS (% TS): $64.4 \pm 1.5\%$; pH: 8.3 ± 0.2 ; volatile fatty acids (VFAs): 300 mg eq. acetate L^{-1} ; and ammonium content: 2.1 g $\text{N-NH}_4^+ \text{L}^{-1}$.

Table IV.1. The main chemical properties of the PLA used in this study.

Parameters	Values (\pm S.D)
VS (%TS)	98
C (%TS)	51.8
H (%TS)	6.0
N (%TS)	0
S (%TS)	0
O (%TS)	40.2
COD_{th} gCOD g^{-1}VS	1.492
BMP_{th} NLCH ₄ kg^{-1}VS	522

2.2) Pretreatment of PLA

Mechanical pretreatment was carried out using liquid nitrogen and a centrifugal mill (ZM 100, Retsch GmbH & Co., Haan, Germany) at a screen size of 2 mm. The powder was then sieved to

different sizes by sequential use of molecular sieves of 2 mm, 1 mm, 800 μm , 500 μm , and 300 μm . The thermal and thermo-chemical pre-treatments were performed in batch mode with a total solid content of 50 gTS L⁻¹. The pretreatments were carried out in 35 mL Pyrex glass tubes heated in a heat system with magnetic agitation (Hei-Tec, Heidolph Instruments, Schwabach, Germany). Several pretreatment series were performed: 1) variation of the temperature (70 °C, 90 °C, 120 °C, and 150 °C) at different residence times (1 h, 6 h, 24 h, and 48 h), 2) impact of chemical addition (*i.e.*, Ca(OH)₂ and H₃PO₄) at concentrations of 5% w/v in addition to thermal treatment at the indicated temperatures, 3) optimization of the Ca(OH)₂ concentration (0.5, 1.25, 2.5, and 5% w/v) at 70 °C and 90 °C for 48 h.

2.3) Physicochemical analysis

The pH was measured using a 340i pH meter fitted with a Sentix[®] electrode (WTW, Weilheim, Germany). The VFAs and N-NH₄⁺ concentrations were determined using a previously described protocol (Monlau et al., 2021). The elemental composition of the feedstock was assessed using an elemental apparatus (vario Micro V4.0.2, Elementar[®], Germany). The size of the various fractions of the mechanically pretreated PLA was determined using a particle size analyzer (Mastersizer 3000, Malvern Panalytical, Malvern, United Kingdom). Differential scanning calorimetry (DSC) was performed on granules of PLA and on the various fractions of the mechanically pretreated PLA using a DSC Q100 (TA Instruments, New Castle, USA) with a heating and cooling rate of 10 °C min⁻¹. Analysis were performed using about 7 mg of samples placed in standard aluminum pans. The samples were heated from 20 °C to 260 °C under a nitrogen flow rate of 50 mL min⁻¹. Then, cooled to 20°C and finally a second heating scan was performed from 20°C to 260°C. The melting enthalpy (ΔH) was calculated by measuring the area under the curve during the first heating cycle. The crystallinity of the samples was calculated using Equation 1.

$$\text{Crystallinity (\%)} = \frac{\Delta H}{\Delta H_{100}} \times 100 \text{ (Eq. 1)}$$

where ΔH is the measured melting enthalpy and ΔH_{100} is the theoretical melting enthalpy of a 100% crystalline PLA (93.1 J.g⁻¹) (Fischer et al., 1973).

The theoretical chemical oxygen demand (COD) of the PLA was then calculated based on Equation 2 (Haandel and Lubbe, 2007)

$$COD_{th} (g\ COD/g\ C_xH_yO_z) = 8 \times \frac{4x+y-2z}{12x+4+16z} \text{ (Eq. 2)}$$

where x, y, and z represent the amount of carbon, hydrogen, and oxygen, respectively.

The pretreatment supernatant from thermal and thermo-chemical pretreatments was centrifuged at 13,000 rpm for 20 min using a Mini Spin® (Eppendorf, Hamburg, Germany) and filtrate to 0.7 µm in order to collect the soluble fraction. The soluble COD was then analyzed using commercial kits (Spectroquant® 14,155, Merck, Germany). The concentrations ranged from 500 to 10,000 mg COD.L⁻¹. All of the analyses were performed in duplicate for each sample. Briefly, 1 mL aliquots of the supernatant fluids were placed in the commercial tubes. The tubes were then heated to 148 °C in a preheated thermoreactor for 120 min. Finally, the COD was measured using an automatic spectrophotometer (photoLab® S6, WTW, Weilheim, Germany).

The percentage solubilization of the PLA is the ratio between the experimental solubilized COD and the theoretical BMP, calculated using Equation 3.

$$PLA\ solubilization\ (\%) = \frac{Soluble\ COD}{COD_{th}} \text{ (Eq. 3)}$$

SEM observations were performed using a Desktop SEM (Phenom ProX, Fondis Bioritech, France) with an acceleration voltage ranging between 5 and 10kV secondary electrons. PLA granules (neat and pre-treated) were directly mounted on stub using carbon conductive tape and then coated with Gold/Palladium during 45s at 20mA (4 nm thick) by ion sputtering (Mini Sputter Coater SC7620, Quorum Technologies Ltd, UK).

2.4) Biochemical Methane Potential

BMP tests were carried out in duplicate under mesophilic conditions by filling 500 mL reactors with 300 mL of an inoculum/substrate ratio at 2.85 g VS g⁻¹ VS. The pH of the reactors was balanced between 7 and 8 when necessary, by adding HCl or NaOH (1M). After filling, each bottle was flushed with N₂ for 30 seconds, incubated at 38 °C, and degassed after 1 h. Each day, manual homogenization was performed and the biogas production was measured using an electronic manometer device (Digitron 2023P, Digital Instrumentation Ltd, Worthing, United Kingdom) and expressed in normal-liters (at 0 °C, 1.013 hPa). Once a week, the gas composition was analyzed by gas chromatography (Varian GC-CP4900, Agilent, Germany) using an instrument equipped with two columns. Molsieve 5A PLOT column was used at 110 °C for O₂, N₂ and CH₄ analysis and a

HayeSep A fixed at 70 °C was used to measure CO₂. The injector and detector temperatures were set at 110 °C and 55 °C, respectively. Two standard gases for calibration were used: one composed of 9.5% CO₂, 0.5% O₂, 81% N₂, and 10% CH₄ and the other of 35% CO₂, 5% O₂, 20% N₂, and 40% CH₄ (special gas from Air Liquide®). The BMP tests were terminated when the biogas production reached a stationary state and did not vary by more than 0.5% over three consecutive days. Duplicate blank (inoculum only) and positive controls (cellulose, Tembec®) were run in parallel.

The theoretical BMP was calculated based on the elemental characterization (C_xH_yO_zN_nS_s) according to Equation 4 (Achinas and Euverink, 2016b)

$$BMP_{th} (L CH_4 \text{ kg}^{-1} VS) = \frac{22.4 \times \left(\frac{x}{2} + \frac{y}{8} - \frac{z}{4} - \frac{3n}{8} - \frac{s}{4} \right)}{12x + y + 16z + 14n + 32s} \text{ (Eq. 4)}$$

where 22.4 is the molar volume of an ideal gas.

Finally, the percentage biodegradation is the ratio between the experimental BMP and the theoretical BMP (Equation 5).

$$Biodegradation (\%) = \frac{BMP_{exp}}{BMP_{th}} \text{ (Eq. 5)}$$

In order to quantitatively evaluate the biomethane production, the cumulative methane data were fit by the modified Gompertz model (Equation 6). The parameters of the model were determined using R software version 3.6.2 and the nonlinear least-squares method (nlsLM function from minpack.lm package). Statistical comparison of the parameters was performed on R using anova and tuckeyHSD at a probability of significance level $P \leq 0.05$.

$$G(t) = G(0) * \exp \left[- \exp \left(\frac{R_{max} * \exp(1)}{G(0)} * (\lambda - t) + 1 \right) \right] \text{ (Eq.6)}$$

where:

- G(t) is the cumulative methane production at time t in L CH₄.kg⁻¹ VS
- G(0) is the ultimate amount of methane produced in L CH₄.kg⁻¹ VS
- λ is the time lag in days
- R_{max} is the methane production rate in L CH₄.kg⁻¹ VS d⁻¹

3) Results and Discussion

3.1) Impact of mechanical pretreatment

Mechanical pretreatment (size reduction by centrifugal milling) was performed to improve the biodegradability of PLA under mesophilic anaerobic conditions. The sizes distribution of the mechanically pretreated PLA particles are indicated in **Table IV.2**. After mechanical pretreatment and separation by molecular sieves, five fractions were obtained with mean sizes of 1,660 μm , 1,140 μm , 808 μm , 502 μm , and 272 μm , respectively. The percentage of crystallinity reported in **Table IV.2** indicate no significant changes since similar values ranging from 26.6% to 28.9% were obtained for the different fractions.

Table IV.2. The crystallinity (%) and the size of the particles of the various PLA samples before and after mechanical size reduction.

Sieve size separation	Crystallinity (%)	Laser granulometry (mean in μm)			
		dv10	dv50	dv90	Mean size
Granule	28.5	-	-	-	-
1 - 2 mm	28.9	1,100	1,580	2,320	1,660
800 μm - 1 mm	26.6	784	1,080	1,570	1,140
500 - 800 μm	28.6	537	771	1,130	808
300 – 500 μm	28.3	293	479	751	502
50 μm – 300 μm	28.3	138	254	434	272

The cumulative methane curves and the methane production rate (during the first 25 days of the digestion) for the various fractions are shown in **Figure IV.1**. Additionally, in order to quantitatively evaluate the methane production kinetics, the Gompertz model was employed to fit the cumulative methane potential. Such models have been implemented successfully in the past to model the cumulative methane potential (Ryan et al., 2016). The results of this modeling are presented in **Table IV.4**. After 520 days of digestion, the methane potential ranged from 429 L CH_4 kg^{-1} VS (PLA granules) to 460 L CH_4 . kg^{-1} VS (mean PLA size of 272 μm). The particle size had a clear effect on the biodegradation kinetics but it had only a limited effect on the ultimate methane production. As revealed by the statistical analyses (Anova and TuckeyHSD), the ultimate methane production predicted by the Gompertz modeling ($G(0)$, **Table IV.3**) was only significantly different for the smallest particle size PLA (mean size of 272 μm) and the bigger size (mean size of 1660 μm and untreated granule). Similarly, the predicted ultimate methane production from PLA of 502 μm

and the untreated granule were statistically different. Very few studies to date have investigated to what extent mechanical pretreatment and size reduction can improve anaerobic digestion of biodegradable plastics (Ryan et al., 2016; Yagi et al., 2012b, 2009). For instance, Yagi et al. (2009) have investigated methane production from PCL at different particle sizes (< 125 μm ; 125-250 μm , and 250-500 μm) and they reported similar biodegradation yields for the various tested fractions. Nonetheless, smaller particles clearly improved the degradation time, as the < 125 μm fraction reached a stable phase after 30 days versus 65 days for the 250-500 μm fraction. Similarly, Ryan et al. (2016) have investigated the biodegradability in mesophilic anaerobic digestion of different particle sizes of PHBV that varied from 10 μm to 3,900 μm . Interestingly, they found that a reduction in particle size did not affect the ultimate methane potential (around 600 L CH_4 kg^{-1} VS), although the time required to reach the ultimate amount of methane produced was significantly reduced for the smaller particles sizes (25 days for particles of 10 μm versus 40 days for particles of 3,900 μm). The improved biodegradation kinetics can be explained by an increase in the accessible surface area, which results in more attack sites for microorganisms (Barakat et al., 2013; Ryan et al., 2016). Furthermore, below a particle size of 0.8 mm, the rates of hydrolysis and acetogenesis exceeded the rates of methanogenesis, with the accumulation of intermediates leading to a temporary inhibition of CH_4 production (Ryan et al., 2016).

Interestingly, the anaerobic digestion of the PLA started with a short lag phase (less than one day) followed by a phase of intense methane production (**Figure IV.1 B**). After several days (6-7 days), the methane production rate decreased for the various particle sizes of PLA that were digested. After a certain storage time, the surface of the PLA granules became coated with a fatty compound, presumably due to the loss (by exudation) of an additive. Therefore, the high level of methane production in the early days of the digestion could be due to degradation of this additive (Chen et al., 2021). The magnitude of the high methane production phase increased as the size of the PLA particles decreased (**Figure IV.1B**). Presumably, smaller PLA particle sizes release the additive more rapidly due to a greater accessible surface area. As shown in **Table IV.2**, the crystallinity was not a significant parameter as similar crystallinity values were observed for the various fractions. The biodegradation yields of PLA at different times (30 days, 100 days, and 520 days) are presented in **Figure IV.2**. These times (31 and 100 days) correspond generally to the HRTs applied in biogas plants that treat biowastes or OFMSW (20-30 days) and agricultural residues or

co-products (approximately 60-100 days) (Bátori et al., 2018b; Cucina et al., 2021b; Ruile et al., 2015). At 31 and 100 days, the biodegradation yield of particles was influenced by their size, with smaller sizes being degraded more rapidly. At 31 days, the biodegradation yield varied between 4.1% (PLA granules) to 16.9% (mean PLA size of 272 μm). At 100 days, the biodegradation yield was higher, with values ranging from 15.3% (PLA granules) to 37.9% (mean PLA size of 272 μm). After 520 days of mesophilic anaerobic digestion, the biodegradation yield was very close for all sizes, with values ranging from 82.4% to 88.4%.

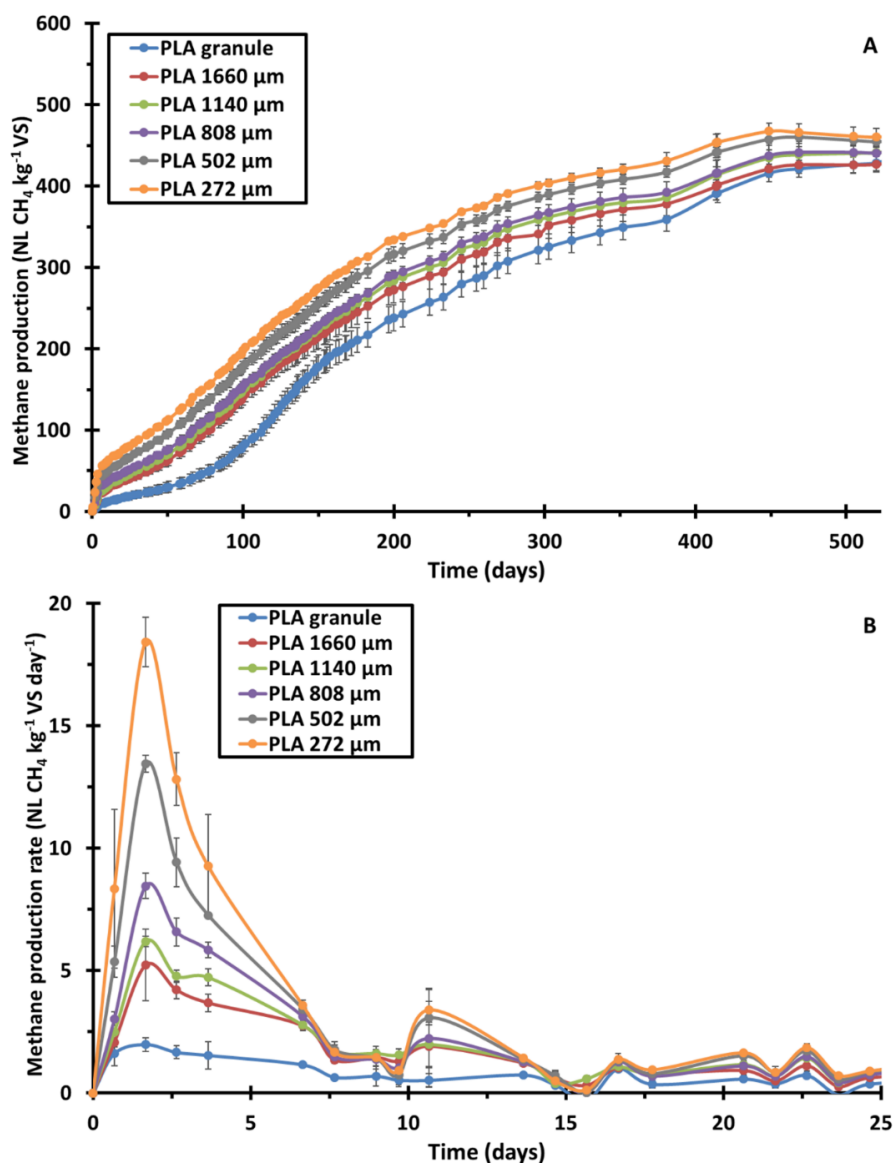


Figure IV.1. The cumulative methane production ($\text{L CH}_4 \text{ kg}^{-1} \text{ VS d}^{-1}$) (A) and the methane production rate ($\text{L CH}_4 \text{ kg}^{-1} \text{ VS d}^{-1}$) (B) for the various PLA sizes. The error bars represent the standard deviations of the replicates.

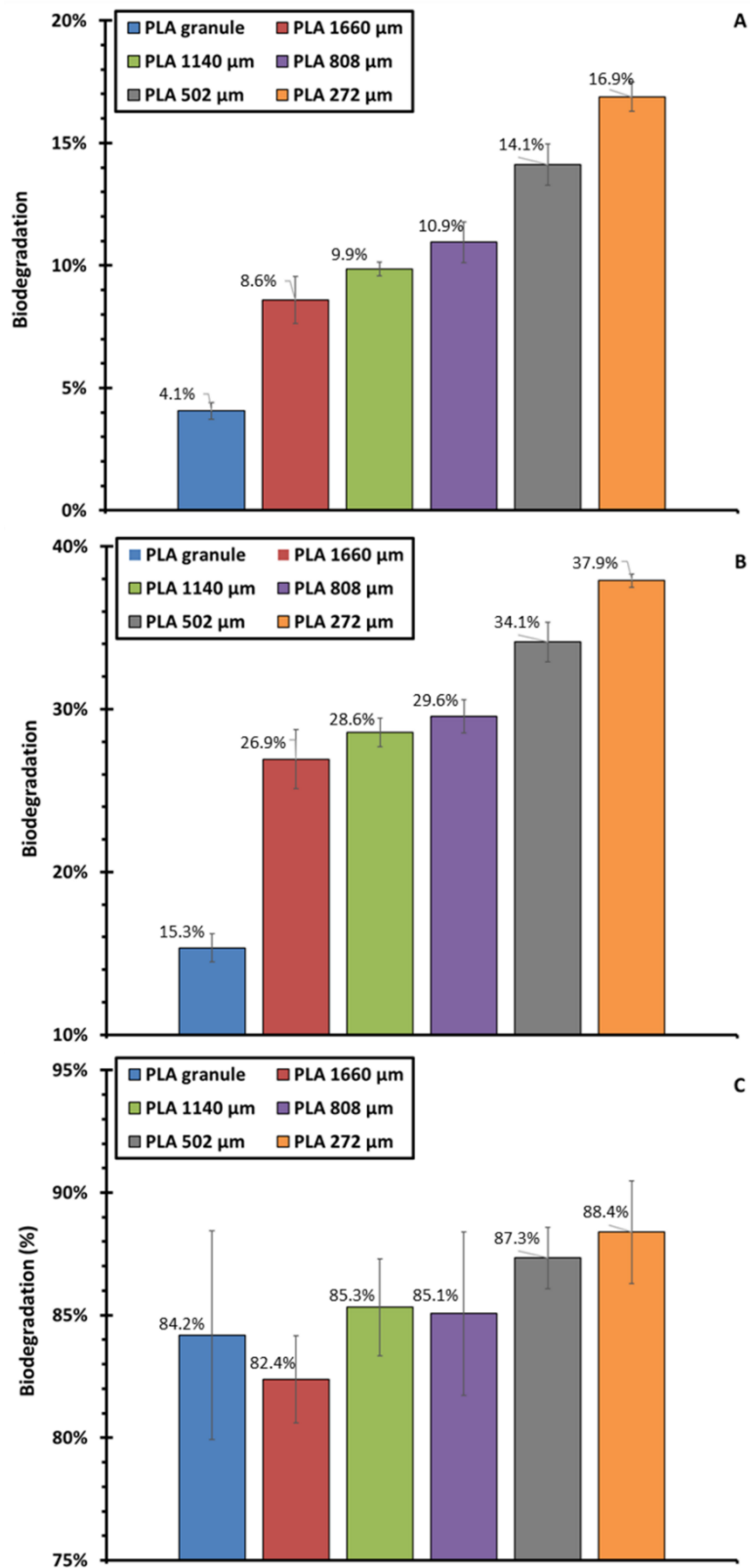


Figure IV.2. The biodegradation yields (%) for the various PLA sizes at different incubation times (31, 100, and 520 days). The error bars represent the standard deviations of the replicates.

3.2) Impact of thermo-chemical pretreatment

In parallel to mechanical pretreatment, thermo-chemical pretreatments were also investigated in order to improve anaerobic degradation of PLA granules. The PLA solubilization for various pretreatment durations (1 h, 6 h, 24 h, and 48 h) and temperatures (70 °C, 90 °C, 120 °C, and 150 °C) are presented in **Figure IV.3A**. A high level of PLA solubilization was achieved at high temperatures of 120 °C and 150 °C with pretreatment durations of at least 6 h. PLA solubilization levels of 64%, 63%, and 65% were achieved for thermal pretreatments at 150 °C for 6 h, 150 °C for 24 h, and 120 °C for 24 h, respectively. As shown in **Figure IV.3B**, similar trends were observed for thermo-acid pretreatment. For instance, a similar PLA solubilization level of 65% was noted for thermal pretreatment (120 °C, 24 h) and thermo-acid pretreatment (120 °C, 24 h, 5% w/v H₃PO₄). These results can be explained by the fact that thermal pretreatment led to the release of acidic substances (mainly lactic acid). Indeed, the pH was acidic (1.55 and 3.43) for 24 h at 120 °C and 24 h at 120 °C with 5% w/v H₃PO₄, respectively. Mu et al. (2021) reported a PLA solubilization level of just 14.9% using hydrothermal pretreatment at 120 °C for shorter pretreatment durations (between 10 and 120 min) whereas higher solubilization levels of 91.9% and 96.2% were noted at 200 °C and 240 °C, respectively. Thermo-alkaline pretreatment can also significantly improve PLA solubilization compared with thermal and thermo-acid pretreatments, as shown in **Figure IV.3C**. Indeed, satisfactory levels of PLA solubilization were obtained at lower temperatures (70 °C and 90 °C) and time durations of 24 h and 48 h. For instance, PLA solubilization levels of 62 and 60% were observed for 48 h at 90 °C and 48 h at 70 °C, respectively. In parallel, thermo-alkaline pretreatment allowed 72% of the PLA to be solubilized in just 1 h at 150 °C. Mu et al. (2021) reported a similar solubilization level of 68.5% after thermo-alkaline pretreatment of PLA at 160 °C for 1 h. In order to improve the PLA solubilization rate during thermo-alkaline pretreatment, a dual strategy can be applied comprising high temperature (120 – 150 °C) and a low residence time (1 – 6 h) or low temperature (70 – 90 °C) and a longer pre-treatment time (24 – 48 h).

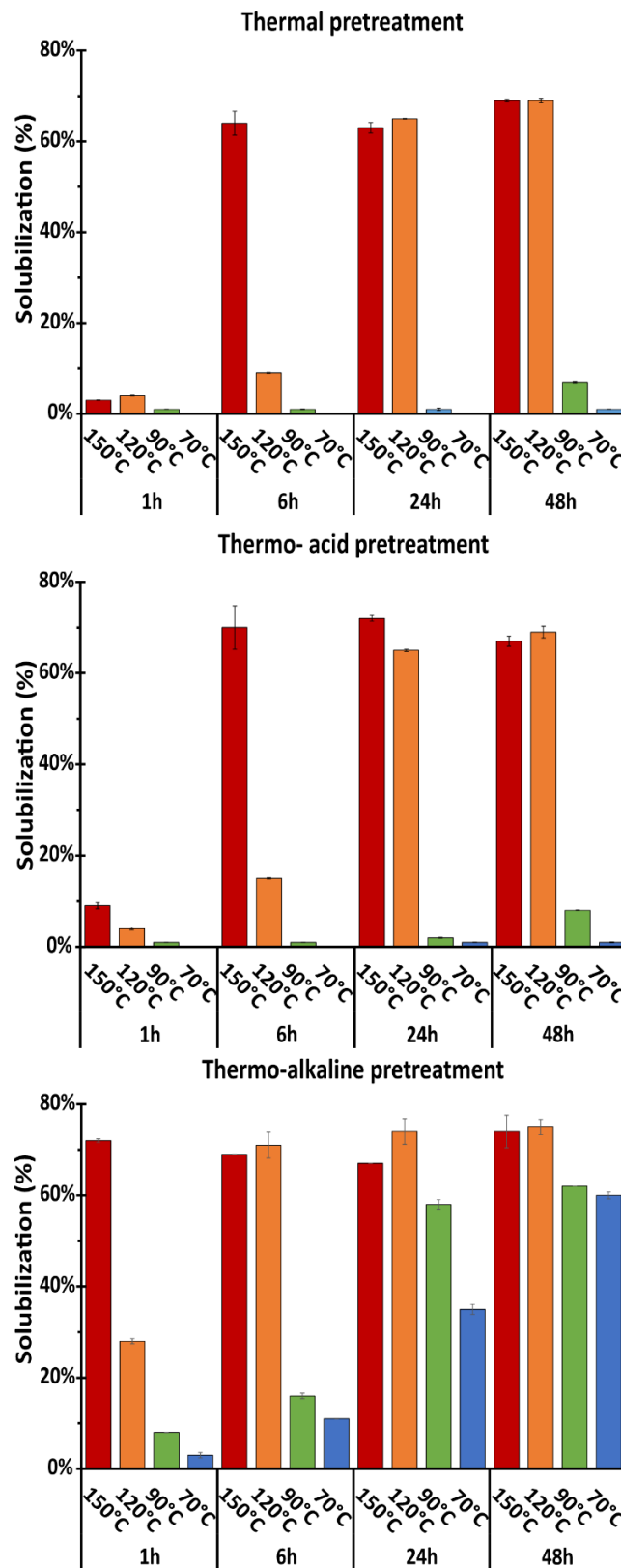


Figure IV.3. PLA solubilization (%) during: A) thermal pretreatment at 70 °C, 90 °C, 120 °C, and 150 °C; B) thermo-acid pretreatment with 5% w/v H_3PO_4 ; and C) thermo-alkaline pretreatment with 5% w/v $\text{Ca}(\text{OH})_2$. The error bars represent the standard deviations of the replicates.

Biochemical methane potential tests were performed for some of the pretreatment conditions presented in **Figure IV.3**. The results and the methane potential profiles for untreated PLA, thermal pretreated PLA (6 h at 150 °C, 24 h at 120 °C, 48 h at 90 °C, and 48 h at 70 °C), and thermo-alkaline pretreated PLA with 5% Ca(OH)₂ (1 h at 150 °C, 6 h at 120 °C, 48 h at 90 °C, and 48 h at 70 °C) are presented in **Figure IV.4**. Additionally, the result of Gompertz modeling is presented in **Table IV.4**. First, it can be seen that PLA granules achieved a very low methane potential of 14 L CH₄ kg⁻¹ VS after 24 days. This result is in keeping with those previously described in this paper as well as with the data in the literature listed in **Table IV.3** (Battista et al., 2021; Cucina et al., 2021b; Narancic et al., 2018). Indeed, methane potentials of 29 L CH₄ kg⁻¹ VS after 12 days (Mu et al., 2021), 34 L CH₄ kg⁻¹ VS after 60 days (Cucina et al., 2021b), and 130 L CH₄ kg⁻¹ VS after 45 days (Battista et al., 2021) were reported. It is interesting to note that methane production increased gradually as the temperature increased from 70 °C to 150 °C. Indeed, methane potentials of 24, 147, 370, and 381 L CH₄ kg⁻¹ VS were observed for 48 h at 70 °C, 48 h at 90 °C, 24 h at 120 °C, and 6 h at 150 °C, respectively. At high temperatures (120 and 150 °C), the methane potential was associated with a biodegradation yield of 71-73%. Mu et al. (2021) have reported similar results, with methane production gradually increasing as the temperature increased from 120 °C to 160 and 200 °C, and correspondingly the average methane yields increased from 139.8 L CH₄ kg⁻¹ VS to 321.8 L CH₄ kg⁻¹ VS and 440.3 L CH₄ kg⁻¹ VS, respectively. Vargas et al. (2009) also reported enhancement of the methane potential of PLA after steam exposure (3 h, 120 °C), with values increasing from 2 L CH₄ kg⁻¹ VS to 90 L CH₄ kg⁻¹ VS.

For low temperatures (*i.e.*, 70 and 90 °C), thermo-chemical pretreatments allowed enhancement of the methane potential compared with thermal pretreatment alone. Indeed, at 70 °C, the biodegradation yield in the presence of Ca(OH)₂ was 62% compared with 2.5% without Ca(OH)₂. Similarly, at 90 °C, thermo-alkaline pretreatment enhanced the level of biodegradation from 28% to 62%. At higher temperatures (120 °C and 150 °C), the addition of Ca(OH)₂ allowed similar methane potentials and biodegradation rates to be obtained, but with a shorter pretreatment period. Indeed, biodegradation yields of 73% and 75% were observed for 150 °C, 1 h, 5% w/v Ca(OH)₂ and 150 °C, 6 h, respectively. High temperature pretreatments (150 °C or 120 °C, with or without addition of Ca(OH)₂) and low temperature pretreatment (90 °C or 70 °C) with the addition of Ca(OH)₂ resulted in non-statistically different ultimate methane production (G(0)) and

biodegradation rate (R_{max}) (**Table IV.4**). In the literature, a contrasting impact of thermo-alkaline pretreatment has been noted for PLA. Battista et al. (2021) reported no effect, while Mu et al. (2021), Hobbs et al. (2019), and Benn and Zitomer (2018) found that there was a positive effect. For instance, Benn and Zitomer, (2018) reported enhancement of the methane potential of PLA from 1 NL $\text{CH}_4 \text{ kg}^{-1}$ COD to 86 NL $\text{CH}_4 \text{ kg}^{-1}$ COD after thermo-alkaline pretreatment (90 °C, pH=10, 48 h). Analogously, Mu et al., (2021) reported an increase in the methane potential from 28.6 L $\text{CH}_4 \text{ kg}^{-1}$ VS to 432 L $\text{CH}_4 \text{ kg}^{-1}$ VS after alkaline pretreatment of PLA at 160 °C for 1 h and 10% NaOH. Hobbs et al. (2019) similarly reported an improvement of 35% after alkaline pretreatment (21 °C, 15 days, pH > 11) of PLA. All these publications are in agreement with our results and suggest that thermo-alkaline pretreatment can be an efficient strategy to improve the PLA methane potential by the application of high temperature (> 120 °C) with a low residence time (1-3 hours) or a low temperature (20 °C-90 °C) for higher residence times (several hours or days).

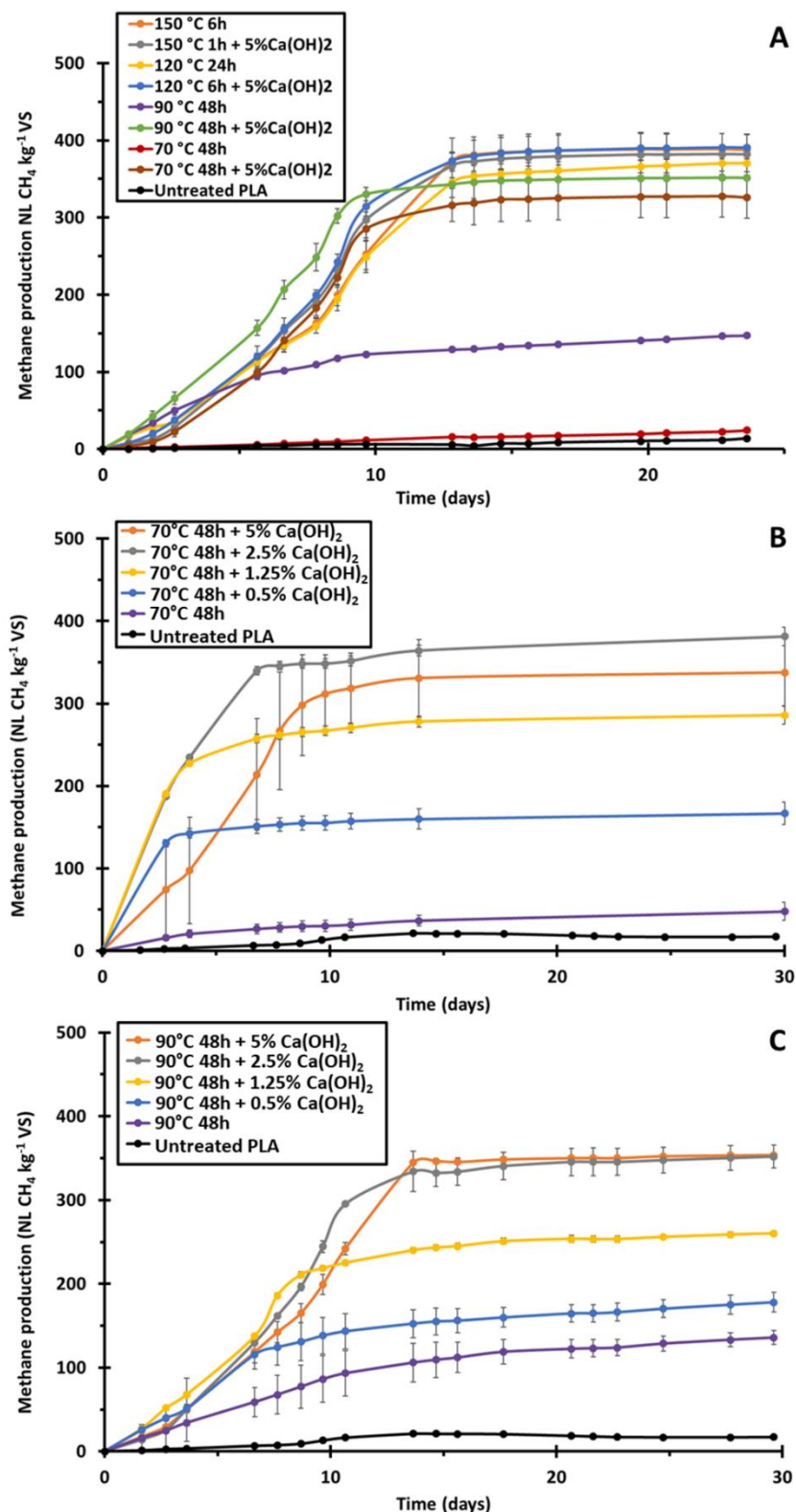


Figure IV.4. The cumulative methane production (L CH₄ kg⁻¹ VS): (A) for untreated PLA, thermal pretreated PLA (150 °C 6 h, 120 °C 24 h, 90 °C 48 h, and 70 °C 48 h) and thermo-alkaline pretreated PLA with 5% Ca(OH)₂ (150 °C 1 h, 120 °C 6 h, 90 °C 48 h, and 70 °C 48 h); (B) Pretreated PLA at 70°C and (C) at 90°C with different amounts of Ca(OH)₂ (0.5, 1.25, 2.5, and 5% w/v). The error bars represent the standard deviations of the replicates.

3.3) Optimization of the Ca(OH)₂ concentration to increase anaerobic digestion

In the last section of this study, the impact of the Ca(OH)₂ concentration was investigated, with concentrations ranging from 0.5% w/v to 5% w/v, as presented in **Figure IV.4**. Temperatures of 70 °C and 90 °C and a duration time of 48 h were applied. The main results obtained are presented in **Table IV.3**. As demonstrated previously, thermo-alkaline pretreatment greatly enhanced PLA solubilization. Interestingly, the PLA solubilization paralleled the increase in the Ca(OH)₂ concentration. Nonetheless, at both 70 °C and 90 °C, the PLA solubilization was relatively close for 2.5% w/v and 5% w/v Ca(OH)₂. Indeed, at 70 °C, the PLA solubilization reached 49% and 56% for 2.5% w/v and 5% w/v Ca(OH)₂, respectively, whereas at 90 °C, the PLA solubilization was 54% and 68% for 2.5% w/v and 5% w/v Ca(OH)₂.

The methane potential profiles are represented in **Figure IV.4**. As was the case previously, PLA granules exhibited a very low methane potential of 21 L CH₄ kg⁻¹ VS after 30 days of incubation. Thermal pretreatment at 70 °C and 90 °C resulted in methane potentials of 48 L CH₄ kg⁻¹ VS and 136 L CH₄ kg⁻¹ VS, respectively, corresponding to biodegradation yields of 9% and 26%, respectively. At both temperatures, the addition of Ca(OH)₂ during the pretreatment enhanced the methane potential and the biodegradation rate, as shown in **Figure IV.4** and **Table IV.4**. The increase in the methane potential was proportional to the concentration of Ca(OH)₂ used during the thermo-chemical pretreatment, and the higher the concentration, the greater the enhancement was of the methane potential. However, at concentrations of 2.5% and 5% w/v Ca(OH)₂, no significant difference in terms of the methane potential was observed for both temperatures. According to these results, and in light of the intention for industrial implementation, the condition of 70 °C with 2.5% w/v Ca(OH)₂ appears to be the most appropriate. Such conditions will be implemented in further studies on a semi-continuous scale in order to validate the batch results and the stability and performance processes.

The results of Gompertz modeling are presented in **Table IV.4**. The high correlation coefficients ($R^2 = 0.94\text{--}0.99$) indicate that the Gompertz model accurately described the cumulative methane yields of pretreated and untreated PLA products. First, a very short lag time was observed for all conditions (< 4 days), demonstrating that the inoculum is well acclimated and able to degrade soluble compounds (mainly lactic acid) from PLA. The methane production rate (R_{max}) was

enhanced at both 70 °C and at 90 °C when Ca(OH)₂ was added, although it was not possible to compare the series at the two temperatures as they were started a different times. For instance, the R_{max} of 9.92 and 40.27 L CH₄ kg⁻¹ VS d⁻¹ were calculated for 90 °C and for 90 °C with 2.5% w/v Ca(OH)₂. Finally, the ultimate methane production (G(0)) clearly indicates the benefits of thermo-alkaline pretreatment instead of thermal pretreatment alone (at 70 and 90°C) and no treatment on PLA granules.

Scanning electron micrographs (SEM), presented in **Figure IV.5**, were also used to investigate the impact of thermo-alkaline pretreatment at 70 °C. The untreated PLA presented a smooth surface, and thermal pretreatment at 70 °C started to create a degree of roughness at the surface. We also observed that thermo-alkaline pretreatment resulted in visible surface erosion, increased porosity, and the presence of numerous holes on the surface. This effect was even more pronounced at higher concentrations of Ca(OH)₂. These structural changes occurring at the surface of the PLA granules can be explained by the breakdown in the polymer chains during the pretreatment step (Ren et al., 2019). These observations are in agreement with the enhancement of PLA solubilization and the methane potential reported in **Figure IV.4** and **Table IV.4**. Indeed, the greater porosity and accessible surface area by pretreatment increased the available binding sites for biological enzymatic and microbial degradation of the polymers, as previously reported (Barakat et al., 2014; Benn and Zitomer, 2018). Finally, a good correlation ($R^2 = 0.90$) was found between the solubilization rate of PLA and its further biodegradation by mesophilic anaerobic digestion (**Figure IV.6**). Mu et al. (2021) have also noted a similar correlation ($R^2 = 0.89$) between enhancement of the methane yield and the PLA solubilization rate after hydrothermal pretreatment of PLA. Thus, it is clear that PLA solubilization is a key parameter for enhancing methane production from PLA. Nonetheless, solubilization is certainly not the only parameter responsible for the improved methane production, and thermo-alkaline pretreatment definitely changes physical parameters such as the molecular weight, crystallinity, accessible surface area, and porosity that are known to be key parameters that affect the biodegradability of plastics by anaerobic digestion (Elsawy et al., 2017; Folino et al., 2020; Mu et al., 2021).

Table IV.3. Summary of the main studies to date that investigated the methane potential of PLA and pretreated PLA.

Nature of the biodegradable plastic	Form	Temperature (°C)	Pretreatment	Time (days)	Methane potential	Biodegradation yield (%)	Methane improvement	Reference
PLA (Ingeo)	Pieces of plastic cups <1 cm ²	35	-	90	0 mL CH ₄ .g ⁻¹ VS	0%	-	Vasmara and Marchetti, 2016
PLA (Unitika)	125-250 µm	37	-	277		29-49%	-	Yagi et al., 2014
PLA (Natureworks)	1-2 mm-wide pellets	37 ± 2	-	20		< 5%	-	Greene, 2018
PLA	20x40 mm film	35	-	100		0%	-	Shin et al., 1997
PLA (Argonne A)	6x5 cm film	35	-	40		10%	-	Day et al., 1994
PLA (Argonne B)	6x5 cm film	35	-	40		15%	-	Day et al., 1994
PLA	Granules	37	-	100		60%	-	Itävaara et al., 2002
PLA (Natureworks, Cargill)	2x2 cm film, 20 µm thickness	35	-	28		0%	-	Massardier-Nageotte et al., 2006
PLA (Biopolymer-4043D, Nature Works)	< 2x2 cm	35 ± 2	-	56		0%	-	Narancic et al., 2018
PLA film	1x1 cm ² film	35	-	65		18.8%	-	Zhang et al., 2018
PLA blend	Pellets	35	-	65		2.6%	-	Zhang et al., 2018
PLA (plastic cup)	2x2x0.5 mm	37 ± 0.5	-	280	564 L biogas kg ⁻¹ VS	66%	-	Bernat et al., 2021
PLA Total Oil Company	10 mesh sieve	37	-	150	57.4 L CH ₄ kg ⁻¹ VS		-	Mu et al., 2021
PLA	PLA items cut	37	-	50	130 L CH ₄ kg ⁻¹ VS	26%	-	Battista et al., 2021
PLA	Pieces of 5x5 cm	37 ± 2	-	60	34 L CH ₄ kg ⁻¹ VS		-	Cucina et al., 2021
PLA NatureWorks 4032D	nd	35	-		24 L CH ₄ kg ⁻¹ VS	8.6%	-	Ren et al., 2019
PLA	Commercial items	38	Steam exposure, 3 h 120 °C	56	90 L CH ₄ kg ⁻¹ VS		*45	Vargas et al., 2009
PLA	Commercial items	37 ± 1	48 h, addition of HCl pH = 2	250	125 L CH ₄ kg ⁻¹ VS		None	Battista et al., 2021
PLA	Commercial items	37 ± 1	48 h, addition of NaOH pH = 12	250	101 L CH ₄ kg ⁻¹ VS		None	Battista et al., 2021
Crystalline PLA	2x2 cm cups	37 ± 1	21 °C, pH > 11, 15 days	70	1.021 L CH ₄		+ 35%	Hobbs et al., 2019

Table IV.4. Summary of the pretreatments applied (condition, pH variation, solubilization) and of the anaerobic digestion test (duration, methane potential, biodegradation and parameters of the Gompertz model) for the various pretreatments. Results are presented as mean values (\pm standard deviation).

	Duration of pretreatment	pH initial	pH final	Solubilisation (%)	Duration of the BMP test	Methane Potential (L CH ₄ kg ⁻¹ VS)	Biod. (%)	G(0) (L CH ₄ kg ⁻¹ VS)	Gompertz model		
									R max (L CH ₄ kg ⁻¹ VS)	λ (day)	R ²
Untreated PLA	-	-	-	-	520	429 (\pm 21)	82% (\pm 4)	415 (\pm 22)	1.54 (\pm 0.06)	43.33 (\pm 2.05)	0.993 (\pm 0.001)
1660 μ m	-	-	-	-	520	427 (\pm 9)	82% (\pm 2)	419 (\pm 10)	1.51 (\pm 0.10)	9.73 (\pm 3.59)	0.996 (\pm 0.001)
1140 μ m	-	-	-	-	520	441 (\pm 10)	84% (\pm 2)	432 (\pm 10)	1.54 (\pm 0.04)	5.76 (\pm 1.06)	0.996 (\pm 0.000)
808 μ m	-	-	-	-	520	441 (\pm 17)	84% (\pm 3)	438 (\pm 17)	1.53 (\pm 0.01)	1.61 (\pm 2.29)	0.997 (\pm 0.000)
502 μ m	-	-	-	-	520	455 (\pm 7)	87% (\pm 1)	455 (\pm 7)	1.62 (\pm 0.05)	-8.25 (\pm 1.41)	0.996 (\pm 0.000)
272 μ m	-	-	-	-	520	460 (\pm 11)	88% (\pm 2)	462 (\pm 11)	1.67 (\pm 0.01)	-16.99 (\pm 1.96)	0.996 (\pm 0.000)
Untreated PLA	-	-	-	-	25	14 (\pm 4)	3% (\pm 1)	15 (\pm 1)	0.50 (\pm 0.01)	0.33 (\pm 0.05)	0.941 (\pm 0.004)
150	6h	6.09	2.19	64 (\pm 2.64)	25	389 (\pm 20)	75% (\pm 4)	405 (\pm 22)	45.28 (\pm 5.40)	3.58 (\pm 0.88)	0.986 (\pm 0.004)
150°C + 5% Ca(OH) ₂	1h	11.51	12.23	72 (\pm 0.38)	25	382 (\pm 27)	73% (\pm 5)	391 (\pm 29)	49.96 (\pm 2.76)	3.52 (\pm 0.06)	0.995 (\pm 0.002)
120	24h	6.57	3.43	65 (\pm 0.05)	25	370 (\pm 11)	71% (\pm 2)	384 (\pm 10)	39.44 (\pm 0.31)	3.11 (\pm 0.46)	0.989 (\pm 0.002)
120°C + 5% Ca(OH) ₂	6h	11.66	12.75	71 (\pm 2.82)	25	391 (\pm 3)	75% (\pm 1)	399 (\pm 1)	50.33 (\pm 4.09)	3.35 (\pm 0.62)	0.993 (\pm 0.003)
90°C	48h	7.5	2.6	7 (\pm 0.15)	25	147 (\pm 0)	28% (\pm 0)	140 (\pm 0)	16.32 (\pm 0.16)	-0.12 (\pm 0.03)	0.990 (\pm 0.000)
90°C + 5% Ca(OH) ₂	48h	12.4	12.02	62 (\pm 0)	25	351 (\pm 25)	67% (\pm 5)	357 (\pm 25)	45.41 (\pm 3.88)	1.60 (\pm 0.63)	0.990 (\pm 0.001)
70°C	48h	7.7	3.49	1 (\pm 0.02)	25	24 (\pm 4)	5% (\pm 1)	26 (\pm 6)	1.27 (\pm 0.10)	1.27 (\pm 0.25)	0.990 (\pm 0.002)
70°C + 5% Ca(OH) ₂	48h	12.67	12.2	60 (\pm 0.75)	25	328 (\pm 27)	63% (\pm 5)	330 (\pm 30)	51.44 (\pm 0.13)	3.84 (\pm 0.27)	0.995 (\pm 0.002)
Untreated PLA	-	-	-	-	30	21 (\pm 17)	4% (\pm 3)	19 (\pm 2)	2.97 (\pm 0.96)	4.38 (\pm 1.93)	0.929 (\pm 0.02)
90°C	48h	7.5	2.6	3 (\pm 0)	30	136 (\pm 8)	26% (\pm 2)	134 (\pm 2)	9.92 (\pm 4.2)	0.42 (\pm 0.59)	0.996 (\pm 0.00)
90°C + 5% Ca(OH) ₂	48h	13.12	12.01	68 (\pm 2)	30	354 (\pm 1)	68% (\pm 0)	364 (\pm 3)	37.48 (\pm 8.06)	3.47 (\pm 1.6)	0.986 (\pm 0.01)
90°C + 2.5% Ca(OH) ₂	48h	13.18	11.97	54 (\pm 1)	30	352 (\pm 14)	67% (\pm 3)	353 (\pm 18)	40.27 (\pm 0.52)	3.23 (\pm 0.14)	0.993 (\pm 0.00)
90°C + 1.25% Ca(OH) ₂	48h	13.18	10.8	36 (\pm 0)	30	260 (\pm 3)	50% (\pm 1)	256 (\pm 4)	30.23 (\pm 0.66)	1.29 (\pm 0.18)	0.994 (\pm 0.00)
90°C + 0.5% Ca(OH) ₂	48h	13.1	4.67	21 (\pm 1)	30	178 (\pm 11)	34% (\pm 2)	168 (\pm 10)	18.67 (\pm 5.46)	0.56 (\pm 0.43)	0.991 (\pm 0.00)
Untreated PLA	-	-	-	-	30	21 (\pm 17)	4% (\pm 3)	19 (\pm 2)	2.97 (\pm 0.96)	4.38 (\pm 1.93)	0.929 (\pm 0.02)
70°C	48h	7.7	3.49	1 (\pm 0)	30	48 (\pm 4)	9% (\pm 1)	46 (\pm 4)	2.92 (\pm 0.30)	-1.9 (\pm 0.60)	0.941 (\pm 0.02)
70°C + 5% Ca(OH) ₂	48h	13.12	12.3	56 (\pm 3)	30	338 (\pm 41)	65% (\pm 8)	343 (\pm 41)	49.49 (\pm 4.89)	1.84 (\pm 1.29)	0.993 (\pm 0.00)
70°C + 2.5% Ca(OH) ₂	48h	13.18	12.29	49 (\pm 6)	30	381 (\pm 11)	73% (\pm 2)	363 (\pm 8)	74.65 (\pm 1.79)	0.41 (\pm 0.03)	0.993 (\pm 0.00)
70°C + 1.25% Ca(OH) ₂	48h	13.18	11.9	35 (\pm 2)	30	286 (\pm 14)	55% (\pm 3)	270 (\pm 10)	83.12 (\pm 0.43)	0.42 (\pm 0.02)	0.992 (\pm 0.00)
70°C + 0.5% Ca(OH) ₂	48h	13.1	11.2	17 (\pm 1)	30	167 (\pm 11)	32% (\pm 2)	157 (\pm 7)	63.41 (\pm 0.28)	0.42 (\pm 0.01)	0.998 (\pm 0.00)

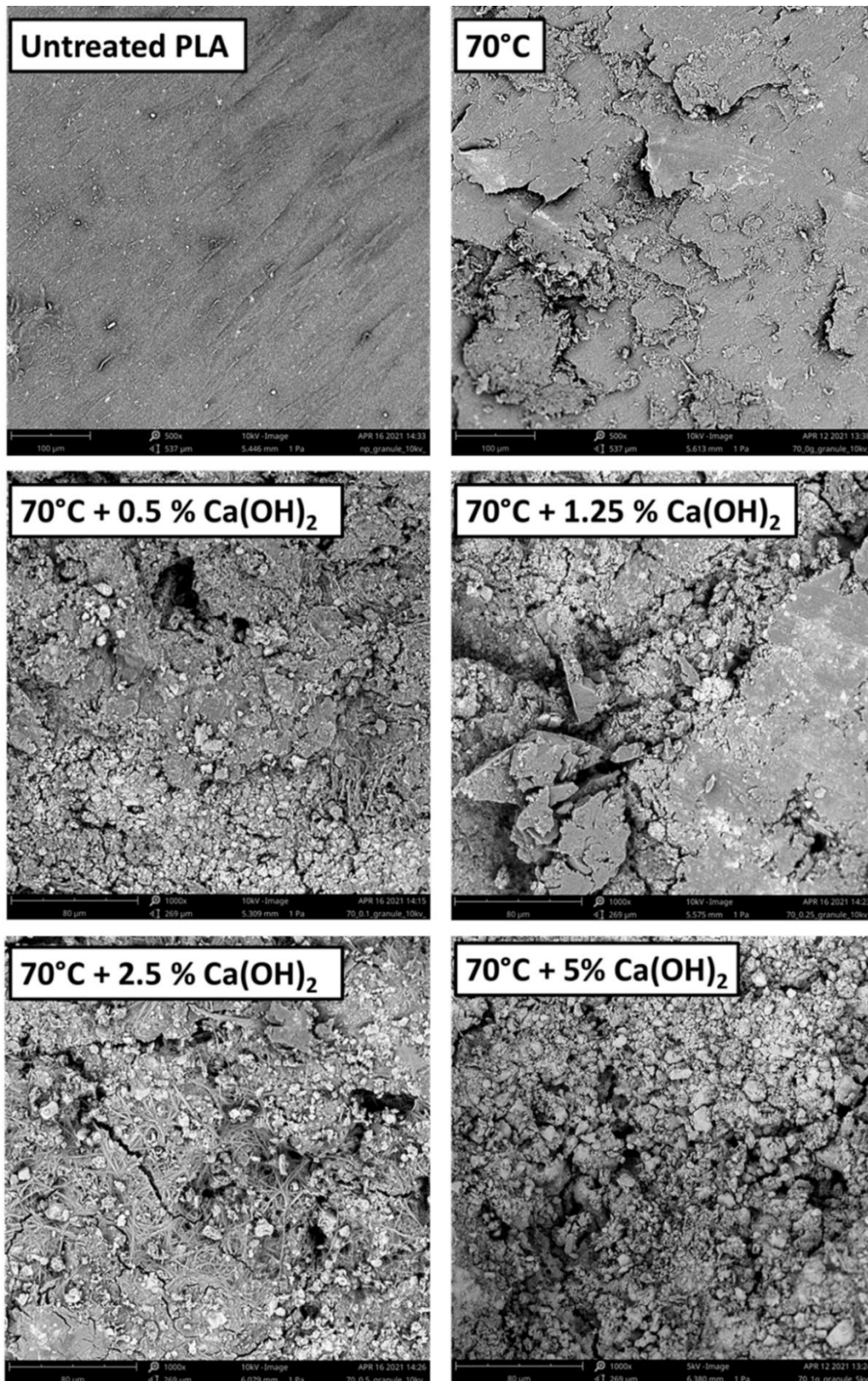


Figure IV.5. Scanning electron micrographs at 500x magnification for untreated PLA and PLA pretreated at 70 °C without Ca(OH)_2 and at x1000 for PLA pretreated at 70 °C with different concentrations of Ca(OH)_2 .

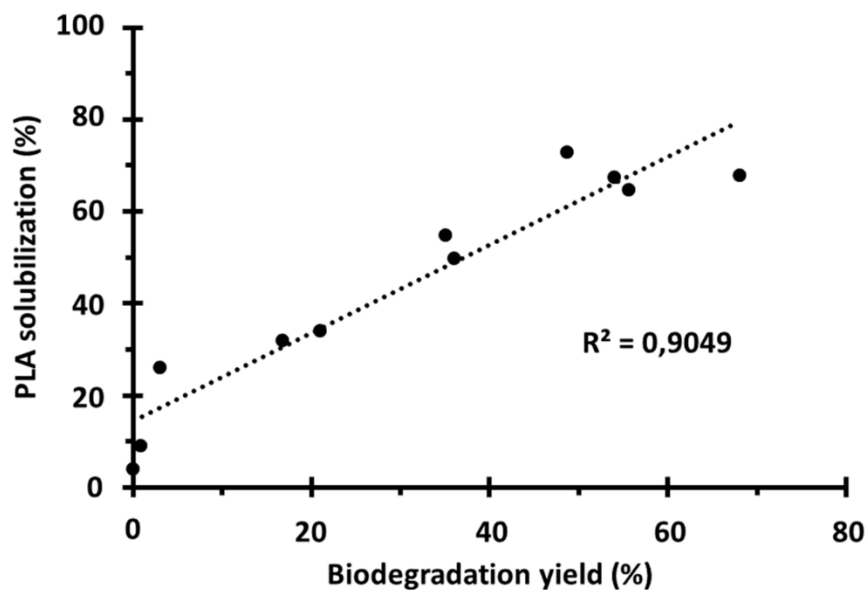


Figure IV.6. Correlation between biodegradation yields (%) after 25-30 days of digestion and the PLA solubilization (%) obtained after pretreatment.

4) Conclusion

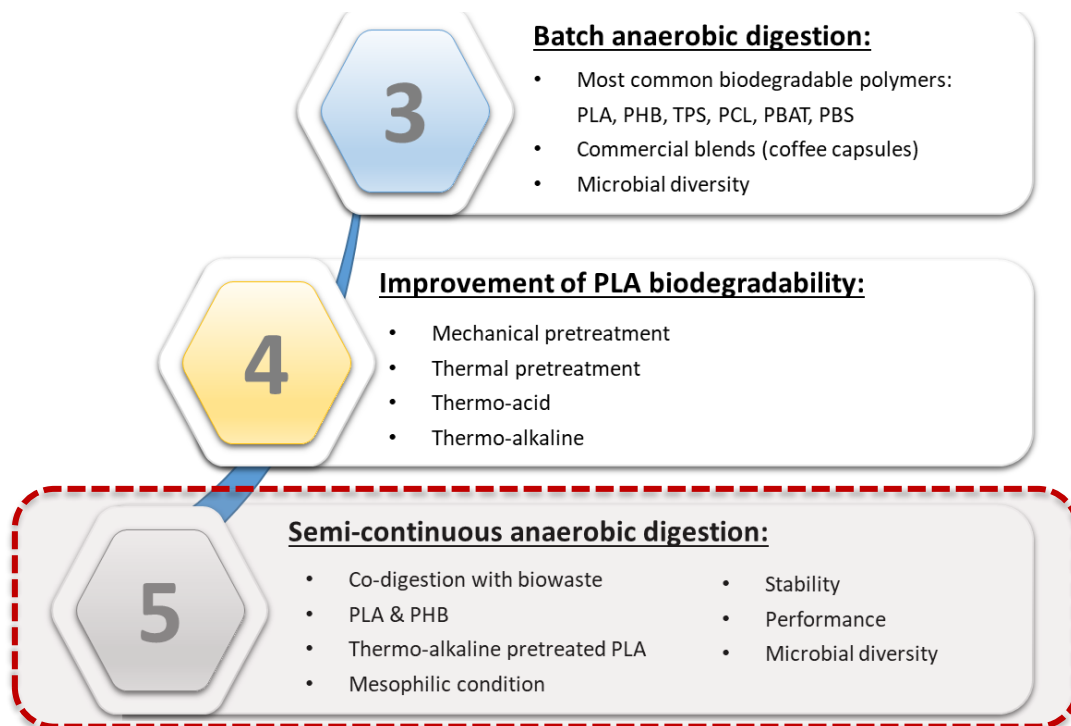
The impact of various pretreatments (mechanical, thermal, thermo-chemical) was investigated in this study in order to improve the efficacy of mesophilic anaerobic biodegradation. Mechanical pretreatment affected the methane production rate but led to a similar final methane potential. However, biodegradation remained insufficient for implementation at an industrial scale and fit with the HRTs of industrial biogas plants. In parallel, thermo-alkaline pretreatment was found to improve PLA solubilization and it increased the methane potential. The most suitable condition of pretreatment was at 70 °C for 48 h in the presence of 2.5% w/v $\text{Ca}(\text{OH})_2$, resulting in a methane potential of 381 L CH_4 kg^{-1} VS and a biodegradation yield of 73% in 30 days. These results should be confirmed by semi-continuous pilot-scale testing in order to validate the stability, the performances, and digestate quality.

CHAPTER V

Semi-continuous anaerobic digestion

This chapter is based on a modified version of a scientific paper in preparation:

Cazaudehore, G., Guyoneaud, R., Cauzi, P., Gassie, C., Lallement, A., Monlau, F., 2022. Simulation of the co-digestion of biowastes and biodegradable plastics in semi-continuous reactors: performances and microbial communities. (in preparation).



Foreword

As previously highlighted, Poly (lactic acid) (PLA) and Poly (hydroxybutyrate) (PHB) are among the most promising polymer to substitute to conventional plastics (Boey et al., 2021; Naser et al., 2021). Unfortunately, only PHB achieve high biodegradation (between 57 and 80.3%) within a relatively short period (25-50 days) under both mesophilic and thermophilic anaerobic digestion conditions. By contrast, PLA has a very slow biodegradation rate under mesophilic conditions. However, it has been shown in the previous chapter, that it was possible to significantly enhance the methane production rate from PLA under mesophilic condition by applying thermal or thermo-alkaline pretreatment. Thermal pretreatments only achieved substantial solubilization (> 60%) at higher temperatures (120 and 150 °C). At lower temperatures (70 and 90 °C), negligible solubilization (between 1 and 6%) occurred after 48 h. By contrast, coupling of thermal and alkaline pretreatment significantly increased solubilization at the lower temperatures (70 and 90 °C). The best pretreatment to reduce the concentration of Ca(OH)_2 and the temperature used, while maintaining high methane production kinetics was achieved at 70 °C using 2.5% w/v Ca(OH)_2 for 48 h. This pretreatment resulted in a PLA biodegradation yield of 73% after 15 days in comparison to 4% in the same time for untreated PLA.

The selective biowastes collection by 2024 in France will generate new organic flows for anaerobic digestion. These biowaste flows will probably be mixed in the future with biodegradable plastics and for this purpose, it is important to generate scientific knowledge on the fate of these biodegradable plastics in co-digestion with biowastes in the anaerobic digestion process. To anticipate the overall impact and methane yields of such a diverse range of substrates on large-scale, continuous-flow laboratory-scale experimental methods have been developed (Labatut et al., 2011). Semi-continuous laboratory pilots are designed to emulate the conditions of commercial-scale digesters and studied their overall performance over time (Labatut et al., 2011). All the results presented earlier in this work were obtained from BMP experiments, which may not provide a true picture of the performance of full-scale, especially about the stability of the process over long-term (Sambusiti et al., 2013a).

That is the reason why the chapter 5 is dedicated to the investigation of the biological stability and performances of the co-digestion of the two selected biodegradable plastics (PLA and PHB) with food wastes fed semi-continuously under mesophilic conditions. The mesophilic condition was selected for this study as mesophilic reactors are more widespread than thermophilic reactors (Kasinski, 2020; Van et al., 2019). Moreover, the PLA is introduced in two forms: as powder (1mm) or as thermo-alkaline pretreated granule (70°C, 48h with 2.5% w/v Ca(OH)₂) to assess the impact of the pretreatment on the stability of the process and its performances. Finally, the active microbial diversity of the different reactors is also described in order to better understand which microbial communities are active in the biodegradation of biodegradable plastics.

Abstract:

The development of the selective biowastes collection in most European countries opens new perspectives for the anaerobic digestion sector. In parallel, a strong development of biodegradable plastics supports (*e.g.*, bags, food packaging) in replacement of conventional plastics is observed in the last decades. Up to date, data available on biodegradability of biodegradable supports has been mainly provided in batch test, which may fail in truly predicting full-scale anaerobic reactors performance. For this purpose, this study has investigated the anaerobic co-digestion in semi-continuous reactors of biowastes (75% VS) and biodegradable plastics (25% VS, pretreated or untreated poly(lactic acid) and poly(hydroxybutyrate)). The addition of biodegradable plastics resulted in a more stable process (in comparison with the reactor only fed with biowaste) and no negative effects could be detected. PHB was estimated to be fully biodegraded in the reactors. By contrast, PLA was accumulating in the reactor, and an average biodegradation of $47.6 \pm 17.9\%$ was estimated during the third HRT. Pretreatment of PLA, by thermo-alkaline hydrolysis at 70 °C, with 2.5 w/v of Ca(OH)₂ during 48 h, improved the biodegradation yield of PLA to $77.5 \pm 9.3\%$. The identification of specific microorganisms specifically implicated in the biodegradable plastic degradation was complicated since the majority of the observed microorganisms were implicated in the AD of the biowaste, due to the low proportion of biodegradable plastics introduced. Finally, all the digestates obtained for the various conditions demonstrated to have agronomic and sanitary parameters in adequation with the French Regulation. Alkaline pretreatment can thus represent a promising method allowing a significant improvement of the PLA degradation even if its industrial integration must be thought out in the future.

1) Introduction

The development of source separation of biowastes is a good opportunity for the development of the anaerobic digestion industry by the diversification of their feedstocks. The sorting at source of biowaste will be mandatory from 2024 for all in Europe, as provided by the European directive on waste even if it is already operational in some European countries (*Directive (EU) 2018/851*, 2018; European Environment Agency, 2020). Up to now, in France, only large producers were concerned. However, the anti-waste law, by transposing the directive, extends it to all producers. The use of biodegradable plastic collection bags could be a useful tool for achieving higher food waste collection, especially at a household level, as householders perceive plastic bags as convenient and hygienically safe (Dolci et al., 2021; European Bioplastics, 2016; Kern et al., 2018). Currently, the treatment in anaerobic digestion (AD) requires the removal of the biodegradable plastic bags before the digestion process, especially when wet or semi-dry technologies are employed, because they cannot be separated from conventional plastic bags, their management can cause hydraulic problems in the plant (Dolci et al., 2021). Moreover, the biodegradation time of biodegradable plastics bags are generally much higher than biowastes (Bátori et al., 2018b). Nonetheless, such approach can lead the loss of a non-negligible amount of organic waste contained in the bags and therefore to a loss of profitability of the biogas plant (Dolci et al., 2021). Ideally, the use of products made of biodegradable plastic capable of being digested in the same period as other biowaste would be a great advantage allowing to avoid costly deconditioning steps and to recover more biowaste.

Polyhydroxybutyrate (PHB) and poly(lactic acid) (PLA) are among the most studied polymers to replace petroleum-derived plastics (Boey et al., 2021; Naser et al., 2021). PLA represent approximately 25% of the biodegradable plastic production in 2018 (European Bioplastics, 2019). The biodegradation rate of the PLA is very low under mesophilic AD (i.e., 35-40 °C), to the point that very long digestion period (280 days) were required to reach a biodegradation level between 29 and 66% (Bernat et al., 2021; Yagi et al., 2014b). Anaerobic digestion of PLA under thermophilic conditions (i.e., 55-60 °C) was much faster than under mesophilic conditions resulting in high conversion to methane (between 82 and 90%) in a mean digestion time of 90 days (Hegde et al., 2018; Narancic et al., 2018; Šmejkalová et al., 2016; Yagi et al., 2013c, 2009). However, it is possible to improve the biodegradation kinetics of the PLA by applying thermal or thermo-alkaline

pretreatments (Benn and Zitomer, 2018; Hobbs et al., 2019; Vargas et al., 2009). Hobbs et al., (2019) observed a near complete solubilization (97-99%) of two PLA during an alkaline pretreatment (21°C, pH > 11, 15 days). On the other hand, PHB represents a relatively small portion of the biodegradable plastics market (approximately 2% of the biodegradable plastic production in 2019), but the production capacities are expected to more than triple in the next five years due to their better biodegradability capacity (European Bioplastics, 2020). Indeed, PHB has ample potential for being rapidly converted into methane under either mesophilic (Abou-Zeid et al., 2004; Benn and Zitomer, 2018; Narancic et al., 2018) or thermophilic conditions (Itävaara et al., 2002; Narancic et al., 2018; Yagi et al., 2013c).

Most of the studies investigating the anaerobic digestion of the biodegradable plastics were performed in batch mode at a laboratory scale generally without co-digestion process (Greene, 2018b; Narancic et al., 2018; Yagi et al., 2014b, 2013c). However, experiments at a pilot scale performing co-digestion with a semi-continuous or continuous feeding are more representative of the full-scale process, giving a better appreciation of the biodegradation kinetics, the interaction between the co-substrate and on the process stability over a long period (Lavergne et al., 2018). Another important parameter when considering biodegradable plastics is their co-digestion with other wastes and in particular biowaste or food wastes. The low carbon to nitrogen (C/N) ratio of biowastes is a challenge to anaerobic digestion, but co-digestion with higher C/N biodegradable plastics can efficiently improve the biogas plant stability and biogas production in the system (Benn and Zitomer, 2018). Moreover, the addition of biodegradable plastics can help reducing the proportion of highly biodegradable matter that can lead to acidification (Abraham et al., 2021). Indeed, Tonanzi et al. (2020) reported that food wastes mono-digestion showed progressive system instability caused by the inhibition of methanogens, which resulted in volatile fatty acid accumulation and process failure even at low organic loading rate (OLR). Up to date, little experiments were reported in the literature on the anaerobic digestion or co-digestion of biodegradable plastics (Benn and Zitomer, 2018; Dolci et al., 2022; Venkiteshwaran et al., 2019; Wang et al., 2012; Zhang et al., 2018). Recently, Dolci et al. (2022) and Kern et al. (2018) evaluated the anaerobic digestion of Mater-bi® biodegradable bags at bench scale and at full scale, respectively. Dolci et al. (2022) performed semi-continuous co-digestion experiment with synthetic food waste under thermophilic condition (50 °C). Mater-bi® bags exhibited a relatively

low biodegradation (*i.e.*, 27% based on Carbon Oxygen Demand), using a 20 days hydrolytic retention time (HRT). Kern et al., (2018) investigated the biodegradation of Mater-Bi® biodegradable bags in four anaerobic digestion plants in Germany: a batch thermophilic and mesophilic plant, and two plug flow thermophilic plants. On one hand, 21 days of thermophilic anaerobic digestion resulted in a reduction of the presence of the compostable bags from 39% to 50%. On the other hand, 21 days of mesophilic anaerobic digestion resulted in no reduction of the presence of the Mater-bi® bags. A composting step was employed on the digestate, after an active composting phase (8 days) and a maturation phase (35 days) of the compost, full biodegradation of the bags was observed.

Due to the low biodegradability of most of biodegradable plastics under the classical HRT of biogas plant treating biowastes (generally around 30 days), several pretreatments strategies can be carried out in order to boost the degradation of biodegradable plastics. Up to date, pretreatment on biodegradable plastics has mainly focus on mechanical, thermal and thermos-chemical (Battista et al., 2021; Benn and Zitomer, 2018; Hobbs et al., 2019; Vargas et al., 2009). The impact of pretreatment in enhancing methane production of biodegradable plastics has been documented in batch tests but less information are available on semi-continuous pilot scale (Benn and Zitomer, 2018). For instance, Benn and Zitomer, (2018) and Venkiteshwaran et al. (2019) performed co-digestion of two pretreated and untreated PHBs with synthetic municipal primary sludge under mesophilic conditions. Untreated PHB was biodegraded to 79-93%, while pretreatment consisting of thermo-alkaline hydrolysis (pH 12, 55 °C for 24 or 48 h) resulted in a 5% increase in the methane conversion of the PHB.

Besides the performances and stability of the biodegradable plastics digestion, it is crucial to determine the impact of the plastic addition on the microbial communities and to identify the microorganisms implicated in their biodegradation. A relatively low amount of work investigating the microorganisms involved in the AD process of biodegradable plastics have been published and most of them has been obtained from batch tests (Bandini et al., 2020; Cazaudehore et al., 2021; Peng et al., 2022; Tseng et al., 2019; Venkiteshwaran et al., 2019). Venkiteshwaran et al. (2019) explored the microbial ecology during continuous co-digestion of PHB and biowaste but the analysis did not bring to light previously known PHB degraders. Recently, Tseng et al. (2020, 2019) and Cazaudehore et al. (2021) highlighted the importance of lactate utilizing bacteria from the

genus *Tepidimicrobium* during the thermophilic AD of PLA and of PLA blends, respectively. A better understanding of these microorganisms is however a key parameter that could lead to the development of selection or bioaugmentation strategies capable of improving the conversion of certain biodegradable plastics into methane.

In view of the scientific literature, there is currently a lack of information on co-digestion of biodegradable plastics with biowaste. The progressive implementation of selective biowaste collection in several European countries will generate new sources of biomass for the anaerobic digestion sector. For this purpose, it is important to collect more information on the stability and performance of biowaste anaerobic digestion with biodegradable plastics carried out in semi-continuous scale, which are more representative of future industrial biogas plants than batch tests. For this purpose, this study intend to investigate the stability and performance of the co-digestion of two selected biodegradables plastics (PLA from NaturePlast and PHB from KD Feddersen) with biowaste fed semi-continuously under mesophilic conditions. In parallel, the impact of a thermo-alkaline pretreatment on PLA in order to improve it biodegradation rate was also investigated. Finally, the microbial ecology of the different reactors were described using amplicon sequencing of the 16S rRNA transcripts.

2) Materials and Methods

2.1) Samples preparation and characterization

PHB (KD Feddersen, Paris, France) and PLA (SF 141, Natureplast®, IFS, France) pellets were purchased on the market. Biodegradable plastic samples were ground at a sieve of 1mm using a centrifugal mill (ZM 100, Retsch, Haan, Germany) after a short immersion in liquid nitrogen. Unground PLA pellets were used for PLA thermo-alkaline pretreatment. Briefly, 50 g TS L⁻¹ of PLA pellets, 2.5% w/v calcium hydroxide and distilled water were introduced into 35 mL Pyrex glass tubes heated in a heat system with magnetic agitation (Hei-Tec, Heidolph Instruments, Schwabach, Germany) at 70°C for 48 hours. The biowastes were collected at the entrance of a local composting plant (Suez Organique, Soumoulou, France) before being mixed with compost. The biowastes consisted of fruits, vegetables and meats from food services. An homogenized biowaste soup was obtained by a successive passage in a plant shredder (GHE 355, Stihl®, Waiblingen, Germany) and in a meat grinder (IE12 Tech Eco, EMYG, Carnoux-en-Provence, France) with a final plates size (6

mm). The soup of biowaste was aliquoted in 2 L container and stored at -20 °C to avoid biological modification during the storage period.

Total solids (TS) and volatile solids (VS) of the different substrates were determined using APHA standard methods (American Public Health Association., 2005). Analysis of the elemental composition (determination of the carbon, hydrogen, nitrogen and sulphur content) was performed using an Elemental Vario Macro Cube analyser (Elementar, Langenselbold, Germany). Oxygen content was calculated by difference between the VS content and the sum of the carbon, hydrogen nitrogen, and sulphur content. The theoretical methane production (BMP_{th}) was calculated from the elemental composition using the Eq.1 (Achinas and Euverink, 2016a; Boyle, 1977; Buswell and Mueller, 1952). **Table V.1** summarize the characterization of the inocula and samples used.

$$\text{Eq.1: BMP th (L CH}_4\text{. g}^{-1}\text{ C}_x\text{H}_y\text{O}_z\text{N}_n\text{S}_s) = \frac{22.4 \times \left(\frac{x}{2} + \frac{y}{8} - \frac{z}{4} - \frac{3n}{8} - \frac{s}{4} \right)}{12x + y + 16z + 14n + 32s}$$

Total Kjeldahl Nitrogen (TKN) of the digestates was determined according to the Kjeldahl method (Kjeldahl, 1883), by using a mineralizator (BUCHI digestion unit K 438) and a BUCHI 370-K distillator/titrator. Moreover, N-NH₄⁺ content of the liquid digestate was determined by the titrimetric method after distillation using a BUCHI 370-K distillatory (Rodier, 1975).

Other nutrients content (P, K, Mg, S, Ca and Na) were determined by the Inductively Coupled Plasma Mass Spectrometry (ThermoFisher Scientific, XSeries 2 ICP-MS) equipped with a cooled spray chamber, a quadruple mass spectrometer and a collision cell. The ICP-MS settings were as follows: Nebulizer flow of 0.82 L min⁻¹, auxiliary flow of 0.80 L min⁻¹, cool flow of 13 L min⁻¹, forward power 1400 Watts, cell gas flow He/ H of 0.0045 L min⁻¹. For this purpose, a microwave-assisted mineralization of the digestate was performed after the addition of nitric acid (65%) and hydrogen peroxide (30%). The reaction was conducted for 30 min at room temperature, and then the mixtures were placed in the microwave reactor (Flexiwave, Milestone, USA) and heated for 20 min to reach 210 °C, which was maintained for 20 min and then cooled for 25 min. The obtained solutions were filtered using 0.2 µm filters then analyzed with ICP-MS.

The metal content in the digestate was determined with an Agilent 7900 ICPMS (Agilent, Tokyo, Japan) following acid digestion in a Milestone UltraWAVE digestion system (Milestone Srl, Sorisole, Italy). 5 mL of HNO₃ (Baker, Deventer, Netherlands) were added to 300 mg of sample. After digestion the volume was made up to 15 mL with ultrapure water. Samples were further diluted 10, 100 or 1000 times with HNO₃ 2 % (v/v) before analysis. Digestions were made in triplicate. Blanks were run in parallel with the samples.

2.2) Semi-continuous anaerobic digesters

Five liters' anaerobic digesters with 3L working volume were operated for 19 weeks. Digesters were continuously stirred-tank reactors (CSTRs), mixed at 350 rpm using a magnetic stir bar and heated to 38 °C by hot water circulation in a double casing. Reactors were inoculated using digestate from a mesophilic digester treating agricultural wastes (Méthalayou, Préchacq-Navarrenx, France) sieved to 2mm in order to reduce the residual undegraded organic fraction. Feeding was semi-continuous (five alimentation per weeks), the TS solid content of the ration was equilibrated with water at 8%. The organic loading rate (OLR) was increased gradually in the first three weeks of the operation, starting from 1.5 g VS L⁻¹ d⁻¹ to 2 g VS L⁻¹ d⁻¹ and finally to 2.5 g VS L⁻¹ d⁻¹. The reactors were operated in two stages. During the first stage, a reactor was fed with biowaste; another with a mixture of PHB powder (25% VS) and biowaste (75% VS); the last with untreated PLA powder (25% VS) and biowaste (75% VS). Control reactor fed only with biowaste showed sign of overload after the week 11, therefore the organic loading rate was decreased to 2 g VS L⁻¹ d⁻¹. During the second stage, another control reactor was operated with only biowastes, this time the OLR was maintained at 2.5 g VS L⁻¹ d⁻¹ despite process imbalance. Due to instability, several purges were performed during the trial: half of the volume of the reactor was removed at the beginning of the week 12 and replaced with fresh inoculum, all of the reactor volume was emptied at the beginning of week 15 and replaced with fresh inoculum. Additionally a reactor fed with a mix of thermo-alkaline pretreated PLA pellets (25% VS) and biowastes (75% VS) was run. The HRTs were 30 and 38 days for reactors fed at an OLR of 2.5 and 2.0 g VS L⁻¹ d⁻¹, respectively. Biodegradable plastic accounted for 25% of the intake VS, in term of raw weight the plastic biowaste ratio was 7:93. Kern et al., (2018) performed full scale anaerobic digestion experiments on municipal organics collected in the town of Braunschweig (Germany). Biodegradable plastics bags were provided to the households and finally biodegradable plastics represented between 3.5

and 3.8% (by weight) of the waste collected. The proportion of biodegradable plastic used in this study is higher (7% of the fresh weight) in order to facilitate the observation of the plastic addition effect. Moreover, the proportion of biodegradable plastic can be higher in other applications such as coffee capsules.

Biogas production was continuously measured using MilliGascounter (MGC-1 PMMA, Ritter, Bochum, Germany). Once a day, biogas was sampled using a gas sampling bag (Kynar PVDF Bags, Cole-Parmer®, Vernon Hills, USA) and the biogas composition was determined using a gas chromatography (Micro GC 490, Agilent, Santa Clara, USA), as described elsewhere (Cazaudehore et al., 2021b). Once a week, TS and VS of the digestates were measured according to APHA standard methods (American Public Health Association, 2005). The pH and redox potential was measured each day on the digestate using a 340i pH meter fitted with a Sentix® electrode (Xylem Analytics, Weilheim, Germany) and a blue line electrode (32RX, Xylem Analytics, Weilheim, Germany) on a ProfiLine Multi 1970i (Xylem Analytics, Weilheim, Germany), respectively. The following parameters were analysed three times a week (on Monday, Wednesday and Friday): volatile fatty acids (VFAs) concentration, FOS/TAC ratio, ammoniacal nitrogen concentration. The concentration of the different VFAs was determined by gas chromatography (7890B, Agilent, USA) (Monlau et al., 2021). The FOS/TAC ratio was determined by titration using sulfuric acid (0.1N) (Lallement et al., 2021). Ammoniacal nitrogen content was obtained using Spectroquant cells (Spectroquant cells, Sigma Aldrich, USA) and was measured by a photometer (PhotoLab S6 WTW, Xylem Analytics, Weilheim, Germany).

The biodegradation of the mix was calculated using the methane production as described in Eq.2. The biodegradation of the plastic was estimated by assuming that the biowastes were similarly converted into methane in the reactor fed only with biowaste and in co-digestion reactors. The biodegradation level of the biowaste in the reactor fed only with biowaste in phase 1 (OLR of 2 g VS L⁻¹ d⁻¹) was taken as reference since it showed stable performances.

$$\text{Eq.2: Biodegradation of the mix} = \frac{\text{Observed methane production}}{m(\text{Biowaste}) \times \text{BMPth}(\text{Biowaste}) + m(\text{Plastic}) \times \text{BMPth}(\text{plastic})}$$

2.1) Microbial analysis:

Samples of digestate were taken, in triplicate, at the beginning of the experiment (inoculum) and at different moment during the third hydraulic retention time. The specific days are indicated in the **figure V.1**. The methods used for sampling, extraction and amplification of the 16S rRNA transcripts were previously described by Cazaudehore et al. (2021) using the universal V4 V5 primers (515F and 928R, Wang and Qian, 2009). Amplicons were sequenced by the PGTB service (Bordeaux, France) using Illumina MiSeq 250 bp paired-end technology.

Treatment of the sequencing data was performed on Galaxy FROGS pipeline (Afgan et al., 2018) according to the method described by Escudié et al. (2018). Sequences were merged, denoised and dereplicated using the pre-processing tools of the system. Operational Taxonomic Units (OTU) were produced based on a three base aggregation distance. Minor OTUs, consisting of less than 0.0005% of the total sequences, and chimera OTUs were withdrawn. Finally, the taxonomic assignments was produced using the Silva database v128.1 (Pruesse et al., 2007). The number of sequences per samples were normalized to 14,743 sequences.

Statistical analysis were carried out on R version 3.6.2. After an Hellinger transformation of the OTU table, the relationship between environmental variables and the microbial composition of the different samples was explored using redundancy analysis (RDA) (Paliy and Shankar, 2016; Ramette, 2007; Wang et al., 2012). Multivariate regression analysis (function lm and anova) was realised to select the OTUs that are significantly correlated with the variation of the methane production from the different substrate or co-substrate (p -value < 0.05) and to determine the direction (positively or negatively) and magnitude of the correlation (Wang et al., 2012). Finally, heatmaps representing the top 15 positively correlated OTUs with the methane production of each substrate or co-substrate were generated using heatmap.2 package. OTUs were reordered on the heatmaps to match with the phylogenetic tree made using ggtree package.

3) Results and discussions

3.1) Stability of the CSTR reactors

In **Table V.1**, the main physico-chemical properties of the various biomasses used (*i.e.*, biowastes and biodegradable plastics) are presented. Biodegradable plastics are characterized by high carbon

content and very low nitrogen content. Theoretical methane potential (based on Buswell equation) of 522 and 552 L CH₄ kg VS⁻¹ were respectively calculated for PLA and PHB samples. The biowastes content was characterized by a higher N content and thus a lower C/N content of 13.4. As concerns biowastes, a theoretical methane potential of 532 L CH₄ kg VS⁻¹ was determined.

Table V.1: Properties of the inocula and samples. Values are expressed as average (\pm standard deviation)

Parameters	Unit	Inoculum stage 1	Inoculum stage 2	PLA Natureplast SF 141	PHB K.D. Feddersen	Biowaste
pH	-	8.2	8.0	-	-	-
Redox	mV	-310	-358	-	-	-
Ammonia	g N-NH ₄ L ⁻¹	1.9	2.0	-	-	-
FOS/TAC	-	0.12	0.15	-	-	-
VFAs	g _{eq acetate} L ⁻¹	0.05	0.06	-	-	-
TS	% raw mass	6.2 (\pm 0.4)	4.7 (\pm 0.0)	99.8 (\pm 0.00)	99.6 (\pm 0.01)	22.26 (\pm 0.5)
VS	% raw mass	3.7 (\pm 0.1)	2.7 (\pm 0.2)	99.6 (\pm 0.01)	97.7 (\pm 0.01)	20.90 (\pm 1.1)
Carbon	% TS	-	-	51.80 (\pm 0.17)	53.19 (\pm 0.22)	47.72 (\pm 0.95)
Hydrogen	% TS	-	-	6.05 (\pm 0.01)	6.95 (\pm 0.03)	7.11 (\pm 0.27)
Nitrogen	% TS	-	-	0.00 (\pm 0.00)	0.22 (\pm 0.02)	3.56 (\pm 0.21)
Sulfur	% TS	-	-	0.00 (\pm 0.00)	0.10 (\pm 0.03)	0.26 (\pm 0.05)
Oxygen*	% TS	-	-	40.19	39.45	35.23
C/N				-	241	13.4
Theoretical methane potential	L CH ₄ kg VS ⁻¹	-	-	522	552	532

*Calculated O = 100 - C - H - N - S - Ash

All the reactors were operated for more than 3 HRTs (130 days) to determine the stability of the process. During the overall period, several biological parameters (VFAs, pH, ammonium, FOS/TAC) has been monitored and reported weekly. All these data are reported in **Figure V.1** and **Figure V.2**. The average stability indicator values for the third HRTs are represented in **Table V.2**. The digester fed with biowaste at the second stage was not presented because the reactor volume was renewed before the third HRT.

Instabilities, manifesting in the form of an accumulation of VFAs and ammonium, was observed in the reactors fed with biowastes alone at an OLR of $2.5 \text{ g VS L}^{-1} \text{ d}^{-1}$ (**Figure V.1A, 1D and V.2C**). In the case of the reactor in stage 1, which was fed at that OLR between week 3 and 11, the instability of the process was also associated with a perturbation of the acetate/VFAs ratio, suggesting the accumulation of longer chain VFAs (Dasa et al., 2016). The OLR was reduced to $2 \text{ g VS L}^{-1} \text{ d}^{-1}$ from the week 11. That reduction was followed by the consumption of the VFAs, and to low VFAs level afterwards. The reduction of the OLR occurred before the pH drop due to VFAs accumulation, pH was stable during the accumulation phase to 7.6 ± 0.1 (**Figure V.3A**). On the contrary, during phase 2, the OLR was maintained during all the assay at $2.5 \text{ g VS L}^{-1} \text{ d}^{-1}$ to ensure that the instability observed during phase 1, did lead to a failure of the reactor if the loading rate had not been decreased. In this situation, VFAs concentration raised to 21 g L^{-1} and led to a pH drop to 6.6 and a decrease in methane production close to zero (**Figure V.3A**). Half and finally the entire volume of the reactor was replaced with fresh inoculum in week 12 and 15, respectively, in an attempt to remove the inhibition and get stability, but VFAs continued to accumulate (**Figure V.1D**). In view of the results on biowastes mono-digestion, the OLR of $2.5 \text{ g VS L}^{-1} \text{ d}^{-1}$ is not suitable as it lead to instabilities, accumulation of NH_4^+ and VFAs and further failure of the reactor (Banks et al., 2012; Tonanzi et al., 2020; Xu et al., 2018). Similarly, Tonanzi et al., (2020) reported that food wastes mono-digestion showed progressive system instability caused by the inhibition of methanogens, which resulted in VFAs accumulation and process failure even at low OLR. Indeed, Tonanzi et al., (2020) have observed that mesophilic mono-digestion of food wastes at OLR of $0.8 \text{ g VS L}^{-1} \text{ d}^{-1}$ and $1.7 \text{ g VS L}^{-1} \text{ d}^{-1}$ led to progressive system instability and acidification with VFAs accumulation to concentration up to 11 g L^{-1} . A pH drop was also observed with pH value of 5.2 and 6 at respectively OLR of $0.8 \text{ g VS L}^{-1} \text{ d}^{-1}$ and $1.7 \text{ g VS L}^{-1} \text{ d}^{-1}$. Interestingly, co-digestion of food wastes with waste-activated sludge led to better stability and performances highlighting the importance of co-digestion in order to improve the C/N ratio under suitable value for AD process (Hawkes, 1980b; Kwietniewska and Tys, 2014).

At the OLR of $2.5 \text{ g VS L}^{-1} \text{ d}^{-1}$ the reactors fed with a mixture of biowastes and biodegradable plastics exhibited transient instabilities (**Figure V.1**) but these instabilities were overcome without changing the OLR. Concerning the reactor fed with PHB, the VFAs concentration increased throughout the week and decreased during week-end when there was no feeding. The buffer

capacity was enough to maintain the pH in the operational range. The addition of biodegradable plastics (PLA or PHB, pretreated or untreated) provided better stabilities to the digesters than digestion of biowaste alone. This observation could be explained by the improved C/N ratio due to biodegradable plastic addition. Hawkes, (1980) suggested a carbon to nitrogen ratio for anaerobic digestion ranging from 20 to 30 for preventing both nutrient limitation and ammonia toxicity. Biowaste is protein-rich and has low C/N ratio = 13.4 (**Table V.1**). By contrast, the biodegradable plastics are carbohydrates-rich, they contain high levels of carbon but none nitrogen (**Table V.2**). Thus, co-digestion of biodegradable plastics with biowastes can help balancing the C/N ratio and result in a more robust process (Benn and Zitomer, 2018; Rabii et al., 2019; Rajagopal et al., 2013). The mixture PHB/biowaste and PLA/biowaste have C/N ratio of 18.01 and 18.25, respectively in comparison with 13.4 for biowastes alone. The ammonium concentration remained stable (around $2 \text{ gN-NH}_4 \text{ L}^{-1}$) throughout the test for reactors co-digesting biodegradable plastics with biowastes (**Figure V.2**). In parallel to the improvement of the C/N ratio, in our case, the co-digestion strategies has also permitted to reduce the highly biodegradable fraction, that lead to rapid VFAs accumulation, as biodegradable plastics have a slower biodegradation kinetic than biowaste. Finally, the reactor fed with the mix of biowastes and pretreated PLA exhibited good stabilities except at the beginning. Nonetheless, the calcium hydroxide addition, improved the buffer capacity of the digester; no pH variation were observed even at the starting with 8 g L^{-1} of VFAs.

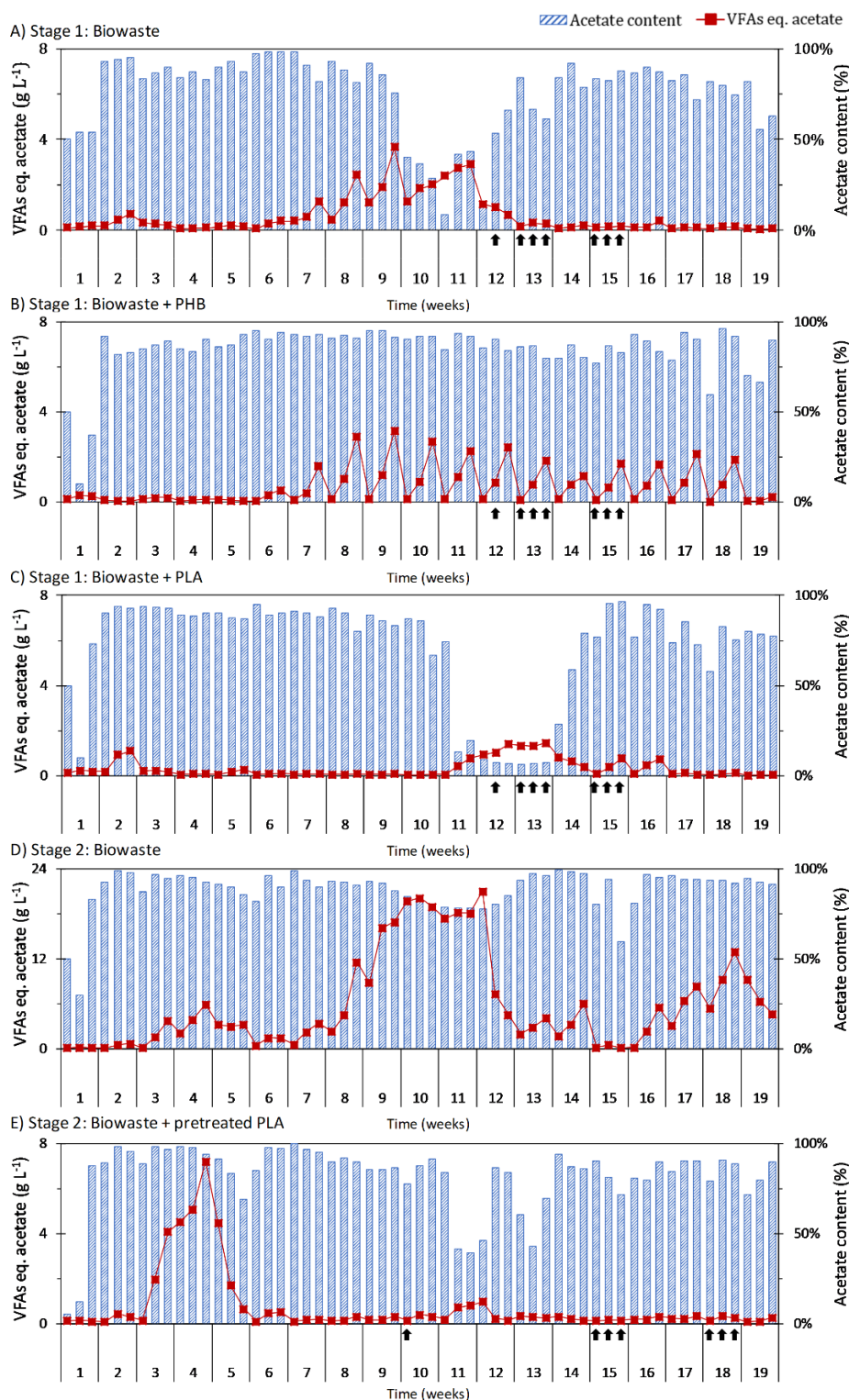


Figure V.1. Evolution of the volatile fatty acids concentration and acetate content in the different reactors. Reactor fed with biowaste in stage 1 was fed at an OLR of 2.5 g VS L⁻¹ d⁻¹ between weeks 3 and 11, after week 11 the OLR was reduced to 2 g VS L⁻¹ d⁻¹. Reactor fed with biowaste in stage 2 was fed throughout the test at an of 2.5 g VS L⁻¹ d⁻¹; half of the volume of the reactor was removed at the beginning of the week 12 and fuel with fresh inoculum, all of the reactor volume was emptied at the beginning of week 15. Arrows indicate the sampling time for microbial analysis.

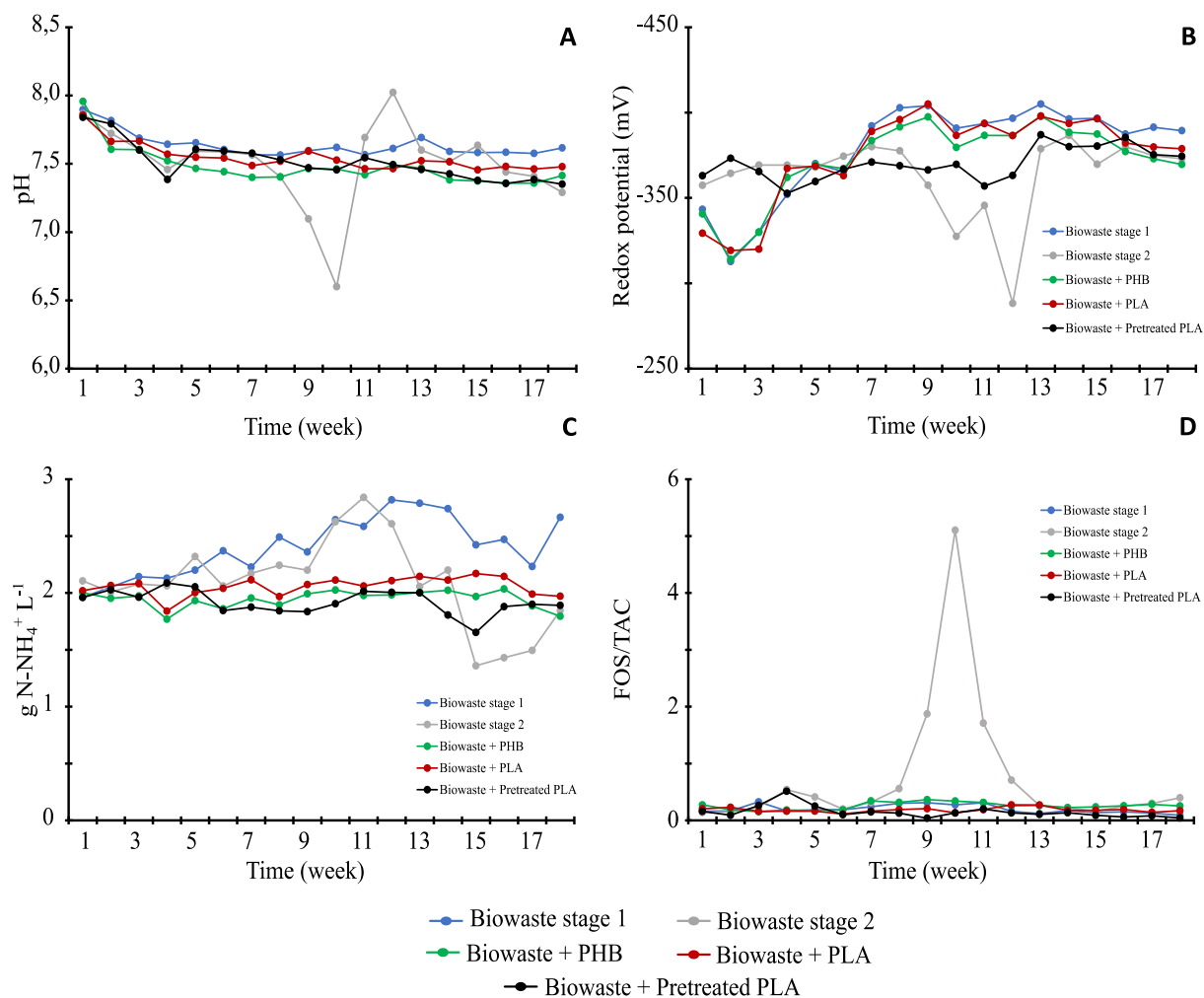


Figure V.2. Evolution of the pH (A), redox potential (B) ammonium concentration (C) and FOS/TAC ratio (D) in the different reactors.

3.2) Performance of the reactors

The observed methane productions in the different anaerobic digesters is presented in **Figure V.3A**. As discussed before, the reactor fed with biowaste only at an OLR of $2.5 \text{ g VS L}^{-1} \text{ d}^{-1}$ throughout the test (Biowastes stage 2), presented an inhibition of the methane production (week 9 to 12). Therefore, the performance of this digester will not be considered for the remainder of this study. On the contrary, the reactor fed only with biowaste at a reduced OLR after the first instabilities (biowaste stage 1), exhibited stable methane production all over the test. The average methane production during the third HRT (week 11-17) was $502 \pm 39 \text{ L kg}^{-1} \text{ VS}$. Based on theoretical methane production, this production corresponded to a biodegradation yield of the biowaste of

93%. Dolci et al. (2022) have also investigated the mono-digestion of food wastes in semi-continuous reactors in thermophilic conditions. At an OLR of 2.20 kg COD m⁻³ d⁻¹, stable methane production was observed with methane value of 380–393 mL CH₄ g⁻¹ VS, corresponding to a good anaerobic degradability of food waste, between 89% and 92%.

The digestion of the PHB and biowaste mixture also resulted in a relative stable methane production over the course of the trial. The average methane production during the third HRT (week 11-15) was 517 ± 16 L kg⁻¹ VS, corresponding to a biodegradation yield of the co-substrates of 96.3 ± 2.3%. When PHB was co-digested, the average methane production increased in comparison with mono-digestion of biowaste. By assuming that the biowaste is converted to methane at the same level in the reactors performing mono-digestion and co-digestion of biowaste, then PHB was fully biodegraded (103 ± 3). Similarly, Benn and Zitomer, (2018) and Venkiteshwaran et al. (2019) found a high level of PHB conversion to methane, between 79 and 98% for two pretreated and untreated PHB, in co-digestion with synthetic municipal primary sludge under mesophilic conditions.

By contrast, co-digestion of PLA and biowaste resulted in a growing methane production over time (**Figure V.3**). At the third HRT (week 11-15) the methane production reached 432 ± 25 L kg⁻¹ VS corresponding to 81.9 ± 4.4 % biodegradation of the co-substrates. Assuming that biowaste were converted to 93% to methane (*i.e.*, conversion yield reached in biowaste-fed reactor at the first stage), the biodegradation of the PLA in the third HRT could be estimate at 47.6 ± 17.9%. Based on data from batch experiment, only a low biodegradation level (around 5-10%) should be expected for PLA using an HRT of 30 days (Cazaudehore et al., 2022). Some visual evidence and a higher VS content in compared with other reactors (**Table V.2**) suggest that PLA particles were accumulated in the reactor. Therefore, due to PLA accumulation, it is supposed that some PLA particles introduced in the early days of the trial undergo conversion to methane latter, explaining the increase in methane production over time. Similarly, Zhang et al. (2018) measured the weight loss of eight biodegradables films (based on PLA, starch or cellulose) and a pellet (made of PLA) during the mesophilic anaerobic digestion with synthetic food wastes and card packaging. Cellulose based films exhibited a high weight loss during the test, between 57.4 and 93.4%, whereas starch-based films had lost only a small part of their weight between 2.1 and 7.9%. PLA film has lost 57.5% of its mass while PLA pellets only lost 3.1% of its mass demonstrating the importance of the initial

morphology of the plastic (W. Zhang et al., 2018). Dolci et al. (2022) performed semi-continuous co-digestion experiment with synthetic food waste under thermophilic condition (50 °C). Mater-bi® bags exhibited a relatively low biodegradation (*i.e.*, 27% based on carbon oxygen demand), using a 20 days HRT. In parallel, Wang et al. (2012) has investigated the co-digestion of kitchen garbage and two plastic bags made of PLA (100% PLA and 70% PLA) in the form of 2x2x0.1 mm pieces. PLA films were converted to 70 % in methane in a single-phase thermophilic system (55 °C) and to 80-83% in a two phase AD system consisting in a hyperthermophilic (80 °C) reactor and a thermophilic (55°C) reactor.

Then, the reactor treating pretreated PLA with biowaste, exhibited stable methane production. At the third HRT (week 11-15) the methane production reached $471 \pm 12 \text{ L kg}^{-1} \text{ VS}$ corresponding to $87 \pm 6.1 \%$ biodegradation of the co-substrates. Pretreated PLA was converted to $77.5 \pm 9.3\%$ to methane. This estimation of the biodegradation of the PLA is in line with the observed biodegradation in batch test, *i.e.*, 72% of biodegradation in 30 days (Cazaudehore et al., 2022). Pretreatment of the PLA increase the conversion in methane and do not have negative effect on process stability. Finally, in **Table V.2**, the VS removal for the various conditions is presented. It was observed that except for the pilot treating biowastes with PLA (VS removal of 69%) all the other pilots exhibited similar VS removal around 80%. The lower VS removal in the pilot treating biowastes in co-digestion with PLA is in accordance with lower methane production observed and an accumulation of PLA due to its low biodegradation in mesophilic condition at the HRT selected for the study.

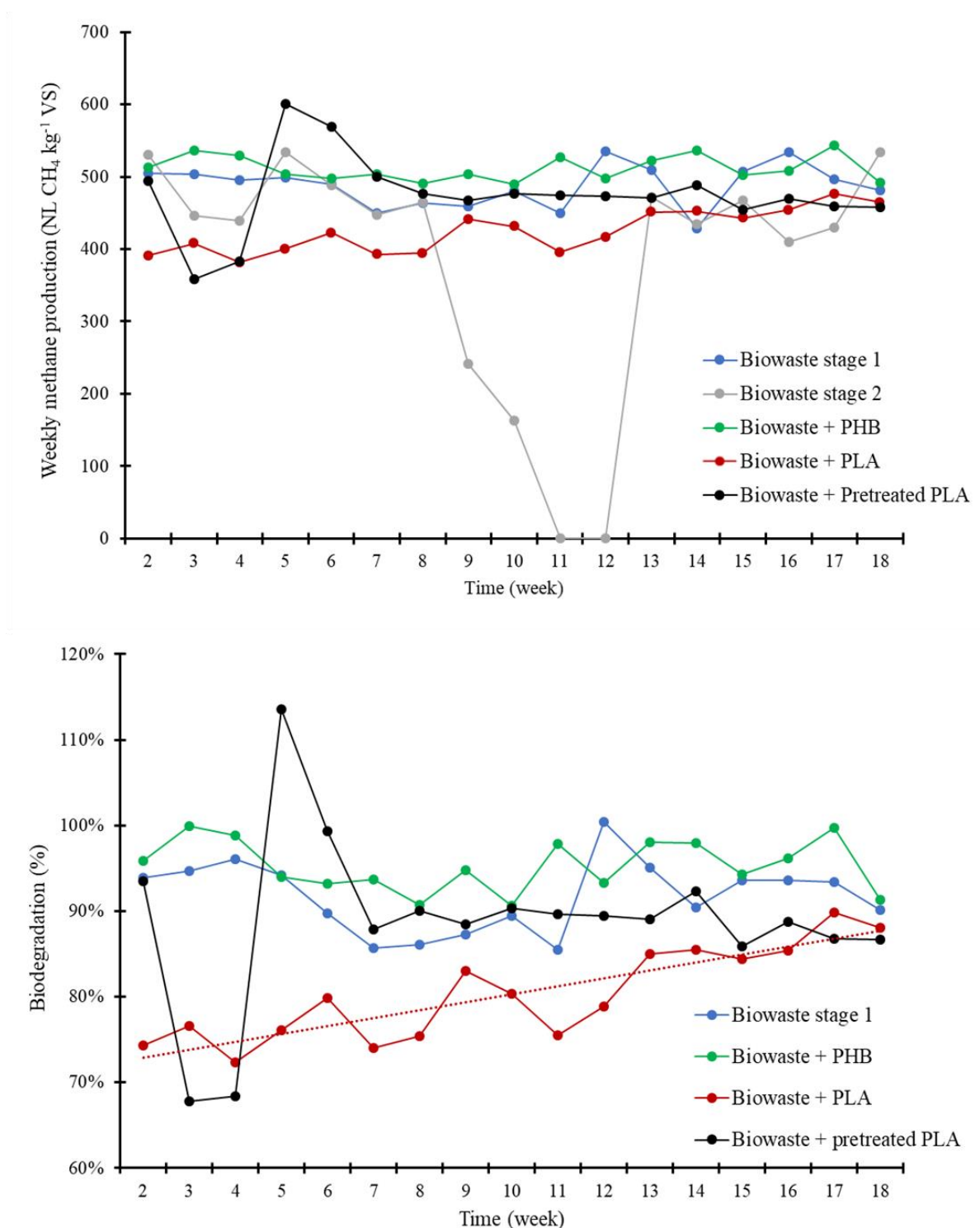


Figure V.3: Weekly methane production (A) and biodegradation (B) from the different co-substrate and from biowaste.

Table V.2. Average stability and performance indicator for the different reactors during the third HRT. Values are expressed as average (\pm standard deviation)

Parameters	Unit	Biowaste stage 1	Biowaste + PHB	Biowaste + PLA	Biowaste + pretreated PLA
pH	-	7.6 (\pm 0.1)	7.4 (\pm 0.1)	7.5 (\pm 0.1)	7.4 (\pm 0.1)
Redox	mV	-396 (\pm 10)	-390 (\pm 14)	-393 (\pm 13)	-379 (\pm 12)
Ammonia	gN-NH ₄ L ⁻¹	2.6 (\pm 0.3)	2.0 (\pm 0.0)	2.1 (\pm 0.1)	1.8 (\pm 0.2)
FOS/TAC	-	0.1 (\pm 0.07)	0.27 (\pm 0.13)	0.15 (\pm 0.12)	0.10 (\pm 0.05)
VFAs	g _{eq acetate} L ⁻¹	0.31 (\pm 0.32)	0.93 (\pm 0.83)	0.87 (\pm 0.45)	0.26 (\pm 0.21)
Acetate	g L ⁻¹	0.18 (\pm 0.13)	0.84 (\pm 0.72)	0.14 (\pm 0.10)	0.18 (\pm 0.09)
Propionate	g L ⁻¹	0.14 (\pm 0.3)	0.14 (\pm 0.14)	0.91 (\pm 0.6)	0.05 (\pm 0.06)
Isobutyrate	g L ⁻¹	0 (\pm 0)	0.01 (\pm 0.01)	0.01 (\pm 0.01)	0.02 (\pm 0.03)
Butyrate	g L ⁻¹	0.01 (\pm 0)	0.03 (\pm 0.03)	0.01 (\pm 0)	0.02 (\pm 0.05)
Isovalerate	g L ⁻¹	0.01 (\pm 0.01)	0.03 (\pm 0.03)	0 (\pm 0)	0.02 (\pm 0.03)
Valerate	g L ⁻¹	0.01 (\pm 0)	0 (\pm 0)	0 (\pm 0)	0.02 (\pm 0.02)
TS	% raw mass	2.2 (\pm 0.1)	2.2 (\pm 0.0)	3.0 (\pm 0)	3.4 (\pm 0.0)
VS	% raw mass	1.5 (\pm 0.1)	1.5 (\pm 0.0)	2.3 (\pm 0.0)	1.6 (\pm 0.0)
VS removal (%)		80	80	69	79
Mean methane production	L CH ₄ kg ⁻¹ VS	502 (\pm 39)	517 (\pm 16)	432 (\pm 25)	471 (\pm 12)
Biodegradation yield of the mix	%	93.1% (\pm 4.6)	96.3% (\pm 2.3)	81.9% (\pm 4.4)	87% (\pm 6.1)

3.3) Microbial diversity

An average richness of 200 ± 12 active OTUs per samples were observed in the different reactors. The alpha diversity indices were not significantly different based on the reactor feeding (Biowaste alone, Biowaste + PHB, Biowaste + PLA and Biowaste + pretreated PLA). Similarly, the alpha diversity was not modified by the sampling time. The microbial communities were highly uneven, suggesting the presence of few dominant active OTUs and a large number of rare active OTUs. At the phylum level, *Bacteroidota* (60-65%), *Firmicutes* (12-17%) and *Cloacimonadota* (5-12%) dominated the microbial communities of the different reactors. Together, the three more

abundant phyla represented 83 to 93% of the collected sequences in the different samples. The composition and the variation of the active microbial communities in the different reactors was explored using redundancy analysis (RDA) (**Figure V.4**).

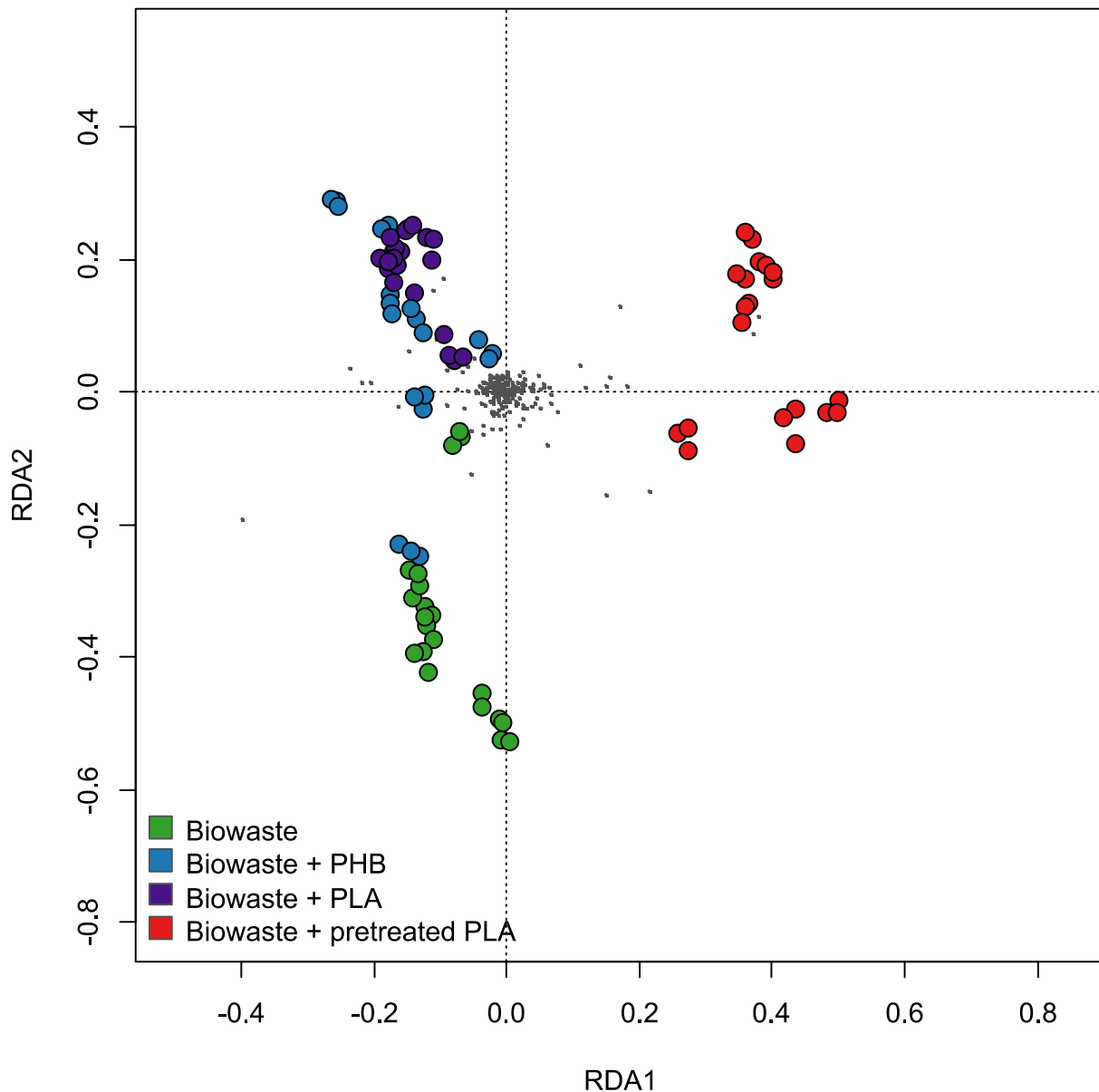


Figure V.4. Redundancy analysis (RDA) representing the OTUs (dots) and samples (colored circles). The RDA (**Figure V.4**) revealed that the active populations from the reactor treating biowaste as only substrate was clearly different from populations of reactors performing co-digestion of biodegradable plastics, suggesting that the active microbial communities were modified by the introduction of biodegradable plastics. By contrast, active microbial communities co-digesting (untreated) PLA and PHB were clustered together (**Figure V.4**) and were clearly distant from the active population from the reactor treating pretreated PLA. It was quite surprising to find closer

active populations in PLA and PHB-fed reactors than in PLA and pretreated PLA-fed reactors. However, the anaerobic digestion experiment on pretreated PLA was performed in stage 2, with a different starting inoculum than during the digestion of untreated PLA and PHB. In addition, due to the thermo-alkaline pretreatment (using $\text{Ca}(\text{OH})_2$), the pretreated PLA supply was more alkaline and contained calcium, making the environmental physico-chemical conditions singular. Multivariate analysis was performed to determine correlations between the active OTUs' relative change in abundance and the methane production from the different substrates or co-substrates. The top 15 OTUs showing the higher change in relative abundance in relation with the methane production from each substrates or co-substrates are displayed on **figure V.5**.

The majority of OTUs correlated with the methane production during the mono-digestion of biowaste were also correlated to the methane production during the plastic (pretreated or untreated PLA, PHB) and biowaste co-digestion. Among these, *Fermentimonas caenicola* was the most abundant OTUs in all the reactors, representing between 15.3 and 31.3% of the samples sequences. *Fermentimonas caenicola* was previously isolated from a mesophilic digester and showed the ability to growth on complex proteinaceous substrate (Hahnke et al., 2016). Wirth et al. (2019) identified *Fermentimonas caenicola* as a core member of the anaerobic digesters fed with *Chlorella* or *maize silage* or a mixture of both. Other OTUs found to be correlated with the methane production from mono or co-digestion of biowaste (such as *Endomicrobium* or *Tisserella*) were also previously identified as populations involved in the anaerobic digestion of food waste (Jo et al., 2021) or other protein-rich substrates (Alauzet et al., 2014; Nolla-Ardèvol et al., 2015; Zheng et al., 2017).

As described before, the microbial communities active during the co-digestion of PLA or PHB were similar. Furthermore, the OTUs correlated with the methane production (from PLA or PHB) were also correlated with methane production during the mono-digestion of biowaste, suggesting the implication of these OTUs in biowaste degradation. Therefore, the multivariate analysis did not reveal notable OTUs specific to PLA or PHB digestion. Probably the active OTUs involved in PHB or PLA degradation were masked by the Active OTUs involved in the degradation of biowaste. Additionally, none of the positively correlated OTUs to the methane production from the mixture of biowaste and PHB were found in the PHA depolymerase database (Knoll et al., 2009). However, *Ilyobacter delafieldii* which was evidenced as a PHB degrading bacteria in batch mode AD

experiment (Cazaudehore et al., 2022; Janssen and Harfoot, 1990), was found to be active in the digester fed with PHB, but represented a relative small relative abundance (0.1 to 0.3% of the sample sequences) of the active microbial diversity in these reactors. Concerning PLA conditions, no previously known PLA degrading microorganisms were evidenced in this analysis (Bubpachat et al., 2018; Cazaudehore et al., 2021; Cazaudehore et al., 2022; Emadian et al., 2017; Kawai, 2010).

The anaerobic digestion of pretreated PLA was marked by the correlation with an OTU related to *Erysipelatoclostridiaceae* UCG-9, which accounted for up to 8.4% of the sequences in the reactor fed with pretreated PLA, in the other reactors the abundance of this OTU was less than 0.7%. Unfortunately, this OTU was uncultured and its implication in anaerobic digestion is yet unknown.

Concerning the Archaea diversity, *Methanoculleus* was predominant in all the reactors and at the different sampling time. It represented an average proportion of $78 \pm 6\%$ of the sequences attributed to archaea, suggesting that the hydrogenotrophic pathway was the main pathway of methane production in the different reactors (Oren et al., 2014). The acetoclastic methanogen *Methanosaeta* and the hydrogenotrophic or methylotrophic methanogen *Methanosillicoccus* were the other abundant archaea, representing an average proportion of $8 \pm 6\%$ and $3 \pm 2\%$, respectively (Boone et al., 2015; Dridi et al., 2012). Hydrogenotrophic dominance was previously reported in mesophilic anaerobic digesters treating various feedstocks (Sundberg et al., 2013; Wirth et al., 2012; Zhu et al., 2011).

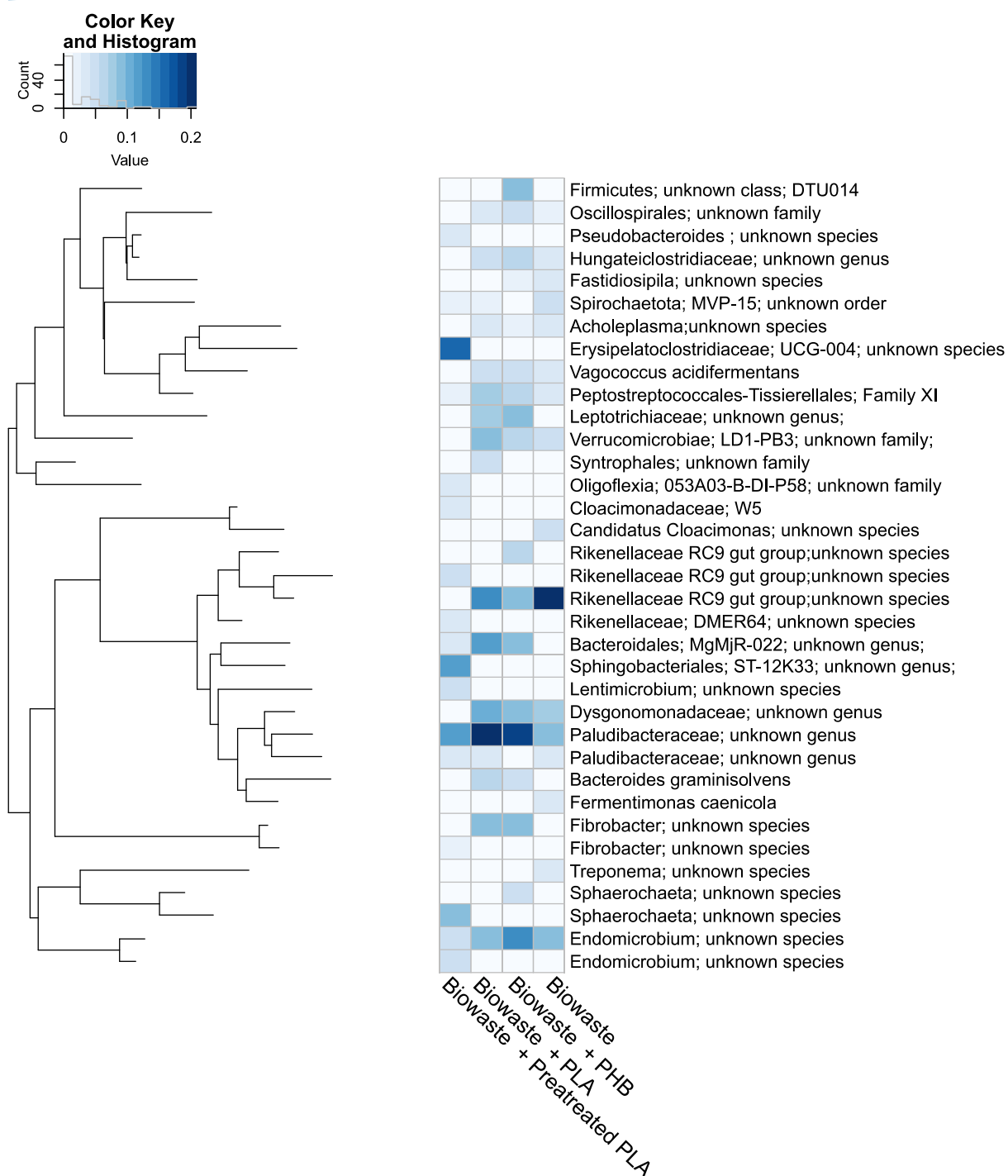


Figure V.5: Heatmap of the multivariate regression analysis. Only the top 15 OTUs having the highest positive correlation with the methane production from the different reactors are displayed. The intensity of the colors correlates with the magnitude of the Hellinger transformed abundance change.

3.4) Agronomic and sanitary parameters of the digestates

In **Table V.3**, the main physico-chemical properties of the digestates obtained from the various conditions are presented. The pH of the different digestates was around 8.5-8.6 which is in adequation with literature (Nkoa, 2014; Tampio et al., 2016b). For instance, Tampio et al. (2016) have reported pH values ranging from 7.6 to 8.3 for various digestates coming from the anaerobic digestion of food wastes and OFMSW. The higher TS content observed for the pilot treating biowastes and PLA can be explained by an accumulation of the PLA during the time due to an insufficient homogenization in the pilot. As concerns the pilot treating biowastes and PLA pretreated, the higher content of TS is due to the addition of $\text{Ca}(\text{OH})_2$ during the pretreatment step. Ammonium concentrations ranging from 31.4 to 69 g N kg TS and NTK concentrations ranging from 76.3 to 158.5 g N kg TS were reported. Then, the ratio $[\text{NH}_4^+] / \text{NTK}$ ranged from 40.7% to 44.3%. This value is in agreement with previous one that reported a ratio of $[\text{NH}_4^+] / \text{NTK}$ on biowastes digestates (Guilayn et al., 2019; Tampio et al., 2016b). The ratio C/N which is also another important agronomic parameter was ranging from 2.1 to 2.9. During the anaerobic digestion process, a large fraction of carbonaceous compounds is converted to methane and carbon dioxide, which are collected as biogas. As a result, the proportion of carbon decreases in the liquid and solid residues while that of nitrogen increases in the form of $\text{NH}_4\text{-N}$, resulting in a lower C/N ratio (Nkoa, 2014). This ratio (5.4) was higher in the pilot treating biowastes in co-digestion with PLA due to the lower biodegradation of PLA than PHB and pretreated PLA.

Comparable results were observed for the other different nutrient sources (*i.e.*, P, K, S, Mg). In contrast, Ca has a much higher concentration in the pilot with biowastes in co-digestion with pretreated PLA due to the addition of $\text{Ca}(\text{OH})_2$ during the thermo-alkaline pretreatment. Indeed, a concentration of 276 g CaO kg^{-1} TS was observed for this condition in comparison to 29.3 to 65 g CaO kg^{-1} TS for the other conditions. Besides nutrients content, the content of the most common heavy metals was also analyzed and is reported in **Table V.3**. All the heavy metals investigated were below the threshold limit recommended by the French (Ministère de l'agriculture et de l'alimentation, 2020; NFU 44051, 2006) and European regulation (Saveyn et al., 2014). Higher values were reported for Cu and Zn. Indeed, the Zn content ranged from 260 to 494 mg kg^{-1} TS. Similar trends were reported elsewhere (Kupper et al., 2014; Tampio et al., 2016b) notably by Tampio et al. (2016) on digestates from food wastes and OFMSW with the highest content of heavy

metals observed for Cu and Zn. Aside from agronomic and safety parameters (heavy metals, organic contaminants), it will be interesting to assess the persistence and the quantification of residual biodegradable microplastics. Indeed, incomplete degradation during anaerobic digestion will lead to the remanence of biodegradable microplastics and potential leakage of bioplastics into the environment if the digestate is used for agronomic application (Cucina et al., 2021a). In parallel, in future work, research activities will focus on the investigation of the potential ecotoxicity of the various digestates produced on several aquatic and terrestrial ecosystems (Li et al., 2012; Ross et al., 2017; Tigini et al., 2016).

Table V.3: Average agronomic and sanitary parameters of the digestates obtained from the different conditions. Values are expressed as average (\pm standard deviation)

Parameters	Unit	Biowaste stage 1	Biowaste + PHB	Biowaste + PLA	Biowaste + pretreated PLA
pH	At 25 °C	8.6 (\pm 0)	8.5 (\pm 0.1)	8.6 (\pm 0.1)	8.5 (\pm 0.1)
TS	% raw mass	2.1 (\pm 0.1)	1.9 (\pm 0.2)	3.2 (\pm 0.6)	3.5 (\pm 0.1)
VS	% TS	65.9 (\pm 1.5)	70.5 (\pm 2.8)	83.2 (\pm 2.5)	44.5 (\pm 0.1)
Conductivity	dS cm ⁻¹	3.4 (\pm 0)	2.7 (\pm 0.4)	2.6 (\pm 0)	2.8 (\pm 3.5)
Organic carbon	% TS	33 (\pm 0.8)	35.2 (\pm 1.4)	41.6 (\pm 1.3)	22.3 (\pm 0.1)
Organic matter	% TS	65.9 (\pm 1.5)	70.4 (\pm 2.8)	83.2 (\pm 2.6)	44.5 (\pm 0.1)
[NH ₄ ⁺]	g N kg TS	69.0 (\pm 7.6)	58.7 (\pm 9.1)	35.1 (\pm 4.5)	31.4 (\pm 1)
NTK	g N kg TS	158.5 (\pm 12)	144 (\pm 15.6)	79.3 (\pm 15.1)	76.3 (\pm 2.3)
C/N	-	2.1 (\pm 0.2)	2.5 (\pm 0.4)	5.4 (\pm 1.2)	2.9 (\pm 0.1)
MO/N orga		7.4 (\pm 0.5)	8.3 (\pm 1)	19.5 (\pm 5.3)	10 (\pm 0.4)
[N _{orga}]	g N kg TS	89.2 (\pm 4)	85 (\pm 7.1)	44.3 (\pm 10.7)	44.8 (\pm 2.3)
[Ca]	g CaO kg ⁻¹ TS	65 (\pm 2)	53.1 (\pm 2.5)	29.3 (\pm 6.8)	273.5 (\pm 9.2)
[K]	g K ₂ O kg ⁻¹ TS	54.1 (\pm 0.7)	45.4 (\pm 1.7)	26.8 (\pm 5.4)	27.5 (\pm 1.1)
[Mg]	g MgO kg ⁻¹ TS	6.9 (\pm 0.2)	5.9 (\pm 0.2)	3.9 (\pm 0.6)	4.6 (\pm 0.1)
[Na]	g Na ₂ O kg ⁻¹ TS	49 (\pm 1.4)	42.9 (\pm 2.3)	24.4 (\pm 4.6)	23.8 (\pm 0.1)
[P]	g P ₂ O ₅ kg ⁻¹ TS	35.1 (\pm 1.4)	29.9 (\pm 1.5)	15.9 (\pm 3.4)	18.0 (\pm 0.4)
[S]	g SO ₃ kg ⁻¹ TS	15.9 (\pm 0)	12.9 (\pm 0.1)	7.2 (\pm 1.9)	7.9 (\pm 0.1)
[Cr]	mg kg ⁻¹ TS	6 (\pm 0.9)	4.8 (\pm 1.8)	4.6 (\pm 0.8)	4.2 (\pm 3)
[Cu]	mg kg ⁻¹ TS	81.6 (\pm 4)	38.4 (\pm 2.5)	56.7 (\pm 3.2)	33.1 (\pm 15.1)
[Zn]	mg kg ⁻¹ TS	494.3 (\pm 16.5)	427.9 (\pm 33.6)	331.6 (\pm 8.7)	259.6 (\pm 60)
[As]	mg kg ⁻¹ TS	1.6 (\pm 0.2)	1.2 (\pm 0.2)	1 (\pm 0.1)	0.8 (\pm 0.4)
[Se]	mg kg ⁻¹ TS	BDL	BDL	BDL	BDL
[Mo]	mg kg ⁻¹ TS	1.9 (\pm 0)	1.8 (\pm 0.4)	1.6 (\pm 0.3)	1.4 (\pm 0.9)
[Cd]	mg kg ⁻¹ TS	2.7 (\pm 0.1)	2.5 (\pm 0.1)	1.9 (\pm 0)	2.9 (\pm 1.1)
[Pb]	mg kg ⁻¹ TS	1 (\pm 1.4)	1.2 (\pm 2.1)	1.2 (\pm 1)	0.6 (\pm 0.3)
[Sn]	mg kg ⁻¹ TS	43.1 (\pm 2.2)	41.6 (\pm 3.1)	34.9 (\pm 0.9)	49.3 (\pm 26.7)
PAH	mg kg ⁻¹ TS	1.2	1.0	0.9	1.1
PCB	mg kg ⁻¹ TS	< 5	< 5	< 5	< 5

BDL: Below Detection Limit

PAH: polycyclic aromatic hydrocarbon

PCB: Polychlorinated biphenyl

4) Conclusion

The co-digestion of biodegradable plastics with biowastes showed no sign of negative effects on the anaerobic digestion process. On the contrary, the co-digestion reactors showed increased stability compared to reactor treating only biowastes. PHB was estimated to be fully converted to methane (103 %) whereas PLA was not fully converted to methane (48 %) and accumulated in the reactor. The implementation of the thermo-alkaline pretreatment on the PLA pellets led to an increase in the biodegradation efficiency of PLA, it was estimated that 77.5% of the pretreated PLA was converted to methane. The identification of specific microorganisms of the biodegradable plastic degradation was complicated, the majority of the microorganisms correlated with the methane production from reactors co-digesting PLA and PHB were implicated in the AD of the biowaste. A previously known PHB degraders, *Ilyobacter delafieldii*, was observed in the reactor co-digesting PHB but showed low abundance.

Conclusion

The growing concern regarding non-biodegradable plastics and their impact on the environment lead gradually to the use of other materials such as biodegradable plastics. Biodegradable plastics offer additional waste management options (*e.g.*, anaerobic digestion or composting) over conventional plastics. Anaerobic digestion (AD) is a waste management method allowing a simultaneous generation of renewable energy (*i.e.*, through biogas generation) and digestate production that can be used in agronomic issues. However, the treatment of biodegradable plastics under anaerobic digestion is only in its infancy. Therefore, the aim of this thesis was to investigate the feasibility and fate of biodegradable plastics in anaerobic digestion systems and the microorganisms involved in the plastic conversion to methane.

The research showed that the inoculum to substrate ratio (ISR) was a key parameter for the estimation of the biodegradation behavior of biodegradable plastics in Biochemical Methane Potential (BMP) tests (**chapter 2**). An increase of the ISR was associated with an improved biodegradation kinetic but also to an increase of variability in methane production. These results pointed out that there is a need to define more precisely the inoculum substrate ratio recommended in the standard test methods for the evaluation of the biodegradability of a plastic in liquid anaerobic digestion (ISO 14853 and ISO 13975). According the results obtained in Chapter 2, we recommend using ISR between 2.85 and 4.

There is a variety of biodegradable polymers on the market and the objective of the chapter 3 was to assess their biodegradability performances. The results of the batch anaerobic digestion experiment on the main biodegradables polymers demonstrated that only Poly(3-hydroxybutyrate) (PHB) and Thermoplastic starch (TPS) exhibited rapid (25-50 days) and important (57-80.3% and 80.2-82.6%, respectively) conversion to methane under both mesophilic and thermophilic condition (**chapter 3 part 1**). Very different biodegradation yield were obtained (57-80.3 %) from two biodegradables plastics made up in PHB, highlighting that the nature of the polymer is not the only factor affecting the biodegradation. Others factors associated with the properties of the plastic can have a significant impact on the biodegradation such as crystallinity, molecular weight, accessible surface area. Methane production rates from poly(lactic acid) (PLA)

and poly(ϵ -caprolactone) (PCL) was very low under mesophilic condition, to such an extent that 500 days were required to reach the ultimate methane production. However, the ultimate methane production was relatively high, corresponding to 49.4% of biodegradation for PCL and 74.7-80.3% for PLA. Methane production rate from PLA was greatly enhanced under thermophilic condition since only 60 to 100 days were required to reach the ultimate methane production, corresponding to 74.6% of PLA biodegradation. The faster biodegradation kinetics under thermophilic conditions (58°C) could be partly attributed to the proximity of the PLA glass transition temperature (around 60 °C), which triggers chemical hydrolysis and facilitates access to microorganisms and enzymes. Unlike PLA, PCL anaerobic digestion was ineffective under thermophilic condition. This observation was not in line with literature data (Narancic et al., 2018; Šmejkalová et al., 2016; Yagi et al., 2013a). Further investigations are needed to understand the observed difference with literature and the mechanisms involved. Poly(butylene adipate/terephthalate) (PBAT) and poly(butylene succinate) (PBS) that are able to undergo complete or near complete biodegradation in relative short period under industrial composting showed minor or zero biodegradation at both temperature even with the 500 days period used under mesophilic condition. This emphasizes the critical need to specify the environment in which a plastic is biodegradable.

Acquiring a better understanding on the microorganisms involved in the anaerobic digestion of the biodegradable plastics could open the way to the use of bioaugmentation/biostimulation methodologies to improve the biodegradation. The microorganisms involved in the process were investigated using 16S rRNA sequencing. Unfortunately, it has been complicated to identify the microorganisms' specific of the biodegradation of a polymer when the biodegradation rate was low. By contrast, lactate-utilizing bacteria such as *Tepidimicrobium*, *Moorella* and *Tepidanaerobacter* were found to be important during the thermophilic digestion of PLA. Similarly, starch-degrading bacteria (from the genus *Clostridium*) were highlighted during TPS digestion at 38 °C and 58°C whereas known PHB degraders (*i.e.*, *Enterobacter*, *Ilyobacter delafieldii* and *Cupriavidus*) were observed during mesophilic and thermophilic AD of PHB.

Commercially available plastics are often blends of different polymers in order to meet the intended technical requirements (Long and Chen, 2009). Therefore, the biodegradation of commercial blends of biodegradable plastic was also explored (**chapter 3, part 2**). The blends,

made of Mater-bi[®], Ecovio[®] and Vegemat[®], were purchased in the form of coffee capsules available on the market and certified biodegradable under industrial composting conditions. The three blends had similar behavior in BMP tests. Mesophilic digestion resulted in a low conversion to methane, between 12 and 24% in 100 days. Thermophilic digestion was much more performant; the biodegradation reached between 47 and 69%, in 100 days. Plastic producers are not transparent about the exact formulation of the blends, making performances and microbial interpretations complicated. For example, *Tepidimicrobium* was found to be dominant in the thermophilic digesters fed with the different biodegradable plastic blends during the high methane production phase. *Tepidimicrobium* was previously identified as a key lactate-utilizing bacteria in PLA fed reactors. However, PLA was not supposed to be a component of all the coffee capsules. Additional analyses on the different plastics used in this manuscript have been undertaken in order to identify the polymers present in the blends (using Fourier transform Infrared spectroscopy and nuclear magnetic resonance) and to make a link between the physico-chemical properties of the plastic with the biodegradation behavior in anaerobic digestion (e.g., crystallinity, glass transition or melting temperature).

The low biodegradation rate of most of the biodegradable plastics in mesophilic anaerobic digesters is a major hindrance to their introduction at industrial scale. Various strategies could be implemented to improve the biodegradation of PLA in anaerobic digesters (**chapter 4**). PLA had been chosen because of its low biodegradation rate (500 days were required to reach the ultimate methane production) and because it represents one of the most used biodegradable plastic (25% of the production). A mechanical reduction of the PLA particle size increased the rate of PLA biodegradation. However, even with very small particles ($\approx 272\mu\text{m}$) the biodegradation kinetic was not satisfactory. Furthermore, such a pretreatment at industrial scale is not technically suitable. By contrast, thermal and thermo-alkaline pretreatments provide a significant improvement in the biodegradation kinetics of PLA. Thermal pretreatments only achieved substantial solubilization (> 60%) of the PLA at higher temperatures (120 and 150 °C). At lower temperatures (70 and 90 °C), negligible solubilization (between 1 and 6%) occurred after 48 h. When coupling thermal and alkaline pretreatment (Ca(OH)_2 addition), a significant increase in solubilization at the lower temperatures (70 and 90 °C) could be achieved. The best pretreatment to reducing the concentration of Ca(OH)_2 and the temperature used, while maintaining high biodegradation

kinetics was achieved at 70 °C using 2.5% w/v Ca(OH)_2 for 48 h. This pretreatment resulted in a PLA biodegradation yield of 73% after 15-20 days; a similar biodegradation yield was obtained after 500 days for untreated PLA. The pretreatment at this temperature is relatively simple to implement at industrial scale, as anaerobic digesters treating biowaste already perform a warming step at 70°C to inactivate biowaste pathogens.

Finally, in order to mimic industrial processes, the anaerobic co-digestion in semi-continuous reactors of both biowastes and biodegradable plastics (pretreated or untreated PLA and PHB) was investigated (**chapter 5**). The addition of biodegradable plastics resulted in a more stable process (in comparison with stand-alone biowastes reactor) and no negative effects could be detected. PHB was estimated to be fully biodegraded in the reactors. By contrast, PLA was accumulated in the reactor, and an average biodegradation of 47.6% was estimated during the third hydraulic retention time. Pretreatment of PLA, consisting of thermo-alkaline hydrolysis at 70 °C, with 2.5 w/v of Ca(OH)_2 during 48 h, improved the biodegradation yield of PLA to 77.5%. The identification of specific microorganisms implicated in the biodegradable plastic degradation was hard to assess. The majority of the microorganisms correlated with the methane production from reactors co-digesting PLA and PHB were implicated in the anaerobic digestion of the biowaste, which can be explained by the low proportion of biodegradable plastics introduced. As an example a previously known PHB degraders, *Ilyobacter delafieldii*, was observed in the reactor co-digesting PHB but showed low abundance. The digestates obtained for the various conditions demonstrated to have agronomic and sanitary parameters in harmony with the French and European regulation.

Perspectives

The treatment of biodegradable plastics in anaerobic digestion is still in its infancy, and some technical, environmental and societal barriers were emphasized during this thesis. These will have to be addressed to consider the introduction of biodegradable plastics in anaerobic digestion facilities.

- 1) **Composition of the biodegradable plastics:** It is clear that the nature of the polymer (*e.g.*, PLA, PHA, PBS, TPS) significantly influences its biodegradation by AD. There has, however, been a paucity of investigations of the biodegradability of commercial biodegradable plastic blends (except Mater-Bi®) as well as a number of emerging polymers such as mcl-PHAs and crystallized poly(lactic acid) by anaerobic digestion. Further research is needed in order to obtain reference values regarding the anaerobic biodegradability of the most common and emerging biodegradable polymers that can be marketed. In parallel, additives are generally added to biodegradable polymers (between 0.05 to 70% (w/w)) to manipulate selected physical properties, such as toughness, flexibility, or barrier properties, thereby improving their functional capabilities (Brebu, 2020; Hahladakis et al., 2018). The most commonly used additives are: plasticizers, flame retardants, antioxidants, acid scavengers, light and heat stabilizers, lubricants, pigments, antistatic agents, slip compounds, and thermal stabilizers, as well as fillers and reinforcements (Hahladakis et al., 2018). For the near future, it will thus be interesting to assess the influence of such additives on the performances and stability of the AD processes, and their influence on the microbial communities. Recently, a number of biobased alternatives to additives have been developed, such as polyphenols (Diouf-Lewis et al., 2018). Nonetheless, the incorporation of such additives, even if they are environmentally friendly, should take into account their impact on the AD process, as such compounds have previously been reported to be inhibitory of anaerobic micro-organisms (Monlau et al., 2014).
- 2) **Morphology and properties of the biodegradable plastics:** The shape and size of biodegradable plastic wastes after their use, and especially their thickness and morphology

(*e.g.*, bags, films, bottles, food packaging.) should be considered to evaluate the biodegradability in AD process. These parameters will influence the biodegradation rate, incorporation in the digester, and the application of eventual pretreatments upstream of the AD process. The typology and the morphology of plastic supports can also affect the process later on by creating obstructions during the introduction in the biogas plants. Consequently, pretreatments can be required in some processes, especially for liquid CSTR system. Furthermore, the rate of biodegradation will also be influenced by the physicochemical properties of the polymers (*e.g.*, the chemical composition, crystallinity, porosity, accessible surface area, molecular weight). A better understanding of the correlation between the physicochemical properties of plastics and their biodegradation rate will be useful to determine key parameters and thus provide guidance to plastic manufacturers with their grading in order to develop plastics that are resistant during their use while still having biodegradability rates suitable to conventional HRT of industrial biogas plants.

- 3) **Pretreatments:** As previously mentioned, biodegradable plastics can be subjected to pretreatment strategies (*e.g.*, mechanical, thermal, and thermo-chemical) to enhance their biodegradability. In the future, results should be consolidated at a pilot scale to validate the promising results obtained in lab-scale and further drawn technico-economic analysis. In parallel, other pretreatment strategies including physical (Garuti et al., 2018) and biological processes (*i.e.*, enzymes, bacteria, and fungi) should be investigated (Brémond et al., 2018). The incorporation of fillers or enzymes is another interesting way for enhancing biodegradability. The incorporation of enzymes is still in its infancy and should be implemented at an industrial scale, although fibres and mineral additives have been shown to enhance the biodegradation of plastics and are hence a way to reduce the price of plastics (Hegde et al., 2018; Ryan et al., 2017b).
- 4) **AD process stability and performances:** Currently, the biodegradability of biodegradable plastics by AD is determined by protocols defined at the European and the international level. The methodology is based on BMP (biochemical methane potential) tests (Holliger et al., 2016; Raposo et al., 2011). Nonetheless, aside from their accuracy, BMP tests are very time-consuming (several days). In recent decades, alternative methods for determining

quickly the anaerobic biodegradability of organic wastes have been developed and can be probably transfer to assess the biodegradability of biodegradable plastics (Jingura and Kamusoko, 2017; Lesteur et al., 2011). Among these methods, the use of near-infrared spectroscopy (Mortreuil et al., 2018; Triolo et al., 2014); chemical composition model (Gunaseelan, 2007; F Monlau et al., 2012), quick respirometric tests (Lesteur et al., 2011), and the Envital kit® (Bellaton et al., 2016) have proven useful. Although all of these technologies are promising tools for fast biodegradability analysis, as they were developed for organic wastes, they need to be tailored to biodegradable plastics. In regard to the biodegradability of plastics by AD, it will also be important to consider the various typologies (CSTR, dry batch, dry continuous, etc.) of biogas plant available. Indeed, the biodegradability and the performances will differ according to the technologies and the operational conditions.

Aside from the technologies used, the operational parameters (*e.g.*, HRT, organic loading rate, temperature) will influence the biodegradation performances of biodegradable plastics. Assays in continuous mode are required, as batch tests that have been mostly used, which may fail to truly predict the performance of full-scale anaerobic reactors (Sambusiti et al., 2013a). The co-digestion of plastics with biowastes should also be better investigated in the future, as biodegradable plastics can counterbalance the low C/N ratio of some organic wastes such as food wastes and thereby further enhance the AD stability and performances. Finally, there is a paucity of information available in the literature regarding the influence of biodegradation of plastics on AD microbial communities.

- 5) **Post-treatment and agronomic quality of digestate:** Last but not least, digestate valorization should be carefully considered in the overall biodegradable plastics-AD chain. Generally, digestates are used for agronomic applications and are separated into solid and liquid phases through various processes (screw press, press filter, centrifuge, vibrating screen, etc.) (Akhiar et al., 2017; Guilayn et al., 2020). The liquid phase is generally used as a fertilizer whereas the solid phase is applied as a soil amender. In parallel, plant phytotoxicity tests of digestates involving biodegradable plastics should be managed in greenhouse and field-scale operations along with the influence of residual plastics and plastic additives on soil microbial communities. Aside from agronomic and safety

parameters (heavy metals, organic contaminants), it will be interesting to assess the influence of different digestate phase separation systems on the distribution of residual plastics. Indeed, incomplete degradation during waste management processes and leakage of bioplastics into the environment are becoming major concerns that need to be further investigated (Cucina et al., 2021a). It is of prime importance to better understand the becoming of the residual biodegradable microplastics present in digestates in a composting process or during soil application (Cucina et al., 2021a; Peng et al., 2022a). In the future, the research priority should be to gain a better understanding of the fate and the potential impact of microplastics and nanoplastics, as this is still largely unknown (Qin et al., 2021). As concerns ecotoxicity parameters, besides residuals biodegradable microplastics in the digestate, a particular attention should also be paid on the additive that can be liberated during the fragmentation of biodegradable plastics. Presently, common types of plastic additives are classified into stabilizers (to prolong lifespan of plastic products), plasticizers (to modify mechanical properties), antioxidants (to delay oxidation of plastics), pro-oxidants (to obtain faster degradation), surfactants (to promote surface properties), and other additives (to improve functionality) based on their different purposes (Qin et al., 2021). Finally, emerging valorization routes of digestates other than for agronomic purposes should also be considered if the digestate is not suitable for agronomic application (Monlau et al., 2015; Sheets et al., 2015).

- 6) **Normalization and standards:** The improvement of a normative and certification system on anaerobic digestion is a main challenge in the coming years in order to improve the perception of the general public and facilitate the comparison of results. The complexity will be to take into account the existing biogas plant diversities (dry batch, dry continuous, wet) and their operating conditions, which differ according to process temperatures and feeding (HRT around 30-50 days for biowastes, 50-120 days for agricultural wastes). If analytical and testing standards exist for the evaluation of biodegradability in both mesophilic and thermophilic conditions, there is clearly a lack of specifications norms (as is the case for composting, soil, and marine environments). Specification norms set the requirements, characteristics, and performance thresholds to be achieved during biodegradation trials.

Aside from the technical feasibility of the end-of-life of biodegradable plastics in the AD process, it is also important to consider the overall sustainability from an economic, environmental, and social perspective. In regard to the environmental impact, there have been few studies in the past several decades (Alarico et al., 2017; Piemonte, 2011) regarding comparison of the life cycle assessments (LCA) of various biodegradable plastics end-of-life (composting and anaerobic digestion) versus conventional plastic disposal (recycling, incineration, landfill, etc.). Alarico et al. (Alarico et al., 2017) investigated the LCA of the end-of-life of biodegradable plastics packaging in various scenarios, including incineration, landfill, composting, and anaerobic digestion. Interestingly, they found that AD had the lowest potential global warming impact among the various scenarios, mainly due to the recovery of a large amount of thermal energy and electricity in the process (Alarico et al., 2017). Piemonte (Piemonte, 2011) investigated the LCA for both PLA and Mater-Bi[®], taking into consideration composting, incineration, anaerobic digestion, and mechanical recycling processes as the final scenarios. They found that incineration, composting, and to a lesser extent anaerobic digestion processes clearly underperformed, from an environmental point of view, relative to mechanical recycling. Nonetheless, if the biodegradable plastics are contaminated with biowastes or with other organic wastes, mechanical recycling becomes complex and, therefore, anaerobic digestion can become an attractive option.



Figure VI.1. The main R&D perspectives that should be addressed in the future to enhance the recovery of biodegradable plastics by anaerobic digestion.

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