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Ulysse Brémond

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE MONTPELLIER SUPAGRO

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École doctorale GAIA – Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau
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(LBE, INRAE UR050)

Optimisation de la gestion des digestats : post-traitements et recirculation pour une amélioration de l'efficacité énergétique des sites de méthanisation

Soutenue par Ulysse Brémond

Le 26 Novembre 2019

Sous la direction d'Hélène Carrere et de Jean-Philippe Steyer

Devant le jury composé de

Rémy BAYARD, Maître de conférence, INSA Lyon

Patrick DABERT, Directeur de recherche, IRSTEA Rennes

Christelle WISNIEWSKI, Professeur, Université de Montpellier

Florian MONLAU, Chargé de recherche, APESA

Hélène CARRERE, Directrice de recherche, INRAE LBE Narbonne

Jean-Philippe STEYER, Directeur de recherche, INRAE LBE Narbonne

Aude BERTRANDIAS, Ingénieur de R&D, Air Liquide

Rapporteur

Rapporteur

Présidente du jury

Examineur

Directrice de thèse

Co-directeur de thèse

Invitée



UNIVERSITÉ
DE MONTPELLIER



Résumé

L'amélioration de la performance des sites de méthanisation agricole est un véritable enjeu visant à augmenter leur rentabilité et permettre ainsi à l'ensemble de la filière un développement pérenne. La gestion des digestats apparaît comme une piste d'amélioration encore relativement inexplorée. Cette thèse étudie la recirculation du digestat solide (éventuellement post-traité) au sein des méthaniseurs agricoles voie liquide. Dans un premier temps, les stratégies de mise en œuvre et l'impact potentiel de la recirculation directe du digestat solide sur six sites de méthanisation ont été étudiés. Les résultats ont montré qu'en fonction de la stratégie choisie, la production de biogaz du site de méthanisation pouvait être augmentée ou bien des pénuries éventuelles en intrants pouvaient être compensées. Dans un second temps, l'intérêt de l'ajout d'un post-traitement afin d'améliorer ces résultats initiaux a été évalué en détail. Les post-traitements thermochimiques et aérobies testés (aération simple, consortia ou champignons ligninolytiques) s'avèrent ne pas être rentables ou efficaces. Au vu de ces résultats, une recirculation rapide et directe du digestat solide sans aucun post-traitement semble être la meilleure option pour une mise en œuvre à grande échelle. Dans un troisième temps, il a été démontré que cette pratique augmente le temps de rétention des solides, la teneur en matière sèche et le coût de l'agitation des sites de méthanisation. Il faut ainsi déterminer au cas par cas, en fonction du site de méthanisation, l'intérêt de cette pratique. À cet effet, une aide à la décision à destination des exploitants a été établie. De là, un essai à grande échelle a été effectué et confirme que la recirculation du digestat solide n'a pas d'impact négatif sur le processus de méthanisation. Pour conclure, cette thèse démontre que la recirculation directe du digestat solide peut être un moyen simple et peu coûteux d'accroître l'efficacité des sites de méthanisation voie liquide.

Summary

Improving the performance of agricultural biogas plants is a key challenge in order to increase their profitability and thus ensure a long-term sustainable development to the entire biogas sector. Digestate management appears to be a way of improvement that has been until now relatively unexplored. This PhD thesis studies the recirculation of the solid digestate (possibly post-treated) within agricultural CSTR biogas plant. As a first step, implementation strategies and the potential impact of direct recirculation of the solid digestate for six biogas plants were studied. The results showed that, depending on the strategy chosen, plant biogas production could be increased or potential feedstocks shortages could be offset. In a second step, the interest of adding post-treatments to improve these initial results was evaluated in detail. The thermochemical and aerobic post-treatments tested (simple aeration, consortia or ligninolytic fungi) are not cost-effective or efficient. Regarding these results, a quick and direct recirculation of the solid digestate without any post-treatment seems to be the best option for full-scale implementation. In a third step, it has been shown that this practice increases the solid retention time, the total solids content and the mixing costs of CSTR biogas plants. The interest in this practice must therefore be determined on a case-by-case basis, depending on the biogas plants. To this end, a decision support for biogas plant operator has been established. Based on that, a full-scale trial was carried out and confirms that the recirculation of the solid digestate does not have any negative impact on the AD process. In conclusion, this PhD thesis demonstrates that direct solid digestate recirculation can be a simple and low-cost way to increase agricultural CSTR biogas plant efficiency.

*“Ideas are like rabbits. You get a couple and learn how to handle them,
and pretty soon you have a dozen.”*

John Steinbeck

« Ah bah mon frangin il a fait toutes les études qu'on pourrait faire, hein !

Il aurait même pu être notaire ou des conneries comme ça.

Enfin il est ingénieur à Grenoble... Je sais pas pourquoi. »

Coluche

« La mer

Qu'on voit danser le long des golfs clairs

[...] A bercé mon cœur pour la vie. »

Charles Trenet

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Résumé détaillé

1. Introduction et objectifs de la thèse

En maintenant les taux actuels d'émissions de gaz à effet de serre (GES), la température moyenne de la planète pourrait augmenter de 2,0 °C peu après 2060. La perte actuelle de biodiversité, l'élévation du niveau de la mer et les catastrophes climatiques majeures seront encore amplifiées par la hausse des températures. Ainsi, selon le rapport du GIEC, l'humanité doit limiter le changement climatique à 1,5°C pour éviter des conséquences majeures sur les écosystèmes et les populations. Cet objectif est toujours réalisable, mais cela implique d'atteindre l'objectif mondial de zéro émission nette de GES d'ici 2050 d'où la nécessité d'une transition énergétique avec entre autres le fort développement des énergies renouvelables (IPCC, 2018).

La méthanisation est une voie de production de ces énergies renouvelables. C'est un processus microbien qui se produit naturellement en l'absence d'oxygène et qui consiste en la conversion/minéralisation du carbone organique par un consortium microbien en biogaz, un mélange de gaz contenant principalement du méthane (50-75%) et du dioxyde de carbone. En Europe ce biogaz est produit dans de grandes installations automatisées (agricoles, territoriales, industrielles, stations de traitement des eaux usées) puis il est valorisé sous forme d'électricité (et de chaleur via cogénération) ou de biométhane, qui sont généralement injectés dans les réseaux nationaux. Outre le biogaz, du digestat (un mélange d'eau, de matières premières résiduelles non digérées, de micro-organismes et de matières inorganiques comme des minéraux) est produit toute l'année et peut-être utilisé comme un engrais organique de substitution aux engrais de synthèse.

Au cours des 20 dernières années, la production mondiale de biogaz a connu une forte croissance. Entre 2000 et 2016, elle a plus que quadruplé, passant de 0,28 à 1,31 exajoules ce qui représente une production annuelle de 61 millions de mètres cubes. L'Union européenne est le chef de file dans ce domaine avec fin 2017, 17783 sites de méthanisation en fonctionnement. À l'heure actuelle, les tendances du marché montrent que le secteur de la méthanisation est à un tournant de son développement. En effet, la méthanisation est en train d'évoluer d'un modèle encore dominé par les cultures énergétiques et la production d'électricité par cogénération vers un modèle plus durable et compétitif. Dans ce nouveau cadre, les biodéchets ménagers, les sous-produits agricoles (fumier, pailles...) ainsi que les cultures intermédiaires à vocation énergétique (CIVE) sont principalement utilisés et le biogaz transformé en biométhane pour diverses applications (transport propre, production de produits chimiques, chauffage...). Par ailleurs, l'âge d'or des tarifs de rachat élevés pour la production de biogaz semble être révolu. À la place, les nouvelles politiques adoptées en Europe visent à encadrer le déploiement du secteur et à réduire progressivement les montants des subventions. Ces politiques encouragent fortement le secteur de la méthanisation à réduire ses coûts de production notamment par : (i) l'utilisation de matières premières moins chères et plus durables ; (ii) l'amélioration du processus de méthanisation ; (iii) le développement de sources de revenus complémentaires (commercialisation des digestats/nutriments, valorisation du CO₂, monétisation des externalités positives...). Par conséquent, la recherche, les industries et le monde agricole doivent se concentrer sur ces

pistes d'amélioration afin d'accroître la rentabilité économique de la méthanisation et assurer un développement durable du secteur pour la période 2020-2030 et au-delà.

C'est dans ce contexte que s'inscrit cette thèse Cifre, fruit d'un partenariat entre Air Liquide et l'INRAE-LBE (Institut national de recherche pour l'agriculture, l'alimentation et l'environnement – Laboratoire de Biotechnologie de L'Environnement) et financée par l'ANRT (Association Nationale Recherche Technologie). Elle se concentre sur la méthanisation agricole en voie liquide et se base sur le constat que les gisements durables de déchets agricoles (fumier, pailles, CIVE) sont riches en lignocellulose, une matière première qui est plus difficilement dégradée lors de la méthanisation. Ainsi, l'optimisation des étapes de préparation des substrats (ex. prétraitements), de co-digestion et de gestion finale du digestat apparaît comme essentielle afin de récupérer un maximum de biogaz de ces matières premières récalcitrantes. Cette suite d'étapes est présentée **figure 1**.

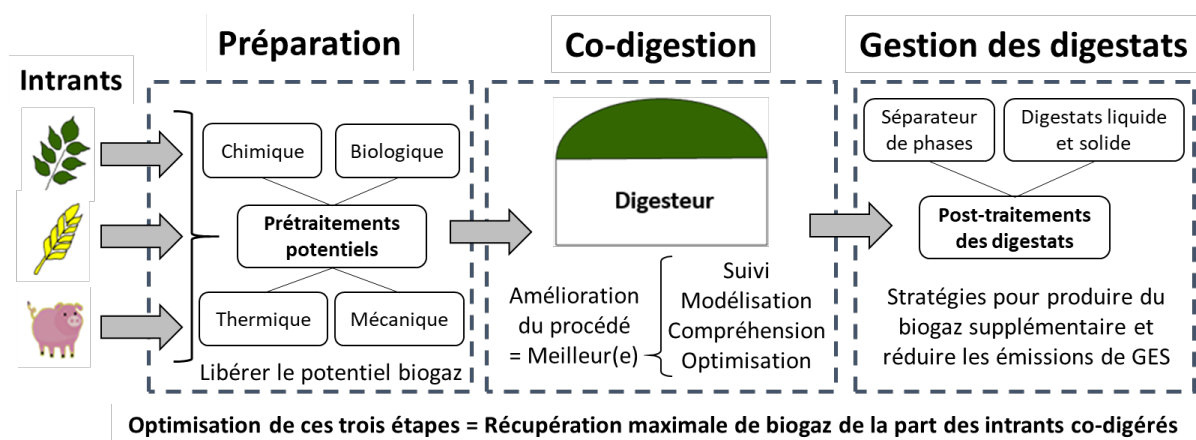


Figure 1: Les trois étapes principales de la méthanisation

Cette thèse s'intéresse plus particulièrement à la dernière étape du procédé et vise à explorer des solutions innovantes de gestion du digestat. Il a été démontré que couvrir et chauffer les cuves de stockage qui contiennent les digestats liquides ou bruts avant épandage sont des bonnes pratiques (Bacchetti et al., 2016; Lijó et al., 2017). En effet, de telles pratiques, aujourd'hui souvent mises en œuvre à grande échelle, permettent de produire davantage de biogaz, ce qui conduit à une plus grande efficacité énergétique et à réduire l'impact environnemental des sites de méthanisation agricole. Cependant, en ce qui concerne la gestion du digestat solide, aucune bonne pratique n'est clairement recommandée. Parmi les stratégies de valorisation existantes, la recirculation du digestat solide a été identifiée comme un moyen relativement peu coûteux et facile à mettre en œuvre qui peut favoriser la méthanisation des matières premières difficilement biodégradables et améliorer l'efficacité des sites de méthanisation (Monlau et al., 2015). Cependant, la viabilité économique des post-traitements appliqués au digestat solide ainsi que les conditions de sa recirculation au sein du méthaniseur ont été relativement peu explorées dans la littérature.

Ainsi, cette thèse a pour but de mieux comprendre l'impact de la recirculation du digestat solide sur les sites de méthanisation agricole en voie liquide. Les résultats obtenus peuvent être regroupés en trois axes de recherche principaux : (i) les stratégies et l'impact de la

recirculation du digestat solide ; (ii) l'intérêt des post-traitements ; (iii) les conditions de la recirculation du digestat solide à grande échelle.

2. Les stratégies et l'impact de la recirculation du digestat solide

Dans un premier temps au sein du Chapitre III les trois principales stratégies de recirculation ont été définies :

- (i) Le digestat solide peut être recirculé en plus de la ration initiale, auquel cas du biogaz supplémentaire peut être produit. Une telle stratégie sera appelée plus loin : « stratégie d'ajout ».

Le digestat solide peut également être intégré à la ration et remplacer certaines matières premières qui sont manquantes (pénurie/non produites volontairement) ou seront stockées dans le cadre d'une utilisation ultérieure (pénurie future prévue).

- (ii) Si une quantité suffisante de digestat solide est recirculée cela permet de compenser totalement la réduction des rations et de maintenir une production de biogaz stable. On parle alors de : « stratégie de remplacement »
- (iii) Si la quantité de digestat solide ne permet pas de compenser entièrement la réduction des rations, elle limite en partie la baisse de production de biogaz. Une telle stratégie sera appelée plus loin : « stratégie de remplacement partiel ».

Par ailleurs, il est important de distinguer la recirculation directe du digestat solide, où le digestat est simplement récupéré au niveau de la presse à vis et directement remis dans la trémie, de la recirculation avec post-traitements, où le digestat solide est sujet à un post-traitement avant d'être renvoyé dans le digesteur.

Les digestats solides provenant de six installations françaises de biogaz agricoles ou territoriales, équipés de presses à vis, ont été analysés (matière sèche, matière volatile, potentiel méthane, teneur en carbone et en azote, fractionnement de la matière organique). En nous basant sur les résultats de ces analyses ainsi que les caractéristiques de chaque site de méthanisation (quantités d'intrants, quantité de biogaz produit, quantité de digestat solide produit, tarif d'achat), nous avons quantifié l'impact potentiel de la recirculation directe sur la production de biogaz du méthaniseur. Nous avons estimé que le méthane produit par la recirculation directe du digestat solide représente entre 0,6 % et 5,7 % de la production totale de méthane du méthaniseur, en fonction du site étudié (taille du site, potentiel méthane du digestat solide) et du pourcentage total de digestat solide recirculé. En fonction de la stratégie choisie les conséquences diffèrent :

- (i) Dans le cas d'une stratégie d'ajout, du biogaz supplémentaire est produit ce qui mène à des gains économiques supplémentaires. Ceux-ci sont évalués entre 8 k€ et 83 k€ par an. Ces gains économiques ne sont pas négligeables pour les agriculteurs. En outre, la stratégie de recirculation directe n'implique pas besoin d'un coût élevé pour être mise en œuvre, car une partie du digestat solide produit est simplement renvoyée dans la trémie. Elle ne nécessite qu'un investissement très limité, composé d'un temps de travail journalier court (quelques minutes) et de l'utilisation d'un tracteur (carburant...).

- (ii) Dans une stratégie de remplacement, aucun gain économique supplémentaire n'est obtenu. En revanche, la recirculation du digestat solide pourrait intégralement compenser la production de méthane de 64 à 1431 tonnes d'intrants (valeurs variant en fonction du site, des intrants utilisés et de la quantité de digestat solide recirculé). Cela apparaît comme une alternative intéressante pour les agriculteurs par rapport à l'achat de matières premières. En effet, le digestat solide est produit à un coût très faible (coût de fonctionnement et amortissement du séparateur de phase).

3. L'intérêt des post-traitements

La deuxième étape de la thèse consistait à évaluer l'intérêt de l'ajout d'un post-traitement afin d'améliorer les résultats initiaux obtenus avec la recirculation directe. Cette partie correspond à une section du chapitre III et aux chapitres IV et V de la thèse. Des post-traitements thermochimiques et biologiques ont été étudiés sur deux digestats solides provenant de deux méthaniseurs agricoles représentatifs des sites traitant des matières premières complexes et difficilement biodégradables (ex. fumier).

Les post-traitements thermo-alcalins testés (NaOH et CaO) améliorent efficacement la biodégradabilité du digestat grâce à la dégradation de la fraction la plus complexe de la matière organique (fraction « ligneuse »). En utilisant les potentiels méthane des digestats post-traités, il a été estimé que par rapport à la recirculation directe de digestat solide, le post-traitement améliore entre 13% et 46% (pourcentage dépendant du digestat solide et du traitement appliqué) les gains économiques supplémentaires ou la quantité d'intrants pouvant être compensée (chapitres III et V). Toutefois, ces augmentations ne compensent pas les dépenses supplémentaires générées par ces post-traitements comme le coût des produits chimiques, la cuve de post-traitement, la main-d'œuvre supplémentaire. Par conséquent, l'ajout d'un post-traitement thermo-alcalin avant la recirculation du digestat solide ne semble pas économiquement viable dans les conditions testées.

Les prétraitements biologiques aérobies seraient moins coûteux et plus respectueux de l'environnement que les prétraitements thermochimiques, tout en présentant des résultats intéressants notamment sur les intrants lignocellulosiques (chapitre I). Il a donc été décidé d'étudier le post-traitement aérobie comme une alternative potentielle plus économique que les post-traitements thermo-alcalins. L'aération à court terme du digestat solide a été testée dans plusieurs conditions : aération simple (chapitre III), ajout d'une solution contenant de la lignine Kraft (chapitre III), ajout de consortia ligninolytiques (chapitre IV), ajout de champignons Basidiomycetes ligninolytiques (chapitre V). Un protocole simplifié est donné pour chacune de ces conditions ci-dessous :

- (i) L'aération simple a été évaluée sur les deux digestats solides (Chapitre III). Des bioréacteurs de 2,5 L équipés d'un système d'aération ont été utilisés. Les digestats solides ont été placés à l'intérieur et plusieurs courtes durées (entre 1,5 et 6 jours) ainsi que des débits d'air (entre 1,5 et 30 L/h/kg de matières sèches) ont été testés. La température a été fixée à 30°C pour éviter la phase thermophile du compostage. Enfin, la perte de masse due à la respiration a été évaluée à l'aide d'une méthode

où le CO₂ émis était piégé dans une solution de soude, dont la conductivité était mesurée.

- (ii) L'ajout en amont de l'étape d'aération simple d'une solution contenant de la lignine Kraft a été évalué sur les deux digestats solides (Chapitre III). En effet la lignine Kraft a été identifiée comme pouvant améliorer les activités endogènes ligninolytiques (Tian et al., 2017). Après son addition une aération de courte durée a été réalisée à 30°C, à faible débit (1.65 L/h/kg de matières sèches) et pendant 3 ou 6 jours toujours dans les bioréacteurs de 2,5 L. La perte de carbone due à la respiration du digestat solide a été mesurée comme précédemment.
- (iii) Les consortias ligninolytiques ont été obtenus à partir de six milieux naturels initiaux susceptibles de contenir des microorganismes d'intérêt (forêt, rumen...). Un enrichissement en continu (en utilisant de la lignine Kraft) et une sélection (sur du bois et de la paille) des microorganismes ligninolytiques aérobies ont été effectués en utilisant un système automatisé de chemostats interconnectés et opérés en aérobies (LAMACs) (Plouchart et al., 2018). Après plusieurs mois d'opération, les consortia sélectionnés ont été récupérés et propagés via des cultures en erlenmeyers. Il est à noter qu'une caractérisation poussée de l'évolution microbienne de ces consortia au cours de ces différentes étapes de sélection a été réalisée. Enfin, ces consortia liquides ont été ajoutés à un digestat solide stérilisé avant de réaliser un post-traitement aérobie à court terme (6 jours, 30°C, sans aération forcée).
- (iv) Finalement, l'impact de deux souches de champignons basidiomycètes filamenteux connues pour leur capacité ligninolytiques sur la paille a été évalué sur un digestat solide. Après une stérilisation nécessaire du digestat solide et en utilisant une dose de mycélium importante, les deux champignons ont été capables de le coloniser durant le post-traitement aérobie à court terme. Celui-ci a été réalisé dans un caisson de culture fermé où l'humidité a été maintenue proche d'un taux de 75 % et la température variait entre 20 et 25 °C. Plusieurs durées de colonisation ont été évaluées comprises entre 5,5 et 21 jours.

À chaque fois, à la suite des différents post-traitements, les potentiels méthane des digestats post-traités furent réalisés en prenant en compte la perte de matière liée à la respiration.

Pour toutes les conditions testées, la biodégradabilité du digestat solide n'a pas été améliorée, car aucune activité spécifique envers la fraction la plus complexe de la matière organique (fraction « ligneuse ») n'a pu être obtenue. La plupart des fractions accessibles de la matière organique du digestat solide (notamment les protéines), qui auraient donné du méthane dans des conditions anaérobies, ont toujours été respirées (perte de carbone sous forme de CO₂) pendant les différents post-traitements aérobies. Cela mène à des digestats solides avec un potentiel méthane réduit. Ces résultats sont dus à la spécificité de la matière organique du digestat solide (riche en protéines) qui pourrait favoriser d'autres activités microbiennes (protéolytiques...) plutôt que ligninolytiques. Par conséquent, le digestat solide ne semble pas

être une matière première appropriée pour les post-traitement aérobie, car le gain potentiel en biodégradabilité ne pourra pas compenser la perte de carbone liée à la respiration.

Au vu de ces résultats, une recirculation rapide et directe du digestat solide sans aucun post-traitement semble être la meilleure condition pour une application à grande échelle.

4. Les conditions de la recirculation du digestat solide à grande échelle

Les conditions de la mise en œuvre à grande échelle de la recirculation directe du digestat solide ont été explorées (Chapitre VI). De nouvelles équations ont été introduites afin de calculer l'impact potentiel de cette pratique sur le temps de rétention hydraulique, sur le temps de rétention des solides ainsi que sur la teneur en matière sèche d'un site de méthanisation. Il a été démontré que la recirculation directe du digestat solide entraîne une augmentation de la teneur en matière sèche de l'installation de biogaz. Cela peut entraîner une augmentation de la viscosité du digestat ainsi que du coût de l'agitation. Cependant, l'ampleur de cette augmentation varie grandement en fonction de la quantité de digestat solide recirculé, des intrants et du système d'agitation du site. La décision concernant l'intérêt de la recirculation du digestat solide pour une installation de biogaz doit être prise au cas par cas. La **figure 2** correspond à un diagramme de décision, basé sur les équations développées dans cette thèse, pour permettre aux opérateurs d'estimer l'intérêt potentiel de cette pratique pour leur méthaniseur.

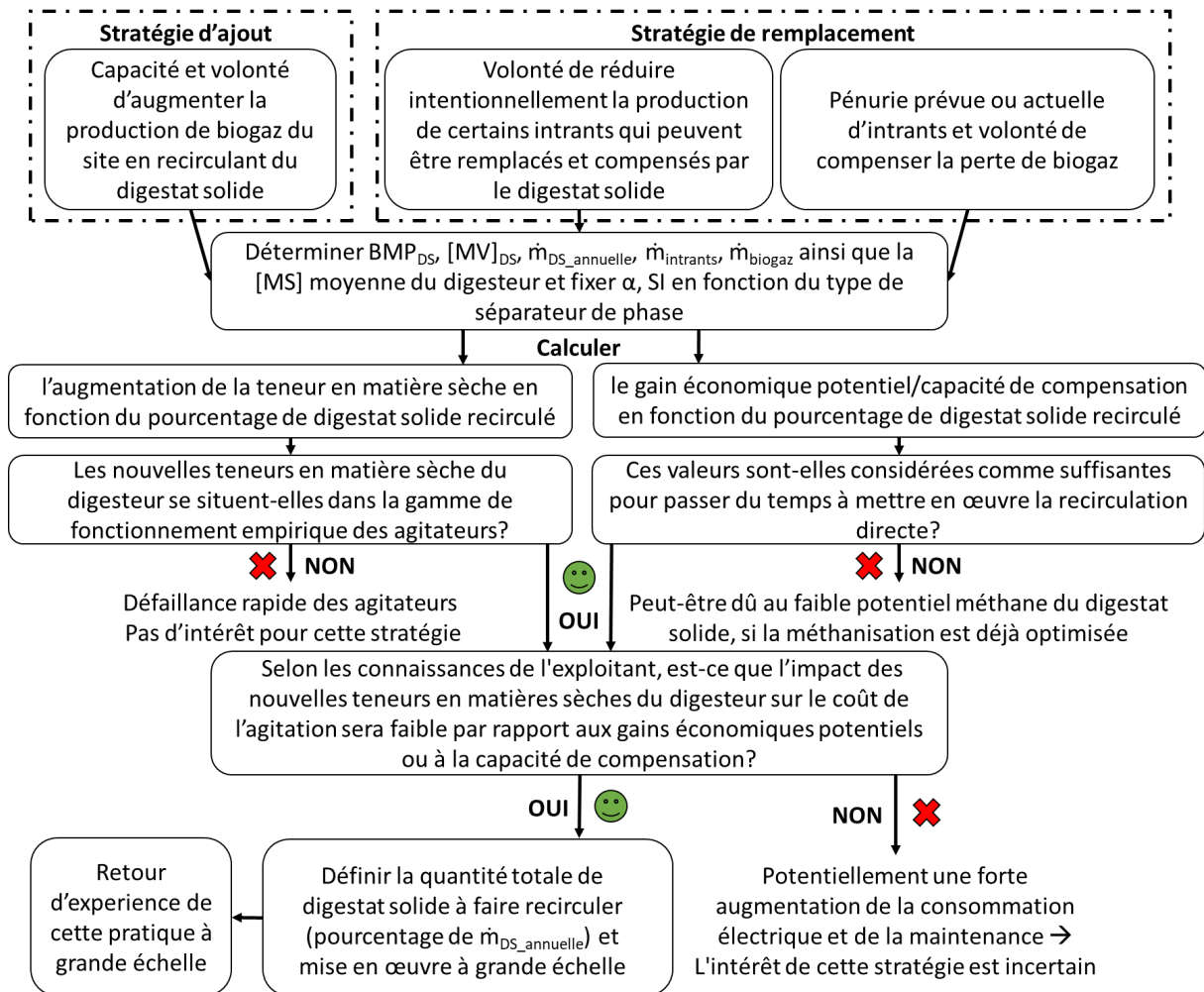


Figure 2 : Outil d'aide à la décision pour connaître l'intérêt de la recirculation directe du digestat solide (DS)

Enfin, sur une période de 10 mois un essai à grande échelle a été mené avec un méthaniseur agricole. Sur cette période le digestat solide a été recirculé tous les jours pendant 4 mois, en remplacement de 8 % des intrants solides selon une stratégie de remplacement partiel. Le principal résultat est que la recirculation directe du digestat solide n'a eu aucun effet négatif sur le fonctionnement du méthaniseur. Parallèlement, d'autres co-bénéfices à la recirculation du digestat solide, comme une hydrolyse accrue ou une capacité tampon plus élevée, peuvent être suspectés aux regards des résultats obtenus à grande échelle.

La figure 3, permet d'obtenir une vision globale du déroulement de la thèse.

Objectif de la thèse: Mieux comprendre l'impact de la recirculation du digestat solide sur les sites de méthanisation.
Trois axes de recherche principaux: (A) Stratégies et impacts
(B) Post-traitements
(C) Grande échelle

Chapitre III

Répond à l'axe de recherche Stratégies et impacts et partiellement à l'axe de recherche Post-traitements

- ✓ Trois stratégies de recirculation SD ont été définies : l'ajout, le remplacement et le remplacement partiel.
- ✓ Pour les six méthaniseurs évalués la recirculation directe du digestat solide permet:
 - A) suivant une stratégie d'ajout, d'augmenter la production de méthane entre 0,6 et 5,7%.
 - B) suivant une stratégie de remplacement, de compenser entre 64 et 1431 tonnes de matières premières.
- ✓ Les coûts des post-traitements thermo-alkalins ne sont pas compensés par les gains supplémentaires en méthane.
- ✓ Le potentiel méthane du digestat solide diminue après un post-traitement aérobie à court terme en raison d'activités endogènes non spécifiques envers la fraction « ligneuse » (la réorientation de l'activité en ajoutant une solution de lignine Kraft n'a pas réussi).

Répond partiellement à l'axe de recherche Post-traitements

Est-ce qu'il serait possible d'améliorer l'efficacité du post-traitement aérobie à court terme en ajoutant:
A) des consortia ligninolytiques microbiens?
B) des champignons ligninolytiques?

Chapitre IV

- ✓ L'ajout des consortia microbiens obtenus après criblage et bioaugmentation n'a pas amélioré l'efficacité du post-traitement aérobie à court terme.
- ✓ Diminution du potentiel méthane du digestat solide observée probablement en raison des activités non spécifiques des consortiums envers la fraction « ligneuse ».
- ✓ Une forte stérilisation par rayons gamma n'a pas été suffisante pour stériliser totalement le SD.

Chapitre V

- ✓ L'ajout de champignons ligninolytiques n'a pas amélioré l'efficacité du post-traitement aérobie à court terme: diminution du potentiel méthane du digestat solide.
- ✓ Les activités fongiques ne sont pas spécifiques envers la fraction « ligneuse » du digestat solide.
- ✓ La colonisation fongique nécessite la stérilisation du digestat solide
- ✓ Le post-traitement thermo-alkalin (utilisé comme moyen de stérilisation) dégrade spécifiquement la fraction « ligneuse » du digestat solide.

- ✓ Les caractéristiques spécifiques du digestat solide (riche en microorganismes endogènes, difficile à stériliser, riche en protéines) en font une matière première inadaptée aux post-traitements aérobies (aucune possibilité d'atteindre des activités spécifiques envers la fraction « ligneuse »).

Répond à l'axe de recherche Post-traitements et partiellement à l'axe de recherche Grande échelle

- ✓ Une recirculation rapide et directe semble être la meilleure condition pour une recirculation SD à grande échelle.
- ✓ Les post-traitements ne semblent pas nécessaires.

Chapitre VI

Répond à l'axe de recherche Grande échelle

- ✓ La recirculation directe du digestat solide augmente la teneur en matière sèche du digesteur, la viscosité du digestat et le coût de l'agitation. Le degré de cette augmentation variera considérablement en fonction de la quantité de digestat solide recirculé et du système d'agitation du méthaniseur.
- ✓ Les équations pour calculer l'augmentation potentielle de la teneur en matière sèche ainsi que l'aide à la décision pour les opérateurs de méthaniseurs ont été fournies. La gamme de fonctionnement du système d'agitation apparaît comme étant le point critique.
- ✓ La recirculation directe du digestat solide n'a eu aucun effet négatif sur le processus de méthanisation. D'autres essais doivent être réalisés pour évaluer pleinement l'intérêt de la recirculation du digestat solide et notamment les co-bénéfices potentiels qui pourraient exister (augmentation de l'efficacité de l'hydrolyse ?, pouvoir tampon).

Figure 3 : Déroulement de la thèse et principaux résultats obtenus

5. Conclusions générales

Cette thèse démontre que la recirculation directe du digestat solide peut être un moyen simple et peu coûteux d'accroître l'efficacité des sites de méthanisation agricole. Elle fournit notamment les clés pour déterminer au cas par cas l'intérêt de cette pratique pour un site donné. Sous certaines conditions, la recirculation du digestat peut être considérée comme une bonne pratique de gestion du digestat qui peut permettre d'augmenter la production de biogaz de l'ordre de quelques pourcents sans investissement supplémentaire et avec un faible

coût de fonctionnement. Cette augmentation potentielle n'est pas négligeable pour les exploitants d'installations de biogaz et peut être considérée comme un moyen pertinent de réduire le coût de production du biogaz.

Enfin, cette thèse souligne l'intérêt pour le secteur de la méthanisation de projets de recherche collaboratifs efficaces entre agriculteurs, recherche publique et industries afin de trouver des moyens d'optimiser les procédés de méthanisations existants et de réduire les coûts de production du biogaz. C'est l'une des clés pour assurer le développement à long terme du secteur de la méthanisation.

6. Perspectives

Suite à ces travaux, plusieurs perspectives émergent. Elles portent soit sur l'approfondissement des connaissances sur la pratique de la recirculation directe du digestat solide soit sur le secteur du biogaz en général :

- Il serait intéressant d'étudier plus précisément les co-bénéfices potentiels de la recirculation du digestat solide. Des études en laboratoire ou à l'échelle pilote devraient être effectuées pour démontrer clairement les activités d'hydrolyse potentiellement plus élevées en lien avec une charge microbienne augmentée ou bien la stabilisation du processus de méthanisation en lien avec le pouvoir tampon du digestat solide. De tels phénomènes seraient difficiles à évaluer à l'échelle réelle, car de nombreux paramètres varient et pourraient interférer.
- D'autres études à grande échelle devraient être effectuées pour confirmer pleinement que la recirculation directe du digestat solide selon une stratégie d'ajout peut améliorer efficacement la production de méthane du site de méthanisation. Les meilleures conditions seraient d'appliquer cette stratégie pendant une période où la ration est stable, avec une quantité de digestat solide recirculée intermédiaire (au moins $R \geq 0.1$, si la teneur en matière sèche du digesteur le permet) et enfin avec une meilleure connaissance des caractéristiques des intrants.
- Par ailleurs, dans cette thèse, nous nous sommes principalement concentrés sur l'impact de la pratique de la recirculation directe du digestat solide sur l'efficacité des méthaniseurs. Cependant, il serait également intéressant d'étudier l'impact de cette pratique sur les émissions potentielles de gaz à effet de serre d'un site de méthanisation, comme cela a été fait pour le digestat liquide (Baldé et al., 2016). Il est probable que cette stratégie sera également bénéfique sous cet angle, car le carbone converti en méthane pendant la recirculation aurait autrement été libéré sous forme de dioxyde de carbone pendant le compostage du digestat solide.
- Une étude similaire sur l'évolution des propriétés agronomiques des digestats lorsque cette pratique est réalisée serait intéressante, notamment à grande échelle. Elle pourrait répondre à la question suivante : "Les digestats solide et liquide récupérés seront-ils plus riches en composés complexes et humifiés et présenteront-ils de meilleures propriétés agronomiques ?"

- Concernant le domaine de la gestion du digestat, la recirculation du digestat liquide a également été identifiée comme une pratique potentiellement intéressante. Elle est déjà réalisée à grande échelle pour homogénéiser la biologie entre les différentes cuves et contrôler la teneur en matière sèche. La recirculation et l'utilisation du digestat liquide comme vecteur biologique pour la pré-hydrolyse de la biomasse lignocellulosique apparaît comme un prétraitement prometteur (Guan et al., 2018; Liu et al., 2019).
- De même que pour la gestion des digestats, l'agitation a été identifiée dans cette thèse comme un paramètre important pour les sites de méthanisation, car son optimisation pourrait conduire à une forte réduction des coûts de fonctionnement (Kress et al., 2018; Nsair et al., 2019). Cependant, les relations entre la teneur en matière sèche, la viscosité du digestat et le coût de l'agitation restent mal comprises, car un grand nombre de paramètres peuvent varier d'un méthaniseur à l'autre (matières premières, prétraitements, taille des particules, mucilage, équipement de mélange, température). Heureusement, ce sujet suscite de plus en plus d'intérêt (Singh et al., 2019). Des études permettant d'adapter et d'appliquer des conditions d'agitation optimales en fonction de la variation de la teneur en matière sèche ainsi que de la viscosité du digestat seraient d'un grand intérêt. En effet, leurs résultats se révéleraient importants pour optimiser les avantages potentiels de la recirculation directe du digestat solide et essentiels pour le secteur de la méthanisation comme moyen de réduire les coûts de production du biogaz.
- L'augmentation de l'efficacité des sites de méthanisation implique principalement de produire plus de biogaz. Cependant, pour les exploitants de méthaniseurs, ce n'est pas une priorité. En effet, aujourd'hui, la plupart des sites effectuant de l'injection, torche une petite partie de leur production, car elles ne peuvent pas injecter dans le réseau de gaz quand celui-ci est saturé. C'est là le principal frein perçu au développement de l'innovation incrémentale et de l'amélioration continue au sein des sites de méthanisation. Pour répondre à la question : « Pourquoi optimiser mon procédé et produire plus de biogaz que je ne le fais déjà, si je ne peux pas le valoriser ? » des solutions émergent actuellement qui peuvent être des installations de stockage du biométhane ou bien des installations de rebours qui rendent les réseaux de gaz bidirectionnels. Ainsi, le système LiLiBox développé par Azola (Bois-Colombes, France), permet de liquéfier et de stocker jusqu'à 60 000 Nm³ de biométhane qui peuvent être réinjectés dès que le réseau n'est plus saturé. Par ailleurs, dans le cadre du projet West Grid Synergy mené par GRTgaz (Bois-Colombes, France), la première installation de rebours en France à Noyal-Pontivy (France) devrait être opérationnelle fin 2019. Ces nouvelles solutions devraient permettre aux exploitants de site de méthanisation, à l'avenir, de se défaire des contraintes du réseau et de s'intéresser davantage à l'optimisation de l'ensemble de la chaîne du procédé de méthanisation (prétraitement, co-digestion, gestion du digestat) afin d'accroître l'efficacité des installations et la production de biogaz.

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List of abbreviations and symbols

α	Mass flow repartition factor between SD and LD
%Total_plant_CH4	Percentage of the total plant methane production
[TS] _{digester}	Concentration in total solids of raw digestate
[TS] _{feedstock}	Concentration in total solids of feedstock
[TS] _{LD}	Concentration in total solids of liquid digestate
[TS] _{SD}	Concentration in total solids of solid digestate
[VS] _{SD}	Volatil content of solid digestate
[VS] _{Spawn}	Volatil content of spawn
[x] _{supply}	Percentage of the feedstock x on the total feedstock supply
⁰ D	Hill number for q =0
¹ D	Hill number for q =1
² D	Hill number for q =2
AD	Anaerobic digestion
ADM1	Anaerobic digestion model n°1
AEg	Additional economic gains
APHA	American public health association
ATEE	Association Technique Energie Environnement
bcm	Billion cubic meters
Bd _{ana}	Anaerobic biodegradability
BMP	Biochemical methane potential
BMP _{direct_codigestion}	Biochemical methane potential of two codigested feedstocks
BMP _{direct_codigestion_Xdays}	Biochemical methane potential of two codigested feedstocks following X days of aeration
BMP _{FPTM}	Biochemical methane potential of final post-treated matter
BMP _{Spawn}	Biochemical methane potential of spawn
BMP _{Aerated_spawn_Xdays}	Biochemical methane potential of spawn aerated X days
BP _{NB}	Beet pulp - fraction non biodegradable
C/N ratio	Carbon/Nitrogen ratio
CAPEX	Capital expenditure
CCHP	Combined cold, heat and power valorization
CC _{NB}	Catch crop - fraction non biodegradable
CCS	Carbon Capture & Storage
CD _{NB}	Cereal dust - fraction non biodegradable
CF	Compost fertilizer
CHP	Combined heat and power valorization
CHP _{power}	Engine nominal power
COD	Chemical oxygen demand
COD _{model_x}	COD feedstock value used in ADM1
COD _{ratio_x}	COD ratio as a function of the feedstock supply
COD _{theoretical_x}	Theoretical COD feedstock value
COD _{tot}	Total chemical oxygen demand
CS _{NB}	Cattle slurry - fraction non biodegradable
CSTR	Continuously stirred tank reactor
d _{CH4}	Methane density
d _{CO2}	Carbon dioxide density

Dig	Digestate
Direct-CF	Direct compost fertilizer
DM	Dry matter
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
E_{elec}	Total electrical energy produced per year
EU	European Union
F1	Forest 1 - ground with wood decomposition
F2	Forest 2 - deep forest litter
F3	Forest 3 - rotten wood
FIT	Feed-in tariff
FM	Fresh matter
FOS	Volatil fatty acids content
GC	Gas chromatography
GES	Gaz à effet de serres
GHG	Green house gases
HHV	Higher heating value
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
iLUC	Indirect land-use change
IPCC	The intergovernmental panel on climate change
IWA	The international water association
k	Extrapolation coefficient
LAMACs	Lab-scale automated and multiplexed anaerobic chemostat system
LBE	Laboratoire de biotechnologie de l'environnement
LCFA	Long-chain fatty acids
LCOE	Leveled cost of electricity
LD	Liquid digestate
LHV	Lower heating value
LiP	Lignin peroxidases
LR	Loading rate
M	Weekly methane production
M9	Mineral culture solution
\dot{m}_{biogas}	Mass of biogas produced per day
MCCAs	Medium-chain carboxylic acids
MCHCA	Microbial consortium with high cellulolytic activity
$\dot{m}_{\text{feedstock}}$	Mass of feedstocks incorporated per day
\dot{m}_{LD}	Mass of liquid digestate produced per day
M_{NB}	Manure - fraction non biodegradable
MnP	Manganese peroxidases
\dot{m}_{out}	Mass of raw digestate leaving the digester per day
\dot{m}_{SD}	Mass of solid digestate produced yearly
\dot{m}_{SDyear}	Mass of solid digestate produced yearly during the initial year
$\dot{m}_{\text{SDyear+n}}$	Mass of solid digestate produced yearly during the year n (\neq initial)
$\dot{m}_{\text{SD_recirculated}}$	Mass of SD recirculated each year
MSW	Municipal solid waste
NEOM	Non-extractable organic matter
NGV	Natural gas vehicle

NMDS	Non-metric multidimensional scaling
OHPA	Obligate hydrogen producing acetogenic bacteria
OLR	Organic loading rate
OM	Organic matter
OPEX	Operational expenditure
OTU	Operational taxonomic unit
P₀	standard pressure (1015 hPa)
Pboiler	Operating power of the boiler
PBS	Phosphate buffer solution
PCA	Principal component analysis
P_{CH4_feedstock_full_scale}	Theoric methane production at full-scale for a given feedstock
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PEM	Proton exchange membrane
PEOM	Poorly extractable organic matter
Petri	Petri dish sample
P_i	Pressure in the flask at time i
PLS	Partial least square
PO	<i>Pleurotus Ostreatus</i>
PPE	Programmation pluriannuelle de l'énergie
PV	Photovoltaic
q	Hill number parameter
Q_{bioCH4}	Flowrate of biomethane injected in the gas grid per week
Q_{bioCH4_hour}	Flowrate of biomethane injected in the gas grid per hour
Q_{flare}	Nominal flow rate of biogas applied to the flare
qPCR	Quantitative polymerase chain reaction
R	Percentage of total solid digestate produced recirculated
RED	Renewable energy directive
REOM	Readily extractable organic matter
RMP	Residual methane potential
RMP_{corrected_SD}	Corrected SD RMP
RMP_{SD}	Residual methane potential of solid digestate
RMP_{treated_SD_Xday}	Residual methane potential of solid digestate aerated X days
RNA	ribonucleic acid
RPM	Rotation per minute
RS	Rotten straw
RU	Sheep rumen
RV_{digester}	Digester volume
RV_{post-digester}	Post-digester volume
S_{amino_acids}	Percentage of amino acids in the dissolved organic matter
SD	Solid digestate
SDR	Super dry reforming
SEOM	Slowly extractable organic matter
S_{fatty_acids}	Percentage of fatty acids in the dissolved organic matter
SI	Separation Index
SP	Screw press
SPOM	soluble extractable fraction from organic matter
SRA	<i>Stropharia rugoso-annulata</i>

SRT	Solid retention time
SS-AD	Solid-state anaerobic digestion
SSF	Solid-state fermentation
S_{sugar}	Percentage of sugar in the dissolved organic matter
T	Temperature
T₀	Standard temperature (0°C)
TAC	Alkalinity
T_{boiler}	Boiler operation ratio
TC	Total carbon
T_{flare}	Flare operation ratio
TN	Total nitrogen
TPAD	Temperature phased anaerobic digestion
TRL	Technological Readiness Level
TS	Total solids
UASB	Upflow anaerobic sludge blanket
V	Volume of the anaerobic digester
V_{biogas_year}	Volume of biogas produced by the plant per year
V_{CH₄_plant}	Volume of methane produced by the plant per year
V_{CH₄_SD}	Volume of methane produced by the solid digestate recirculated per year
VFAs	Volatil fatty acids
V_{headspace}	Headspace volume of the flask
VP	Versatile peroxidases
VS	Volatil solids
w/w	Weight/weight
WRF	White-rot fungi
X_{CH₄} Or X_{CH₄_biogas}	Concentration of methane (in %) in the biogas
X_{CH₄_biomethane}	Concentration of methane (in %) in the injected biomethane
X_{CH₄_offgas}	Concentration of methane (in %) in the offgas
γ_{CH₄i}	Percentage of methane at time i
Δ_{feedstock}	Mass of feedstock that can be offset via recirculation
ΔV_{CH₄}	Volume of methane produced between each GC analysis (BMP test)
η_{CH₄}	Extraction rate of the upgrading unit
Υ_{CH₄}	Average methane yield from feedstock

Publications and communications

Publications

Brémond, U., de Buyer, R., Steyer, J. P., Bernet, N. & Carrère, H., *Biological pretreatments of biomass for improving biogas production: an overview from lab scale to full-scale. Renewable and Sustainable Energy Reviews, 2018, 90, 583-604.*

Brémond, U., Bertrandias, A., Loisel, D., Jimenez, J., Steyer, J. P., Bernet, N. & Carrère, H., *Assessment of fungal and thermo-alkaline post-treatments of solid digestate in a recirculation scheme to increase flexibility in feedstocks supply management of biogas plants. Renewable Energy, 2020, 149, 641-651.*

Brémond, U., Bertrandias, A., Steyer, J. P., Bernet, N. & Carrère, H., *A synthetic vision of European biogas sector development towards 2030 and beyond: Trends and challenges. Submitted to Journal of Cleaner Production, 2020.*

Brémond, U., Bertrandias, A., de Buyer, R., Latrille, E., Jimenez, J., Escudié, R., Steyer, J. P., Bernet, N. & Carrère, H., *Recirculation of solid digestate to enhance energy efficiency of biogas plants: strategies, conditions and impacts. Submitted to Applied Energy, 2020.*

Brémond, U., Bertrandias, A., Hamelin, J., Milferstedt, K., Bru-Adan, V., Steyer, J. P., Bernet, N. & Carrère, H. *Screening and bioaugmentation of ligninolytic microbial consortia to enhance aerobic degradation of solid digestate. To be submitted to FEMS Microbiology Ecology, 2020*

Oral presentations

Brémond, U., Grégory, R., Steyer, J. P., & Carrère, H. *Etat des lieux des prétraitements biologiques pour la production de biogaz : du laboratoire vers une échelle industrielle ? Journées Recherche Innovation (JRI) Biogaz, Beauvais, France, 2017.*

Brémond, U., Bertrandias, A., Steyer, J. P., & Carrère, H., *Post-treatments and recirculation of agricultural solid digestates: impact on full-scale methane yield. 16th IWA World Conference on Anaerobic Digestion (AD 16), Delft, The Netherlands, 2019.*

Brémond, U., Bertrandias, A., Quaak, M., Jimenez, J., Escudié, R., Steyer, J. P., & Carrère, H., *Conditions pour l'implémentation de la recirculation du digestat solide à grande échelle : Théorie et mise en pratique. Journées Recherche Innovation (JRI) Biogaz, Toulouse, France, 2020.*

Brémond, U., Bertrandias, A., Steyer, J. P., & Carrère, H., *Assessment of fungal and thermo-alkaline post-treatments of solid digestate in a recirculation scheme. 8th International Conference on Engineering for Waste and Biomass Valorisation (WasteEng20), Guelph, Canada, 2021.*

Brémond, U., Bertrandias, A., Jimenez, J., Escudié, R., Steyer, J. P., & Carrère, H., *Solid digestate recirculation at full scale in anaerobic digesters. 6th BioProScale Symposium, Berlin, Germany, 2021.*

Introduction

The Special Report on the impacts of global warming of 1.5°C above pre-industrial levels, published in October 2018 by the Intergovernmental Panel on Climate Change (IPCC) is the last-born of a long series of international scientific reports alerting about the impact and the evolution of global warming, that started almost fifty years ago with “The Limits To Growth” from the Club of Rome (IPCC, 2018; Meadows et al., 1972). At the dawn of 2020, it is reported that human activities have caused approximately 1.0°C of global warming above pre-industrial levels. By maintaining current greenhouse gases (GHG) emission rates, average global temperature could reach a 2.0°C increase soon after 2060. Current loss in biodiversity, rising sea levels and major weather disasters will be amplified as temperature is rising. According to the IPCC report, the world needs to limit climate change to 1.5°C to avoid unacceptable consequences on ecosystems and populations. This objective is still doable but it means reaching worldwide net-zero emissions by 2050 and implies a quick, strong and long-term international climate action (IPCC, 2018). The Paris agreement adopted in 2015 by 195 countries and its rulebook decided in the COP24, early 2019, are the first steps towards a global energy transition.

In the wake of the IPCC report, the European Union (EU) presented its strategic long-term vision to reach a climate-neutral Europe by 2050, in which the EU expresses its willingness to be a global leader in the fight against climate change and to be among the first to reach net-zero GHG emissions by 2050 (COM(2018)-773, 2018). A first move was to adopt, in December 2018, a revised version of the renewable energy directive (RED II) with higher targets than initially set for 2030 (DIRECTIVE-(EU)-2018/2001, 2018). Thus by 2030, GHG emissions have to be reduced by at least 40% in comparison to 1990 level and the share of renewable energy is set at a target of at least 32% (instead of 27% and with a clause for a possible upwards revision by 2023). Recent scenarios agreed on the fact that renewable electricity will be essential to fully decarbonize the energy system and it is forecasted that electricity production will at least double, mainly relying on a strong increase in number of solar and on- and off-shore wind facilities (COM(2018)-773, 2018; Eurelectric, 2018; Gas for Climate, 2019a). However, for Dr. Klaus-Dieter Borchardt, Europe’s Director of the Internal Energy Market Integration, pathways towards a decarbonized economy only relying on renewable electricity will not be cost effective and will be technologically impossible mainly due to its storage issue using batteries (Florence School of Regulation, 2019). The optimum solution may lie in a “dual” gas-power network, where “gas infrastructure should become the batteries of the future European energy system”(Bowden, 2019; Brun, 2018). Therefore, integration and decarbonisation of the gas sector is currently at the top of the political agenda in Brussels with notably the preparation of the 2020 gas package that should set a clear vision on the role of gas up to 2050. It will most likely propose renewable gas as well as decarbonised gas targets (Olczak and Piebalgs, 2019). Developments of hydrogen (green and blue) and biomethane production, power-to-gas as well as Carbon Capture & Storage (CCS) are envisioned as the vector of decarbonisation of the gas sector (Gas for Climate, 2019b). In the end of 2017, annual production of biomethane from anaerobic digestion (AD) in Europe corresponded to 2 billion cubic meters (bcm) and it is estimated that it could reach 62 bcm by 2050 in the case of an

optimized gas scenario (Gas for Climate, 2019a). If integration of the gas sector into the EU decarbonisation strategy is validated, AD will continue to grow, but it is clear that the sector is at a turning point that should see a strong shift in its development paradigm.

Current market trends show that the AD sector is undergoing a change from a model still dominated by energy crops and electrical production via combined heat and power (CHP) units towards a model where organic wastes, agricultural by-products as well as sequential crops are mainly used as feedstocks and biogas is upgraded to biomethane for various applications (transportation, chemicals production, heat...). Besides, in Europe, the golden era of high feed-in tariffs for biogas production appears to be over. Instead, observed trends in biogas policies are regulation of sector deployment and progressive reduction of subsidy schemes. These policies are strongly pushing the AD sector to reduce biogas production costs notably by: (i) using cheaper and more sustainable feedstocks; (ii) improving the AD process; (iii) developing additional sources of revenues (commercialization of digestate/nutrients, CO₂ valorization, monetization of co-benefits...). Therefore, research, industries and farmers need to focus on these improvement tracks to increase biogas economic competitiveness and ensure sustainable development of the AD sector on the 2020-2030 period and beyond.

Air Liquide is a world leader in gases, technologies and services for Industry and Health. In the context of its global approach to climate, Air Liquide is engaged to the development of a low-carbon society based on hydrogen and biomethane. As part of the Blue Hydrogen program, Air Liquide produces green or blue hydrogen from water electrolysis using Proton Exchange Membrane (PEM), from biomethane reforming and from natural gas reforming combined with carbon capture and upgrading technology. Both can be used for various purposes and notably clean mobility. Air Liquide currently has more than 100 hydrogen stations worldwide. Concerning biomethane, Air Liquide is a major player in the downstream part of the sector with 100 biogas upgrading units (based on membrane technology) worldwide with an upgrading capacity of 160,000 m³/h and more than 60 bioNGV filling stations in Europe and 10 multi-energy stations. Air Liquide is strongly committed to deploying biomethane for clean mobility. Indeed, in comparison to conventional fossil fuels, running on biomethane can reduce noise by 50%, NO_x emissions by 80%, particulate matter by 90% and display low to even negative CO₂ emissions (in the case of biomethane produced from manure) (European Commission, 2014).

Air Liquide has recently started to move up the value chain and aims to become not only a player in the field of biogas upgrading but also in the field of biogas plant operation. By doing so, Air Liquide would become a biogas producer allowing it to secure its biogas supply. Equity investments in the biogas plant manufacturer Fonroche Biogas (5%) in 2015 and in the agricultural biogas plant of Pot-au-Pin (20% – nearby Bordeaux in France – see **figure 4**) in 2016 are the first steps in this direction. Therefore, since 2015, Air Liquide has been looking for innovative solutions to improve the anaerobic digestion process and reduce biogas production costs. These solutions could be implemented in future biogas plants where Air

Liquide would be involved, or commercialized in addition to the existing upgrading process. This approach is at the origin of this Cifre PhD, that was started by the end of 2016, in collaboration with the INRAE-LBE (Institut national de recherche pour l’agriculture, l’alimentation et l’environnement – Laboratoire de Biotechnologie de L’Environnement). It is a French research unit with 80 full-time equivalents. Research carried out at the INRAE-LBE aims to develop the concept of environmental biorefinery, which consists in treating the by-products of human activities (waste, agricultural residues, effluents) and recovering them as resources of industrial interest (bioenergy, biomolecules, organic amendment), while minimizing their environmental and health impact. In that framework, the INRAE-LBE is notably an academic leader on the topic of anaerobic digestion with more than 25 years of experience.



Figure 4: Pot-au-Pin biogas plant – view from a covered silage pit (@Constant Formé-Bécherat)

The framework of this PhD focuses on gaining a higher biogas yield from agricultural feedstocks and more precisely from lignocellulosic residues, such as straw and manure, that should be more and more incorporated to the ration in future. It is in line with previously stated improvement tracks for the biogas sector as it aims to make use of cheaper lignocellulosic feedstocks and improve the subsequent AD process.

The objective of this PhD is to study original solid digestate management schemes to further anaerobically digest feedstocks and improve biogas yield. Direct recirculation into the digester of solid digestate and the effect of various post-treatments before recirculation were evaluated at lab scale. Their potential effects for several full-scale plants were then evaluated based on these results. Besides, conditions of application of solid digestate recirculation

strategies were also studied and a full-scale trial was performed to evaluate one specific recirculation strategy. Good practices and recommendations for solid digestate management are important outcomes from this PhD.

This manuscript is divided in 7 main sections. Chapter I consists in an introductory literature review, where the biogas sector, principles of anaerobic digestion, identified innovative pre/post-treatments and PhD research questions are described. Subsequently in Chapter II, main materials and methods used in several chapters of this PhD are presented. Chapter III, focuses on solid digestates characterization and economic assessment of direct recirculation as well as three different post-treatments (thermo-alkaline, simple aeration and boosting solution combined with simple aeration) applied before recirculation. Conditions for recirculation of solid digestate are also discussed in this part of the manuscript. Chapter IV, reports screening and selection strategies to obtain ligninolytic microbial consortia. These consortia were further used to enhance aerobic post-treatment of solid digestate before its recirculation. Chapter V investigates the interest of applying a fungal post-treatment on solid digestate to increase its methane yield during subsequent recirculation. Chapter VI discusses the impact of implementation of direct recirculation at full-scale and presents results from a full-scale trial where solid digestate was recirculated, replacing feedstocks in the ration. Finally, Chapter VII ends the manuscript with a discussion regarding results obtained, a summary of the main conclusions and a set of associated perspectives. Appendices briefly describe additional experiments carried out during the PhD. Transitions are inserted between each chapter presenting results, in order to facilitate reading and detail the progress of the thesis.

Chapter I

Literature review and objectives

1. Trends and challenges in the biogas sector: a vision for 2030 and towards

Adapted from Brémond et al., A synthetic vision of European biogas sector development towards 2030 and beyond: Trends and challenges. Submitted to Journal of Cleaner Production, 2020.

1.1. Worldwide and European biogas production

Over the last 20 years, biogas production worldwide from AD has experienced a strong growth. Between 2000 and 2016, global biogas production was more than quadrupled, from 0.28 to 1.31 exajoules (EJ – 10^{18} joules) which corresponds to a global yearly volume of 61 bcm biogas: it is shared mainly between Europe (54%), Asia (30%) and Americas (14%) (World Bioenergy Association, 2018). Biogas production and its utilization display great disparity between developing and developed countries. For the former, biogas is mainly produced from small-scale low-tech household plants in order to provide gas for cooking and lighting and reduce firewood consumption, whereas for the latter biogas is produced from large scale automated and monitored plants and valorized under the form of electricity or biomethane, which are generally injected in national grids (McCabe and Schmidt, 2018; Treichel et al., 2019; Vasco-correa et al., 2018). With 17,783 biogas plants in 2017 and 12.6 GW installed in 2018, Europe is the world leader in biogas electricity production, far ahead the USA (2.4 GW) and China (0.6 GW) (EBA, 2018; IRENA, 2019). European biogas electricity production represents 68% of the global electricity capacity from biogas plants estimated at 18.1 GW. This last value represents only 0.7% share of the worldwide renewable electricity production (2,351 GW) mainly dominated by hydropower (52%), onshore wind (23%) and solar (20%), as represented in **figure 5** (IRENA, 2019).

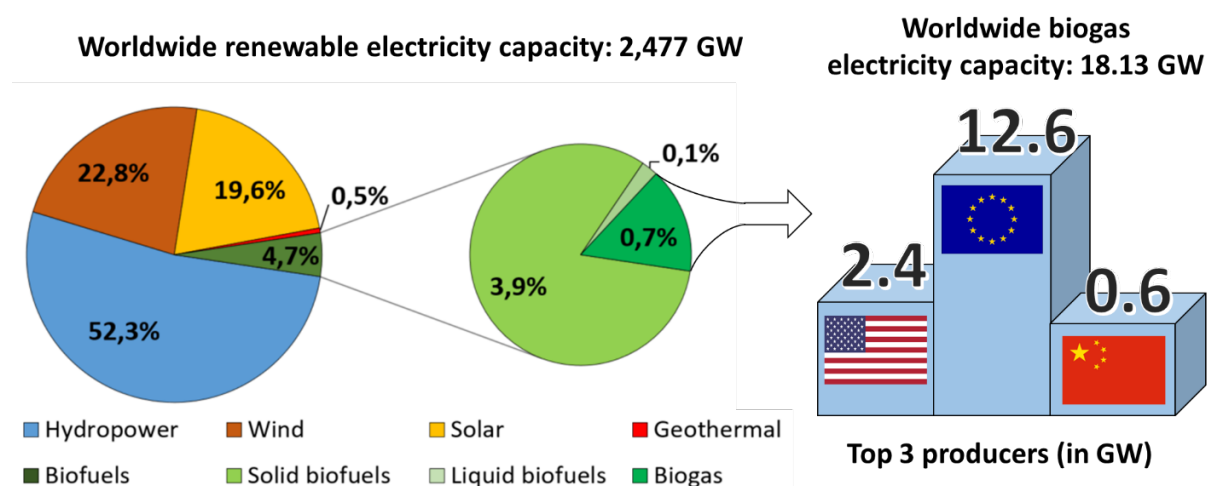


Figure 5: Worldwide renewable electricity capacity and share of biogas sector in 2018

Thus, early 2019 in the EU, over 90% of the biogas is valorized directly on-site, through CHP units (Eyl-Mazzega and Mathieu, 2019; Verbeeck et al., 2018). This is the result of energy policies, set between 2000 and 2015, from several state members based on subsidies incentives for this technology (Eyl-Mazzega and Mathieu, 2019; Vasco-correa et al., 2018). However, recent decline in subsidy levels can explain the slowdown of the biogas industry in

the last few years (EurObserv'ER, 2018). To better understand the possible future of AD in the EU and current market driving trends, it is relevant to look at its recent history in some European countries.

1.2. Market driving trends in biogas sector from relevant European countries

Germany, the European biogas leader with 9,500 biogas plants at the end of 2018, has built its biogas sector on the basis of the Renewable Energies Act that started in 2000 (EBA, 2018). This act forced energy supply companies to buy electricity generated from renewable sources at guaranteed feed-in tariffs (FIT) over a period of 20 years. Combined with strong incentives in 2004 and 2009 for energy crops cultivation, the biogas industry boomed between 2000 and 2012. It was mainly technologically based on a “standard” liquid continuously stirred tank reactor (CSTR) plant using energy crops (especially maize) coupled with cattle liquid manure that permits to ensure high and resilient biogas production valorized via a CHP unit (Theuerl et al., 2019). From this industry scheme, several drawbacks have been pointed out, that are mainly:

- (i) The important use of energy crops has led to the “maizification” phenomenon (wide spreading of maize monoculture over 2.1 million ha) that diminished beneficial environmental impact of biogas production as well as public acceptance of the technology and created agricultural distortions such as the increase of land rental prices, the increase of energy crops prices (also used for cattle feeding) and therefore the difficulty for smaller farms or biogas exploitations to thrive (Appel et al., 2015; Hijazi et al., 2016; Markard et al., 2016).
- (ii) Electricity production cost from biogas remains high in comparison to other types of renewable energies. Indeed, solar photovoltaics (PV) and onshore wind have known since 2010 a strong diminution in their leveled cost of electricity (LCOE) that may reach by 2020 respectively 0.06 and 0.05 USD per kWh, while biogas LCOE has remained quite constant over time close to 0.08-0.09 USD per kWh (IRENA, 2018);
- (iii) A lack of efficient utilization of the heat produced from the CHP as local heat sink is missing, especially in rural areas. It was thus estimated that at least a third of the biogas produced was subject to such lack of heat recovery (Strauch et al., 2013). This is strongly impacting CHP valorization efficiency, biogas plant economic viability and its interest over biogas upgrading valorization to biomethane (Lantz, 2012; Lee, 2017; Pöschl et al., 2010).

These drawbacks have led to regulatory changes for new biogas plant since 2012. Energy crop intake is limited (the maize-cap), FIT progressively reduced and replaced (except for small biogas plants below 150 kW) in 2017 by auctions (Theuerl et al., 2019). The aim was to control sector development following a “deployment corridor” set at 150 MW annually from 2017-2019 and 200 MW annually from 2020-2022 and only allow construction of the most economically competitive plants (Appunn, 2016). All these shifts have led to cease the growth of the biogas sector and auctions in 2017 and 2018 did not reach the targeted power set by the German government (only 76.5 MW in 2018), proof that electricity production from biogas

is suffering competition notably from solar PV (Hill, 2019; Tsanova, 2018). Even flexible biogas plants, using specific equipment (storage facilities, larger generators) that enable them to provide their electricity production offset from wind and solar production, have seen their premium FIT, initially set in 2012, gradually decline, so that today the concept of producing power from biogas flexibly is no longer economically viable (Lauven et al., 2019). By 2020, biogas plants commissioned in 2000 will be the first to see their guaranteed FIT expired and the question of plant shutdown will be raised if no new policy for biogas power generation is undertaken soon (Theuerl et al., 2019). Biogas upgrading to biomethane has been identified as a good alternative to power and heat generation as it can be stored in the natural gas grid. It can then be used for clean transportation or in urban CHP units, where heat is efficiently valorized (Scarlat et al., 2018a). While the biomethane sector remains minor in Germany and has stalled at around 200 biogas plants since the deletion of the biomethane upgrading bonus in 2014, other EU state members have already based their biogas strategy on biomethane instead of power generation (Eyl-Mazzege and Mathieu, 2019; Scarlat et al., 2018a). **Figure 6** displays a short summary of current situations and applied biogas policy in Germany and countries thereafter that are favoring biomethane.

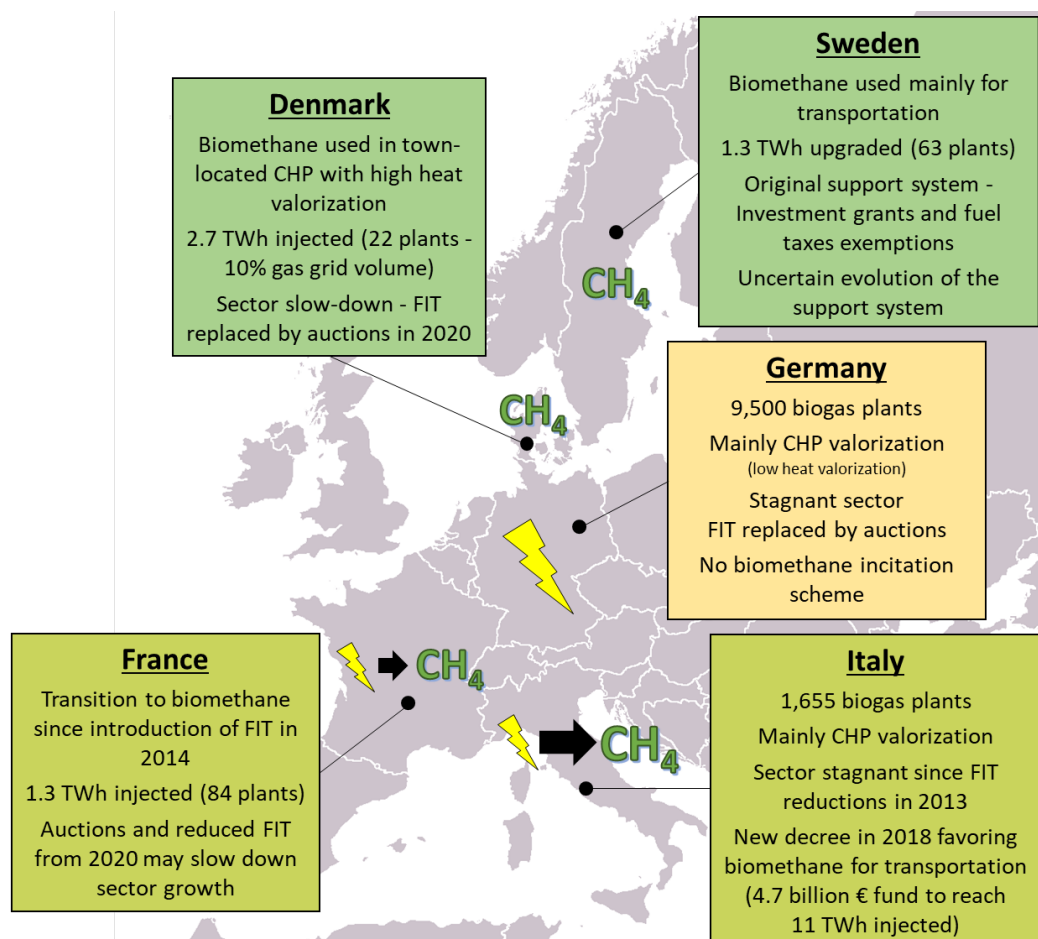


Figure 6: Current biogas sector and applied biogas policy in some EU member states

- : CHP conversion
- : Biogas upgrading
- : Country historically developing CHP
- : Country historically developing upgrading
- : Country shifting from CHP to upgrading

In 2016, biomethane contributed to only 11% of generated energy from biogas in the EU. This is because, the sector started its expansion later than power generation, due to a lack of associated subsidies and a higher cost for upgrading units than CHP units, especially for small plants (Kampman et al., 2016). However, between 2011 and 2018 the number of plants in the EU has tripled going from 187 to more than 540 mainly due to the implementation of incentive policies in several state members (EBA, 2018). Historically Sweden and more recently Denmark have been leaders in development of the biomethane sector.

Sweden has developed biomethane mainly for the transport sector via a network of filling stations as there is no comprehensive gas grid infrastructure in the country. In 2016, 64% of the 2 TWh annual biogas production was used in transportation under the form of compressed or liquefied biomethane provided to a fleet of 55,000 gas vehicles (Sverige Energigas, 2018). Instead of applying FIT, Sweden has historically backed biomethane for transportation via investment grants and exemptions from fuel taxes that make the sector competitive against gasoline and diesel (Larsson et al., 2016). These funding plans are running until 2020, but the Swedish government, despite the new climate policy framework that aims to achieve a 70 percent reduction in emissions from transport by 2030, is still unclear on how the support system will evolve. A proposal biogas production goal for 2030 was recently suggested at 15 TWh (Sverige Energigas, 2018).

Denmark, has a much more developed gas network and had mainly based its strategy on injecting biomethane into the grid that is subsequently used in town-located CHP with district heating systems. The sector has boomed between 2014 and 2018 due to the introduction of 20-year feed-in-premiums for biomethane injection. Annual biomethane capacity has bounced from almost zero in 2014 to 2.7 TWh and now accounts for more than 10% of volumes transported in the natural gas grid (Eyl-Mazzega and Mathieu, 2019). However, this has been accompanied by a massive increase of subsidies granted that reached 215 million euros in 2017. Therefore, a new subsidy scheme part of the Energy Agreement has been adopted in 2018. It will start in 2021 and is based on a yearly 32 million euros pool that will be assigned to new projects in tenders with price ceilings (Ministry of Climate Energy and Utilities, 2018). Danish biomethane sector growth potential will thus be limited from 2020, similarly to the “deployment corridor” in Germany.

In France, future energy policy may also challenge and contain the biomethane sector development. Until now, biomethane has known a strong rise with 84 operating plants early 2019 (+73% increase versus 2017) due to FIT introduced in 2014. The plants are mainly connected to the gas grid and produce a yearly 1.3 TWh (Sia Partners - Energy Lab, 2019). Besides, national studies from ADEME and the négaWatt association demonstrated, in several scenarios, the future importance of anaerobic digestion and biomethane to reach both neutral emissions by 2050 and a 100% renewable gas system (ADEME, 2018; Association négaWatt, 2017). However, the recent proposal of the energy strategy plan for 2030 (Programmation Pluriannuelle de l'Énergie, PPE) has lowered the initial target set at 10% of biomethane of total

gas demand to only 7%, introduced yearly auctions of 700 GWh and sharply decreased FIT by 2023 and 2028. These imply for the sector both limited growth and a quick decrease necessary in biomethane production cost (Ministère de la transition écologique et solidaire, 2019). For instance, FIT of an agricultural plant injecting 200 Nm³/h, may be reduced by 40% in 2028 (from current 101€/MWh to 60€/MWh) and even more for smaller capacity plants. Potential positive externalities of anaerobic digestion (figure 7), which are estimated to represent an additional value comprised between 55 and 75€ per MWh produced in 2030, were not taken into account by the government (Enea Consulting, 2019). Thus, the biomethane sector is currently lobbying, insisting on these positive externalities, to amend the PPE text and render it more favorable, before its adoption by the end of 2019.

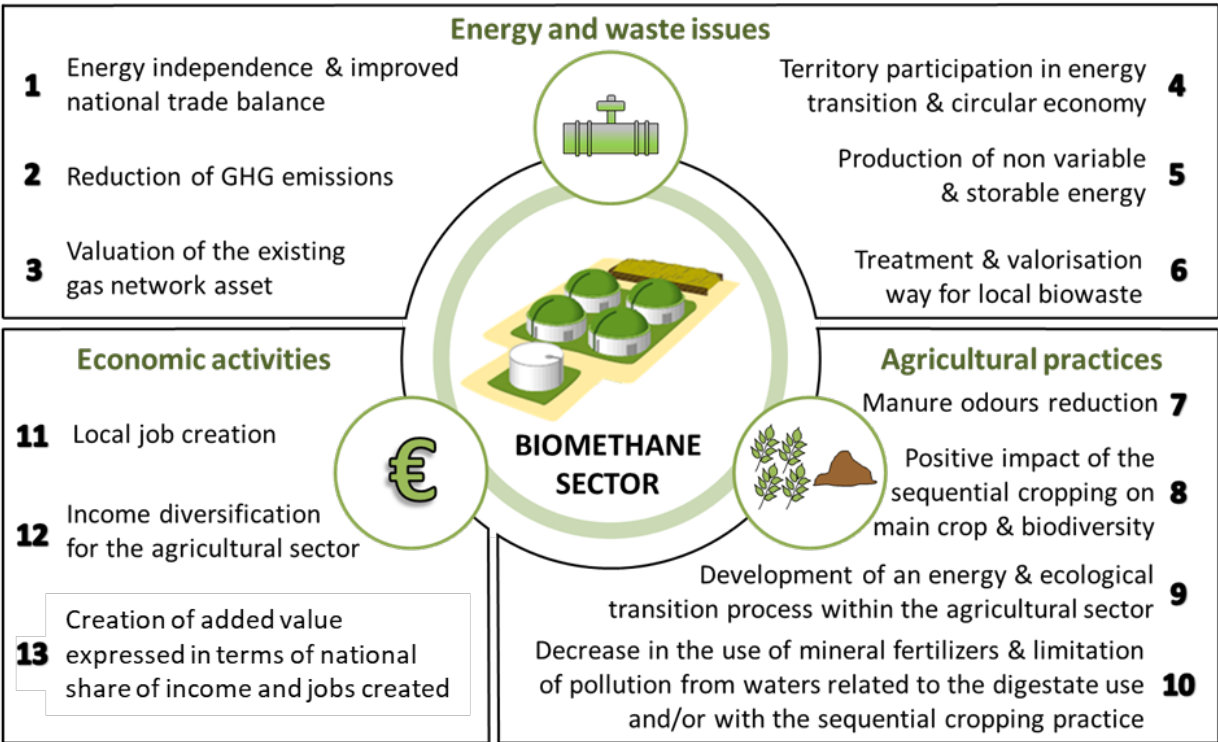


Figure 7: Identified positive externalities of the development of the biomethane sector, adapted from (Enea Consulting, 2019).

Finally, Italy is currently the European country that shows the most willingness to develop biomethane. Historically, Italy has developed its biogas sector similarly to Germany. Strong incentives between 2008 and 2012 for biogas CHP valorization (highest FIT in Europe – 280 €/MWh for plants below 1 MW) allowed a quick development of the sector notably based on energy crops. However, revisions of the subsidy scheme in 2013 (lower FIT and incitation to use by-products instead of energy crops) had slowed down the sector growth. By the end of 2017, 1,655 biogas plants were identified in Italy and only 8 were producing biomethane (EBA, 2018; Eyl-Mazzega and Mathieu, 2019). This reflects a gap in biomethane legislation that has recently been filled. In march 2018 the Biomethane Decree was adopted. It aims to support biomethane development for the transportation sector. It corresponds to a fund of 4.7 billion euros provided by transport fuel suppliers that need to meet increasing biofuel blending obligation. This fund allocated between 2018 and 2022 should cover development of the

biomethane sector (plants and infrastructures such as filling stations) up to 1.1 bcm/year (D'Adamo et al., 2019). Incentives consist in a minimum income for biomethane production that can vary in function of the type and amount of feedstocks used. Interestingly, former biogas plants performing CHP valorization can take advantage of this new subsidy scheme to shift towards injection. A recent study shows that incentives from the Biomethane Decree are making investment in upgrading technologies profitable, while it would have not been the case without (Barbera et al., 2019). Thus, early 2019, already 168 requests, corresponding to a potential of 1 bcm/year biomethane, have been formalized by SNAM (Italian equivalent of GRTgaz or Terega in France) and several new biomethane plants started to operate (about ten) (Eyl-Mazzega and Mathieu, 2019). Rapid growth of biomethane for transportation in Italy will pave the way for biomethane use in other sectors notably through the establishment and development of guarantees of origin for injected biomethane.

What emerges from Sweden, Denmark, France and Italy strategies is that, as for power generation, biomethane upgrading is strongly dependent on subsidies as production cost remains high and makes it currently approximately four times more expensive than natural gas (Eyl-Mazzega and Mathieu, 2019). Recent scenario with a moderate penetration of biomethane technology has estimated 2030 EU potential to reach 18 bcm per year, corresponding to 9 times 2017 production (Prussi et al., 2019). Achieving such an objective for the biomethane sector will depend on the energy market, supporting initiatives from state members, full demonstration of its positive environmental impacts (notably on agriculture) and above all reduction of biogas and biomethane production costs.

1.3. Improvement tracks for the biogas sector and its potential integration to the 2050 renewable gas production system

To answer the production cost challenge, the sector will have to improve all the parts of the industrial value chain and potentially monetize co-benefits. Upstream enhancement will be based on using local and low-cost organic residues combined with improved logistics. Manure, residues from landscape management such as grass, agricultural lignocellulosic residues (straw...) as well as food waste are envisioned to be increasingly used (Gas for Climate, 2019a; Theuerl et al., 2019). But the main feedstock deposit may lay in the future cropping system. While energy crops use is already restricted in many countries due to its competition with the food supply and its associated positive GHG emissions, sequential crops produced according to a newly develop concept in Italy (BiogasDoneRight™) are likely to know a strong expansion in Europe (Gas for Climate, 2019a). With this concept GHG neutral to negative biogas can be produced and at the same time agricultural soil organic carbon is increased (Dale et al., 2016; Valli et al., 2017).

Concerning anaerobic digestion plants an improvement in their efficiency and operation will be necessary to reduce operational costs and maximize gas incomes. This will be possible via: (i) a capacity to handle a wide variety of residues (Theuerl et al., 2019); (ii) methane potential unlocking of feedstocks by applying adequate pretreatments (Carrere et al., 2016); (iii) the use of additives to improve AD performance (Mao et al., 2015); (iv) a better monitoring, modeling

and understanding of the process to avoid operational issues and develop optimal strategies to recover most methane from feedstocks (Theuerl et al., 2019); (v) possible side recovery of biochemicals from the process such as medium-chain carboxylic acids (MCCAs) or alcohols, that have a higher added value than biogas (Angenent et al., 2018; Moscoviz et al., 2018).

Downstream processes are also likely to experience great changes which should provide additional revenues to the sector. Current lack of specific legal framing for digestate use is placing it into the “waste” classification, which is rendering its commercialization difficult (Guilayn et al., 2019a). Adequate legislation as well as an enhancement in digestate post-treatment (dewatering, nitrogen or phosphorus recovery...) may procure additional revenues from its commercialization and/or extraction of-its by-products (Vaneekhaute et al., 2017).

Concerning biogas valorization, several tracks are foresighted. The number of local CHP with no or low heat valorization should be progressively reduced as they are too strongly dependent on subsidies. Instead, large scale biogas plants that perform upgrading to biomethane should develop, especially in countries that are highly relying on natural gas imports. For smaller or remote biogas plants without a close gas grid access, mobile upgrading and storage systems or small-scale bio-liquefied natural gas (bio-LNG) units were recently identified as potential alternatives (Baccioli et al., 2018; Gil-carrera et al., 2019; Hönig et al., 2019). Biomethane obtained can be either used on-site in filling stations, similarly to Sweden or injected into the grid where it can be stored and used at other points of the grid, thanks to guarantees of origins that should develop in Europe. Currently, biomethane sent to the grid is used in distant filling stations, in CHP units with high heat valorizations similar to those in Denmark or directly for heat purpose (domestic or industrial). In the future, biomethane could be used in combined cold, heat and power (CCHP) units to allow cold applications (Angenent et al., 2018), as a C1 carbon source to produce microbial proteins that could be used to feed animals and humans (Pikaar et al., 2018, 2017), or to produce biochemicals and fuels (Verbeeck et al., 2018). On this last aspect, a recent study has shown a promising way to produce biochemicals based on super-dry reforming (SDR). This process combines one molecule of biomethane with three CO₂ molecules produced from industry to form four CO molecules that can be further transformed into chemicals such as methanol. Additional carbon capture and its positive economics without subsidies, in the current context, make this solution particularly attractive. However, the bottleneck remains in the upscaling of the SDR process and more generally of CO₂ to CO technologies (Verbeeck et al., 2018).

Finally, second generation upgrading technologies are likely to bring additional economic benefits to the sector in a near future. Second generation upgrading technologies can be defined as solutions valorizing and possibly trapping the CO₂ contained in the biogas (Villadsen et al., 2019). Biogas can be subject either to reforming in order to produce syngas subsequently used to synthesize liquid organic molecules (i.e. methanol) or to a methanation process that uses hydrogen (green ideally) to produce additional methane that can also be sent into gas the grid. Catalytic methanation via the Sabatier reaction or biological upgrading

via in-situ, ex-situ or hybrid strategies are the two existing processes (Angelidaki et al., 2018). Carbon dioxide can also be recovered at the outlet of the upgrading unit, after being separated from methane, and used in the methanation process or in greenhouse cultivation enhancement (vegetables, microalgae...); chemicals production (methanol...); dry ice production and food applications (carbonation of beverages...) (Esposito et al., 2019; Theuerl et al., 2019).

All these future improvement tracks will allow AD process to become a key technology in the future energy system and bio-based circular economy. To reach such a result and unlock current technological barriers, politics, research institutes, companies and agricultural sectors will have to work hand in hand.

2. Anaerobic digestion

Anaerobic digestion (AD) is a microbial process that occurs naturally in absence of oxygen as well as under physico-chemical conditions compatible with living organisms. It consists in the conversion/mineralization of organic carbon by a microbial consortium into biogas, a mix of gas containing mainly methane (50-75%) and carbon dioxide. Although AD has only been recently acknowledged worldwide as one of the foremost technologies for producing renewable energy and valuable fertilizer from all kinds of organic waste, its origin is much older. In a natural environment, it is probably one of the most ancient metabolisms that appeared on Earth at a time where oxygen was still scarcely present. In nature, these microbial consortia can be found in environments as diverse as swamps, rice fields, lakes or marine sediments, mammalian guts (cows, humans, goats...) and the intestinal tract of termites (Godon, 2016).

Volta in 1776 was the first to describe biogas, by showing combustion of gas from swamps. From 1860, AD started to develop slowly mainly due to empirical observations and progressively spread worldwide notably to recover energy or depollute waste streams. Biological comprehension of the AD process is relatively recent thanks to advances in biochemistry and microbiology (Flotats, 2019). It appears that biogas production comes from a collective and successive work of several groups of microorganisms.

2.1. Anaerobic digestion process

2.1.1. Principles of anaerobic digestion process

AD is a biological transformation process detailed in **figure 8**. From a matrix made of water, minerals and organic matter, microorganisms under the form of consortia will transform organic matter through their metabolism to ensure their growth. Several metabolisms can occur concomitantly under anaerobic conditions. The major metabolism pathway is the AD one, leading to CO₂ and CH₄ through acetogenesis and methanogenesis (described in detail thereafter). Minor metabolic pathways lead to H₂S via sulfate-reducing bacteria that use sulfates or N₂ via bacterial denitrification or bacterial anaerobic oxidation of ammonium (Anammox process). Besides, apart from the lack of oxygen, precise physio-chemical conditions are required to ensure the proper functioning of the microbial community. Intermediate pH range (6.5-8.5) and temperature (15-60°C) are needed to permit microbial growth as well as their enzymatic activities. Low redox potential (-250 to -500mV) and a low range partial pressure of hydrogen (close to 10⁻⁴ atmosphere) are also required to allow thermodynamic feasibility of the methanogenesis step. Finally, cofactors (Fe, Ni, Mg, Ca...) are necessary to ensure good functioning and efficiency of the trophic chain (Godon, 2016).

Regarding organic matter flow during AD, it can be roughly assumed that 5 to 10% of the weight of the organic matter transformed during AD is converted into microbial biomass, while the rest goes into biogas (Bernet and Buffière, 2016). It should also be highlighted that organic matter, especially from the solid matrix, is rarely totally transformed and a non-biodegradable organic fraction remains. Thus, initial organic matter is generally allocated

towards three compartments after AD: biogas, biomass and non-biodegradable remaining organic matter also call digestate. To assess efficiency of AD transformation on a given substrate, chemical oxygen demand (COD) is often used. As AD occurs in the absence of oxygen, COD is conservative, which means that the COD from the initial organic matter is equal to the sum of the COD of the products (biogas + remaining organic matter + biomass). The maximal theoretical yield of CH₄ was defined at 0.35 Nm³ CH₄/kg C. However, this value is at best approached, due to organic matter recalcitrance (especially for solids), AD inhibitions and to a lesser extent deviation towards biomass growth and microorganisms' maintenance (Flotats, 2019). To better understand these phenomena limiting AD yield, insights into the AD metabolic pathways are needed.

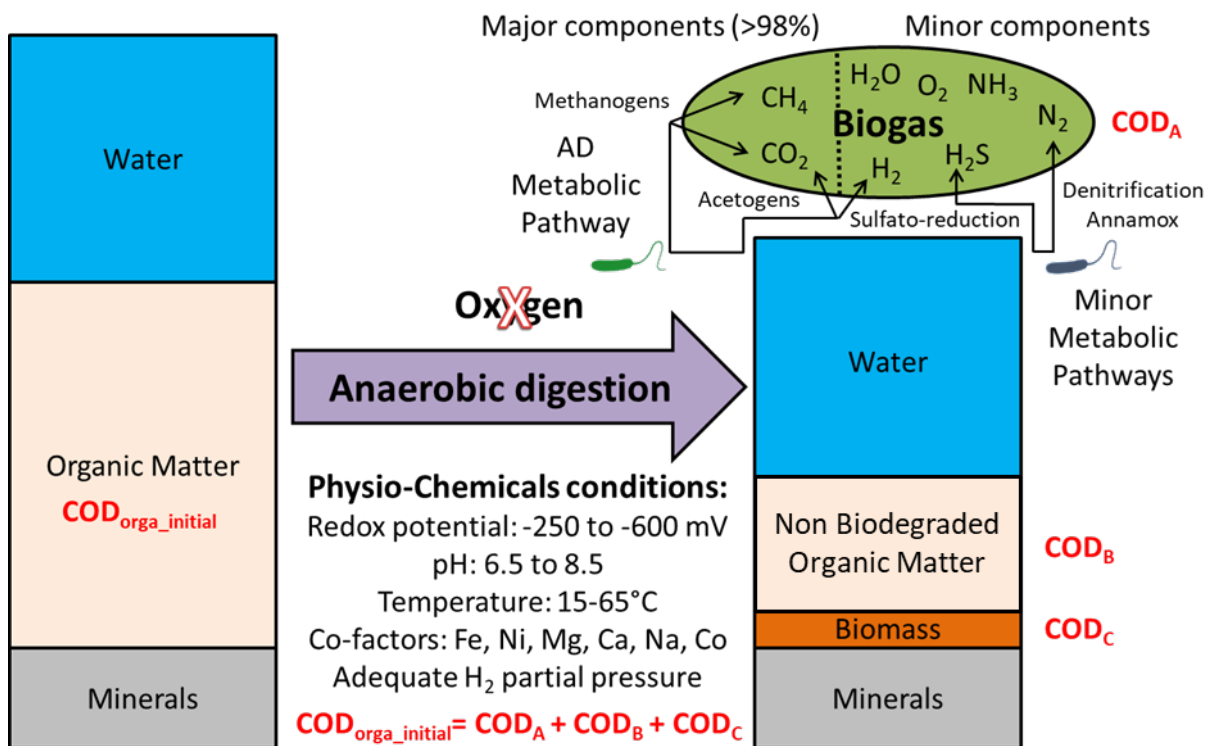


Figure 8: Anaerobic digestion: Matter assessment, operational conditions and biogas formation pathways adapted from (Bernet and Buffière, 2016).

2.1.2. Biochemistry of anaerobic digestion

From a biochemical point of view, AD corresponds to a succession of chemical reactions catalyzed by enzymes produced by the different groups of microorganisms involved. (bacteria, archaea and eukaryote). The different reaction stages, main types of microorganisms involved, and some details about thermodynamics are given in figure 7. AD is composed of four stages, namely, disintegration/hydrolysis, acidogenesis, acetogenesis and methanogenesis.

The first stage (see **figure 9**) is driven by the action of extracellular enzymes that transform complex organic matter compounds into small monomers. In detail, complex organic matter compounds are first disintegrated into macromolecules such as polysaccharides, proteins and lipids. Then, these macromolecules are hydrolyzed into small monomers such as

monosaccharides, amino acids and LCFA, respectively. The hydrolytic enzymes come from a wide range of hydrolytic bacteria that generally have a strict or facultative anaerobic metabolism, such as species part of *Clostridium* or *Bacteroides* genus (Godon, 2016). Some anaerobic and facultative aerobic fungi, existing in ruminant gut and recently identified in full-scale anaerobic digestion plants, are additional actors of the disintegration/hydrolysis step, especially on lignocellulosic biomass (Henske et al., 2017; Young et al., 2018). Despite a rapid growth of the associated microorganisms, this stage is often rate-limiting (Hagos et al., 2017). It was reported that optimal pH for this step is slightly acidic, comprised between 5.5 and 6.5 (Mao et al., 2015; Ward et al., 2008).

Acidogenic bacteria, mainly species coming from *Clostridium*, *Ruminococcus*, *Bacteroides*, *Bacillus*, *Enterobacter* and *Escherichia* genera, convert previous monosaccharides and amino acids into volatile fatty acids (VFAs), alcohols, ammonium, hydrogen and carbon dioxide. The acidogenesis stage (see **figure 9**) is a fast set of reactions (the thermodynamics of these reactions is very favorable under standard conditions) in comparison to the other stages. However, quick accumulation of produced molecules can lead to the acidification of the system and potentially inhibits the subsequent methanogenesis step. Similar to hydrolysis, acidogenesis optimal pH is comprised between 5.5 and 6.5 while acetogenesis and methanogenesis pH should range between 6.5 and 8.5 with an optimal pH close to 7 (Ward et al., 2008). Therefore, spatial separation of the hydrolysis/acidification and acetogenesis /methanogenesis stages has been identified as a way to enhance AD efficiency (see section 3.3.2.1).

The acetogenesis stage (see **figure 9**) transforms chemical intermediates obtained from the two previous stages into direct precursors of methane that are acetate, hydrogen and carbon dioxide. Two types of bacteria are involved: syntrophic bacteria (also called OHPA for Obligate Hydrogen Producing Acetogenic bacteria) and homoacetogenic bacteria. The former catalyzed LCFA, amino acids, alcohols and VFAs transformation into acetate, hydrogen and carbon dioxide. However, these reactions are thermodynamically unfavorable under standard conditions. Direct consumption of hydrogen produced is required to create low hydrogen partial pressure (p_{H_2} between 10^{-4} and 10^{-6} atmosphere) and render these reactions thermodynamically favorable. Methanogens or sulfate-reducing bacteria (in presence of sulfate) are able, due to their metabolic activities to create such conditions. Homoacetogenic bacteria transform monosaccharides or hydrogen combined with carbon dioxide directly into acetate.

Finally, the methanogenesis stage (see **figure 9**) is performed by strict anaerobic archaea following two ways. The fastest way, which accounts generally for 30% of the methane produced, is the hydrogenotrophic methanogenesis that directly transforms hydrogen and carbon dioxide into methane. Catalyzed by a great number of methanogenic microorganisms (*Methanococcus*, *Methanomicrobium*, *Methanosarcina*...), hydrogen consumption following this pathway, has a high importance for the balance of the whole AD process. The second

pathway, acetoclastic methanogenesis is mainly catalyzed by archaea of the genera *Methanosarcina* and *Methanosaeta*. These archaea are very sensitive to environmental conditions (pH notably) and have a slow metabolism and associated growth. They convert acetate directly into methane, which accounts generally for 70% of the methane produced.

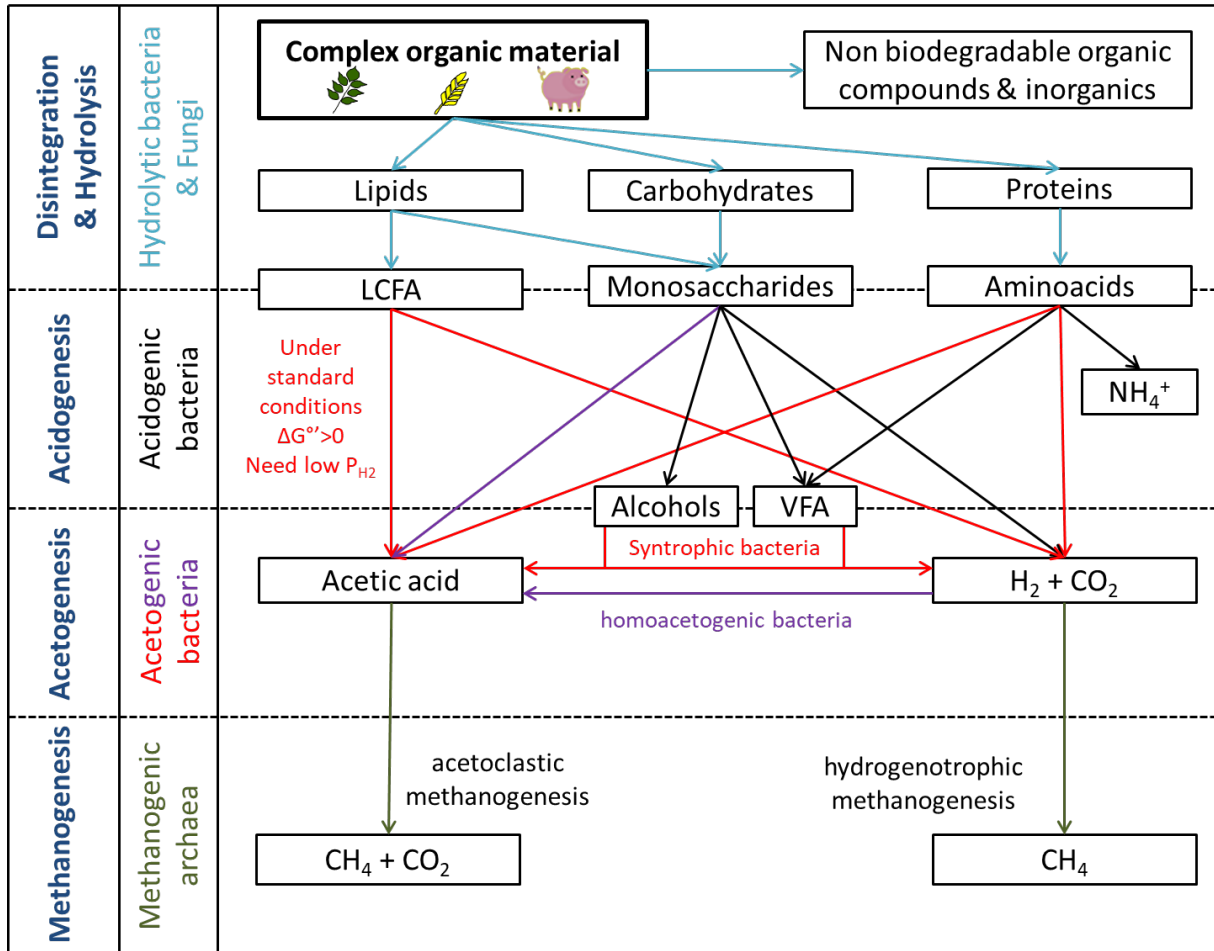


Figure 9: Schematic representation of the anaerobic digestion process (in the case of a low sulfate concentration) adapted from (Batstone et al., 2002).

To summarize, AD is a fantastic and complex metabolic cooperation scheme that allows specific catalytic activities that would otherwise not be thermodynamically feasible under standard conditions. If the last three stages can be at the origin of AD process inhibitions (e.g. OHPA bacteria can be inhibited if methanogens are not consuming hydrogen and themselves can be easily inhibited in case of pH drops due to fast acidogenesis), the disintegration/hydrolysis stage is often the bottleneck of the AD process of solid substrates, limiting feedstock methane recovery (Flotats, 2019). Indeed, the production rate, the amount and the variety of hydrolytic enzymes released by hydrolytic microorganisms are often not sufficient to adequately degrade a given substrate (Merlin Christy et al., 2014). Pretreatments, as we will see thereafter, can be a way to circumvent low feedstock biodegradability and improve efficiency of the disintegration/hydrolysis stage.

2.1.3. Full-scale anaerobic digestion process

At full scale, three main categories of reactors are used to perform AD in function of the total solids content of the feedstock (**figure 10**). Upflow Anaerobic Sludge Blanket (UASB) is the most widespread reactor to digest liquid feedstocks (< 3% TS), that can come for instance from urban waste water and agro-food industries. This technology is based on the capacity of AD consortia to naturally form granules, allowing them to remain in the reactor by settling down, as liquid feedstock is continuously injected at the reactor's bottom and flows upward to exit the system. For solid feedstocks, two strategies exist: Dilute them with water or liquid feedstocks (e.g. whey, slurry...) to reach a TS content between 3 and 15% either directly use them undiluted (TS content > 15%). For the former case, Continuous Stirred-Tank Reactors (CSTR) are mostly used. They consist generally in a succession of heated concrete tanks, equipped with internal stirrers for continuous mixing. For the latter case, called solid-state AD (SS-AD), several technologies exist such as continuous piston reactors or garage systems (batch mode). In terms of market development, CSTR systems have been much more adopted than solid systems and this trend will remain in the future. For instance, currently in France, less than 10% of the total AD plants are based on SS-AD process. Reasons explaining this situation lie in the fact that SS-AD plants are generally more complex to operate, methane yield is lower in most cases (except if process management is excellent), constructors are scarcer, return on investments slower and finally the biogas sector has a lower understanding of the system as numerous technical research questions remains to be solved (e.g. impact of hydric transfers, rheology...) (André et al., 2018; Chiumenti et al., 2018).

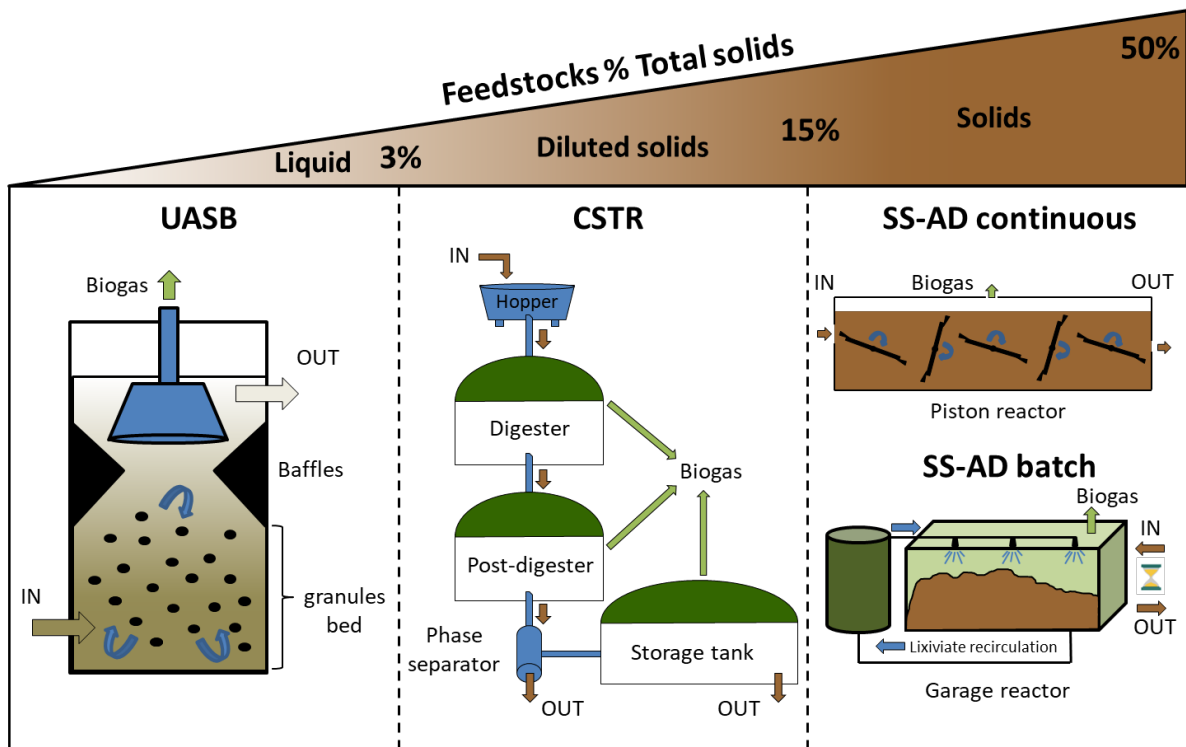


Figure 10: Full-scale main types of reactors in function of feedstocks total solids.

2.1.4. Lab scale methods to evaluate feedstocks biodegradability at full scale

At lab scale, to evaluate biodegradability and maximal biogas production of solid substrates, an internationally standardized test is performed: the biochemical methane potential (BMP) test (Holliger et al., 2016). It is a batch method with a high VS ratio of substrate to inoculum (generally comprised between 0.25 and 1), a diluted environment and a long incubation time (BMP tests terminate when methane production during three consecutive days is <1% of the accumulated volume of methane). Curves of BMP tests are usually modeled by using first order kinetic relations but it cannot be used to evaluate kinetics of continuous reactors except if a more sophisticated modeling procedure is used (Donoso-Bravo et al., 2011). Indeed, at pilot or full-scale, the conditions are totally changed. In the case of CSTR process lower dilution, continuous operation and lower ratio of substrate to inoculum are the main differences. Despite a better adaptation of the inoculum to substrate and the fact that endogenous methane production is included, it is likely that biogas yield at full scale will be lower than results of BMP tests due to a lower degradation time regarding the amount of inoculum. A recent study has shown a correlation between BMP values of given feedstocks ($BMP_{feedstock}$) and their associated methane production at full-scale in co-digestion CSTR AD plants ($P_{CH4_feedstock_full_scale}$) (Holliger et al., 2017). Correlation is calculated according to **Eq. 1**, where k is an extrapolation coefficient comprised between 0.8 to 0.9.

$$P_{CH4_feedstock_full_scale} = k * BMP_{feedstock} \quad (1)$$

2.2. Feedstock categories

AD can treat numerous organic materials. Sewage sludge, food waste, organic fraction of municipal solid waste, unsorted municipal solid waste, industrial waste (e.g. from food industry), animal by-products and agricultural feedstocks are the main groups of feedstocks that can be transformed into biogas. It is important to underline that AD process is unable to convert woody biomass, as it is a very dry and insoluble organic matter and that lignin molecules cannot be degraded by microorganisms forming AD consortia. Gasification process would thus be preferred for such type of feedstocks.

According to the PhD framework we will only focus on agricultural feedstocks as they are the largest deposit of organic matter available and therefore the market for biomethane plants having the highest potential growth. It was estimated that agricultural feedstocks have a potential in Europe of 59.8 bcm biomethane produced annually via AD by 2050, thirty times higher than potential from food waste or sludge (Gas for Climate, 2019a). Moreover in 2018, 71% of all the European plants were treating agricultural feedstocks (12,721 plants) (EBA, 2018). Agricultural feedstocks can be summarily separated between livestock manure, crop related feedstock (energy crops, sequential crops, and crop residues) and, to a lesser extent, some various by-products coming from agricultural production and its potential transformation on-site (pomace, leaves, silos by-product...).

It is estimated that 1,200 million tons (wet weight) of livestock manure are produced every year in the EU and 860 million tons (wet weight) could be collected. From that, a biomethane

potential in the EU from manure was assessed at 16 bcm per year (Scarlat et al., 2018b). Such biomethane potential could be reached by 2050 and would require the creation of 13,800 to 20,000 additional biogas plants (Gas for Climate, 2019a; Scarlat et al., 2018b). **Figure 11**, from Scarlat et al., shows a potential mapping of these new biogas units in Europe according to a constant collection radius of 10 km and where largest plants are concentrating in intensive livestock production areas (e.g. Brittany in France, The Netherlands...). In France, 1,780 additional plants based on manure could be constructed according to this scenario for an additional energy capacity of 1,282 MW.

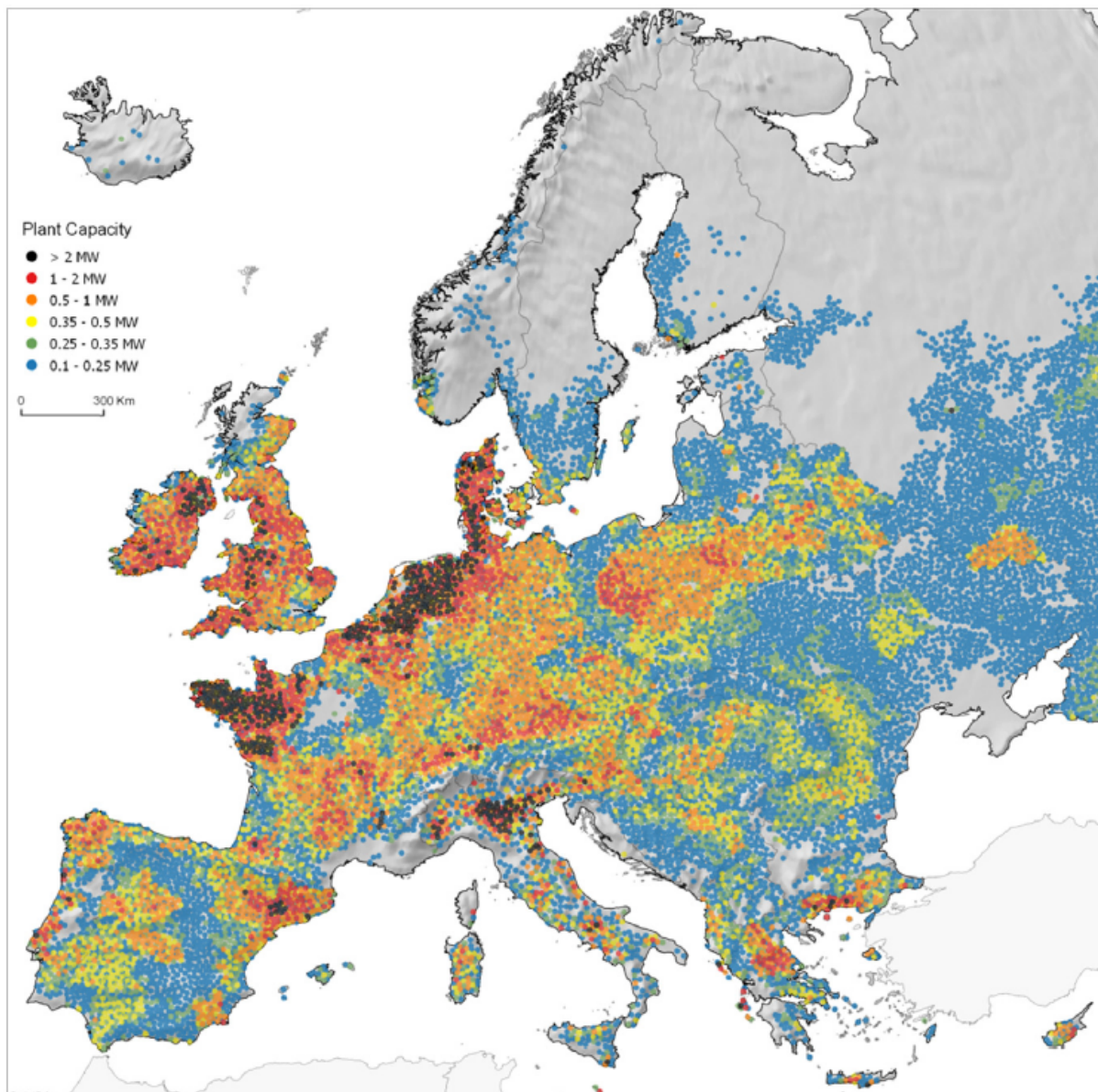


Figure 11: Location in Europe of potential new biogas plant using manure (for a 10 km collection radius) from Scarlat et al., 2018.

Crop related feedstocks have an estimated potential of 46 bcm of biomethane per year by 2050. Due to their low sustainability, energy crops are envisioned to be progressively replaced by sequential crops as agricultural practice should evolve concomitantly with the biogas sector growth. A yearly 147 million tons of sequential crops, such as triticale, sorghum, wheat or

ryegrass, could be produced from 10% of the current EU Utilized Agricultural Area (UAA) in 2050 (Gas for Climate, 2019a). Such amount of sequential crops should produce 41 bcm of biomethane. An additional 5 bcm of biomethane could be produced from crop residues such as straw from cereal or oleaginous crops. The biomass potential from the crop residues in the EU is estimated to range from 70 to 168 million tons of TS and a realistic percentage use for biogas production is estimated close to 30 million tons by 2050 (Gas for Climate, 2019a; Meyer et al., 2018; Scarlat et al., 2019).

2.3. Challenges and improvement tracks for agricultural anaerobic digestion

From an overall perspective, agricultural AD is confronted to two main challenges that are closely linked. Firstly, an environmental challenge that is to develop agricultural practices as well as operational biogas plant strategies to produce very low to negative GHG emissions biogas. Secondly, an economic challenge that is to decrease biogas production costs to reduce subsidies dependency. The finality of answering these challenges is to increase both environmental and economic performance of agricultural biogas plants.

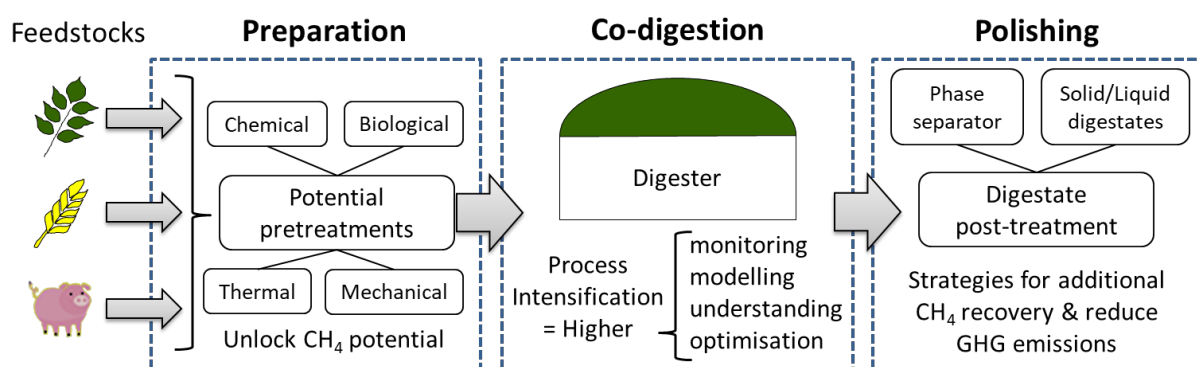
Life-cycle analysis studies on GHG emissions from agricultural biogas plants, despite various methodologies, have made similar conclusions on the most sustainable practices (Bartoli et al., 2019; Hijazi et al., 2016; Valli et al., 2017). Here are some of the most important points:

- (i) Negative GHG emissions are mainly driven by avoided emissions from livestock manure or other by-products/residues that would instead decay and release GHGs into the atmosphere without delivering any energy services;
- (ii) Plants using energy crops are producing biogas with the highest GHG emissions, notably due to their competition with feed production and the phenomenon of indirect land-use change (iLUC) that adds up the carbon footprint of the displaced food production;
- (iii) Sequential cropping developed with the Biogasdoneright™ practice can solve energy crops issues and allow additional agricultural benefits. Higher soil sequestration carbon due to continuous soil coverage as well as additional biomass produced and left in the field (roots and residues), soil protection against erosion and potential nitrogen fixation are the main advantages of this practice;
- (iv) Digestate management is very sensitive to avoid uncontrolled methane, carbon dioxide and nitrogen dioxide emissions as well as nitrogen pollution.

From these observations, the biogas production model, that is sustainably recommended and likely to be the most developed in the future, will be based on anaerobic co-digestion of manure, agricultural by-products/residues and sequential cropping. This model has inherent positive aspects such as the use of low-cost/waste feedstocks or the potential synergies between feedstocks (e.g. adjustment of C/N ratio, inflow of nutrients and water that would be missing in some feedstocks...) that can occur during AD and increase biogas recovery, but also some technological issues coming from a higher AD process complexity (Hagos et al.,

2017). Optimization of the anaerobic co-digestion process is one of the facets that will allow a decrease in the biogas production costs of agricultural biogas plant.

Agricultural anaerobic co-digestion also implies: (i) Upstream, a better capacity to handle a wide variety of feedstocks, notably by applying adequate pretreatments to unlock feedstocks' methane potential; (ii) Downstream, an efficient digestate management to ensure high methane recovery from all feedstocks. Combination of these three steps, described in **figure 12**, should lead to high methane recovery from co-digested feedstocks and potentially decrease biogas production costs, as long as, each step remains cost effective. For the continuation of this literature review, we will explore further pretreatments of agricultural feedstocks and post-treatments of digestate, as a way to improve agricultural AD.



Three stage optimisation = high methane recovery from co-digested feedstocks

Figure 12: Main stages of an optimized agricultural anaerobic co-digestion process

3. Pretreatments of agricultural feedstocks

Agricultural feedstocks can be summarily divided into two main groups: one gathering recalcitrant lignocellulosic feedstocks (agricultural residues, manure rich in straw) and another one gathering easy to degrade feedstocks (mainly energy crops, sequential crops, slurry and various agricultural by-products such as downgraded vegetables or fruits). For the former group, pretreatments will aim to: (i) delignify feedstocks; (ii) reduce polymerization degree of cellulose; (iii) diminish cellulose crystallinity; (iv) increase feedstocks available specific surface area in order to ease the hydrolysis stage (Kim et al., 2016). For the latter group, pretreatments will be more dedicated to: (i) preserving methane potential via optimal storage methods; (ii) enhancing feedstock digestion rates while being careful about acidogenesis risk.

3.1. Pretreatments typology

Four different types of pretreatments can be distinguished: mechanical, thermal, chemical and biological (Carrere et al., 2016; Montgomery and Bochmann, 2014). Mechanical pretreatments generally consist in grinding, shredding or milling. They are largely applied in full-scale agricultural biogas plants as they are necessary to introduce feedstocks into the digester (especially crop related feedstocks). They are generally placed at the outlet of the hopper, making the junction with the digester, such as in **figure 13**. Generally, they do not affect the methane yield and only increase digestion rates due to a higher feedstock specific surface area (Carrere et al., 2016; Kratky and Jirout, 2011).

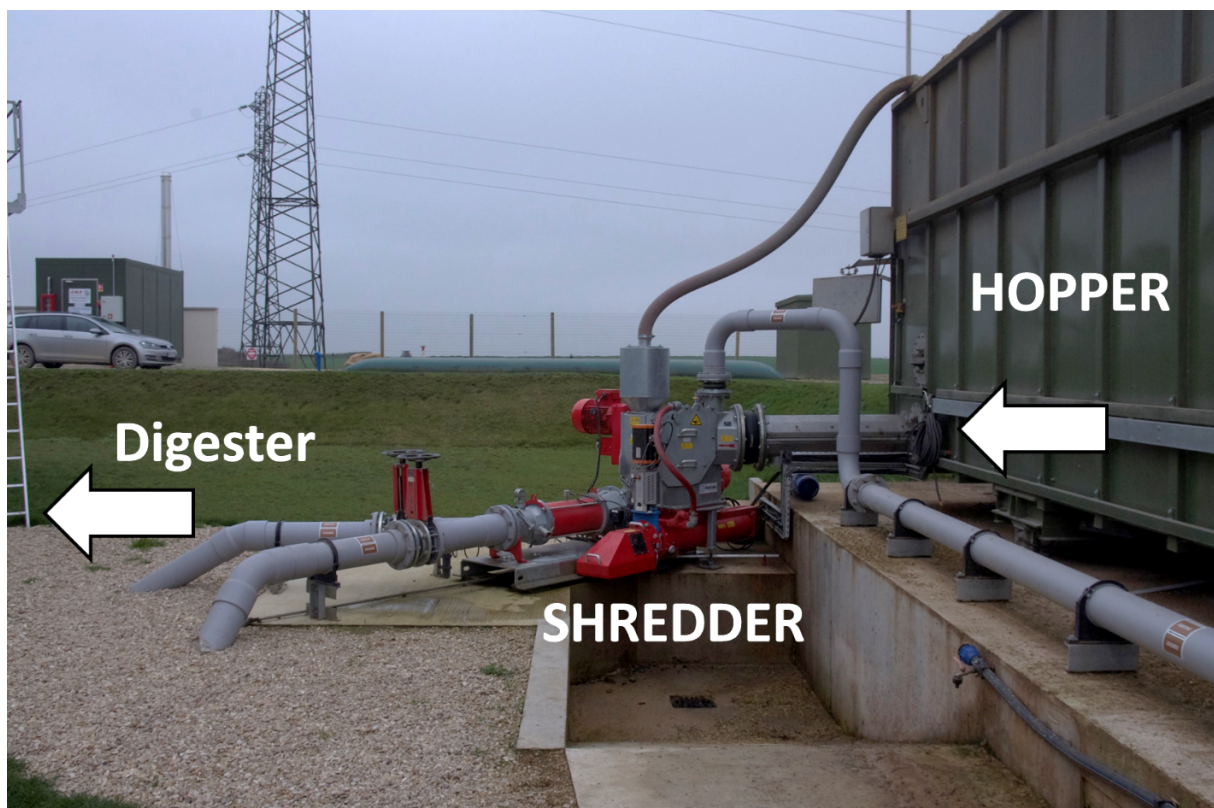


Figure 13: PreMix® incorporator (Vogelsang) including a shredder located in a French agricultural biogas plant

For thermal pretreatments, steam explosion or hydrothermal treatment have been tested on agricultural feedstocks and notably lignocellulosic biomass. It requires fine-tuning as they

could lead to higher methane yields but can also generate AD inhibitors and recalcitrant compounds such as furans and phenols that can only be converted to methane under low concentrations (Monlau et al., 2014). To our knowledge, only one steam explosion technology exists at full scale commercialized by Economizer GmbH (Salzburg, Austria). Agricultural feedstocks are cooked with water between 150-180°C and under high pressure (5-8 bars) for a certain duration before pressure release. At least one plant is equipped with the technology in the UK (**figure 14**). However, wide implementation of such technology in agricultural plants remains highly uncertain due to high energy demand (heat and electricity), additional capital expenditure (CAPEX) and higher process complexity.



Figure 14: Economizer SE in a UK biogas plant built by Future Biogas Ltd – picture from (Future biogas Systems, website)

3.2. Chemical pretreatments

Chemical or thermochemical pretreatments can be categorized as a function of the type of chemicals. Oxidative pretreatments using for instance H_2O_2 or peracetic acid remain marginal and too expensive despite some interesting improvement on methane yield (Ramos-suárez et al., 2017; Venturin et al., 2018). Therefore, they will not be further described here. The most commonly used chemicals are acids and alkalis. Numerous studies have been carried out at lab scale, notably to treat agricultural lignocellulosic feedstocks (e.g. straw, bagasse ...).

Acids such as H_2SO_4 , H_3PO_4 , HNO_3 or HCl are mainly combined with thermal treatment and lead to hemicellulose hydrolysis and lignin disruption that increase accessibility to cellulose, which can then be easily converted into biogas. However, associated costs, GHG emissions from acid production, sensitiveness of methanogenesis towards acidic conditions, necessity to use corrosion-resistant materials and risks for the environment as well as the operator make this kind of chemical pretreatment not adopted in agricultural biogas plants. Innovative

processes such as dry dilute acid pretreatment method (high solid content of feedstock diluted acid and temperature close to 170°C) could nonetheless increase process efficiency and reduce associated economic and environmental burdens (Solarte-toro et al., 2019).

Alkali pretreatments are reported to be more efficient on lignin breakdown. Indeed, hydroxide radicals ($\text{HO}\cdot$) are very reactive oxidants that can interact with lignin structure (phenol rings...) and cause its depolymerization as well as the cleavage of existing linkages with carbohydrates. Additional observed effects are an increase in cellulose accessibility and solubilization of hemicellulose (to a lesser extent with acids) (Carrere et al., 2016). NaOH, KOH, CaO, $\text{Ca}(\text{OH})_2$ and NH_4OH chemicals, combined or not with thermal treatment, have been tested on various lignocellulosic feedstocks and generally leads to methane yield enhancement (Kim et al., 2016; Liu et al., 2015; Xie et al., 2018).

NaOH, despite high efficiency appears to be relatively costly (270-530€/ton) and the presence of sodium can inhibit AD and be detrimental for digestate land applications. A recent study has shown that application of 10% NaOH (VS basis) at 100 °C for 5 min could increase the methane potential of dairy cow manure by 24%. Nevertheless, an economic analysis has also shown that pretreatment was not economically beneficial in comparison to digestion without pretreatment (Passos et al., 2017). A similar economic observation was recently made on NaOH treatment of miscanthus (Fu et al., 2018). KOH can be seen as an emerging alternative to NaOH as potassium brought this way could substitute additional fertilizer purchases and thus reduce its higher cost (450-800€/ton). CaO is also an interesting alternative due to its lower cost (100-150€/ton) but its effect on lignin, on an equivalent molar basis of $\text{OH}\cdot$, is lower to KOH and NaOH due to potential calcium-mediated lignin repolymerization (Thomas et al., 2018). However, as for NaOH, pretreatments using CaO or a combination of CaO and KOH on manure or wheat straw have shown not to be economically beneficial as methane gains did not compensate chemical and potential CAPEX costs (additional pretreatment tank...) (Ramos-suárez et al., 2017; Shen et al., 2019). To our knowledge, only one thermo-alkaline process has been developed at full scale and is currently applied in some agricultural plants treating high amounts of nitrogen-rich feedstocks (i.e. poultry manure). It is commercialized under the trademark NiX™ by the company Xergi (**figure 15**). It consists in a combination of steam cooking with CaO addition that allows partial conversion of nitrogen into ammonia, that is subsequently stripped during the pretreatment. Besides, chemical and physical actions increase manure accessibility as well as partially hydrolyze it, which leads to an enhanced methane potential (Fink, 2013).



Figure 15: Nix® technology (Xergi) integrated in full-scale plant – picture from (Xergi, website)

Alkaline pretreatments appear to be one the most promising tracks to unlock methane potential of lignocellulosic feedstocks. However, additional costs due to chemicals as well as additional process environmental footprint (chemical production, transportation) makes it unrealistic to date. Thus, further process optimization and innovative pretreatment strategies are required to widen its use in the agricultural biogas sector.

3.3. Biological pretreatments

Adapted from Brémond et al., Biological pretreatments of biomass for improving biogas production: an overview from lab scale to full-scale. Renewable and Sustainable Energy Reviews, 2018, 90, 583-604.

Biological pretreatments have been, until now, less explored than previously described pretreatments. They can be divided in three parts: enzymatic, anaerobic and aerobic. Promising and recent research results on agricultural feedstocks will be presented, with a special focus on methane yield enhancements. Furthermore, non-exhaustive examples of associated full-scale products sold by companies will be described in order to provide to the reader a first insight into existing applications at farm scale.

3.3.1. Enzymatic pretreatments

Crop related feedstocks are mainly made of cellulose, hemicelluloses, lignin and proteins in little amounts. Manure is composed of lignocellulosic biomass (straw in some manure coming from mulching of livestock sheds) and feces composed of protein and undigested lignocellulosic rich biomass. Several types of enzymes have been used to pretreat this type of agricultural feedstocks obtaining various results (**Table 1**).

Exogenous enzyme additions during AD in order to improve the hydrolytic step of complex organic substrates have been investigated, with a growing interest since the mid-1980s. From

this literature, it is important to distinguish the four ways to practice enzyme addition as shown in **figure 16**: (i) in a dedicated pretreatment vessel (ii) directly in the digester vessel (single-stage process) (iii) directly in the hydrolysis and acidification vessel (two-stage process) (iv) in the recirculated AD leachate. Besides, it is also worthy to underline that to obtain an increase in biogas production via enzyme addition, parameters such as enzyme activity, specificity to the substrate, quantity, temperature, pH and enzyme stability need to be optimized and are often key parameters in the economic assessment (Divya et al., 2015). This section will give an overview of enzymatic pretreatments with a special focus on results of available commercial solutions. It can be already mentioned that Dupont (Wilmington, USA), Novozymes (Bagsvaerd, Denmark) and DSM (Delft, the Netherlands) are the three key players in the enzyme market. Therefore, enzyme products that will be presented are mostly commercialized by these companies.

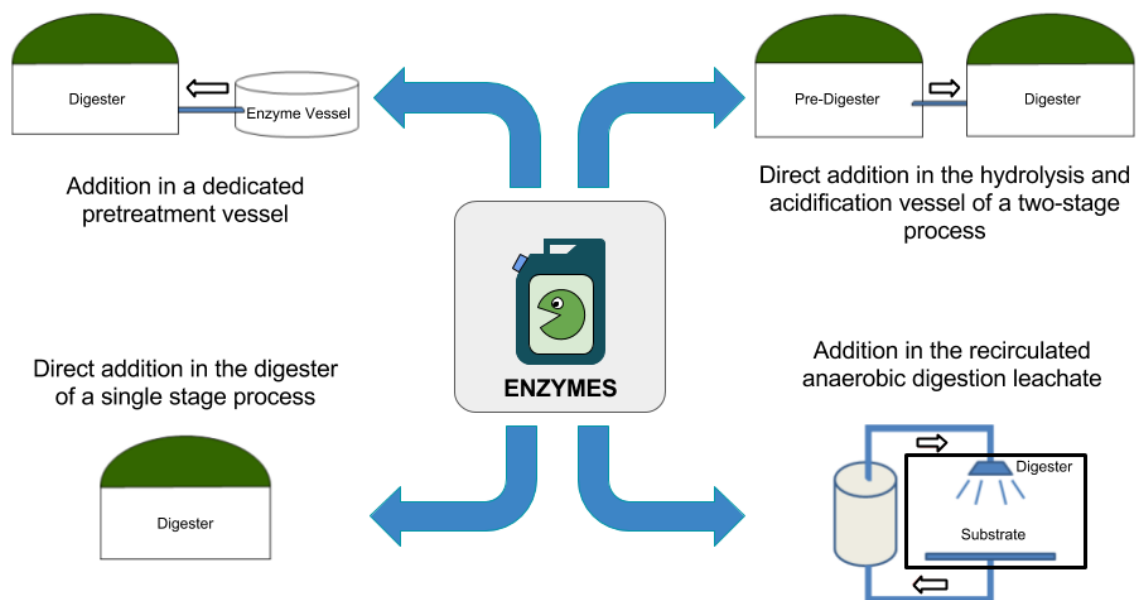


Figure 16: The different ways to practice enzyme addition in order to enhance anaerobic digestion

Proteases have been recently evaluated in studies working on co-digestion of agricultural substrates, where direct addition of proteases in the digester was applied. Muller et al. (Müller et al., 2016) applied different proteases (alkaline, serine and aspartic types) to a mix of maize silage, chicken dung and cow manure in a 2 L BMP test and obtained interesting results. Indeed, proteases increased methane yield between 9 and 52%. Following these results, similar experiments in semi-continuous 12 L digesters were carried out. After digesters stabilization, daily addition of enzymes did not lead to any increase in biogas yield. In addition, when the quantity of enzymes was increased tenfold, the biogas yield decreased between 13% and 36%. Here, the scale up from BMP to semi-continuous AD was not successful, demonstrating that promising BMP results are not ensuring higher scale results. Besides, negative results with BMP were also obtained by Wang et al. (Wang et al., 2016) where direct addition of a protease from Novozymes to a mix of cow manure and straw did not lead to any increase in biogas yield. Several reasons are given in these studies to explain these negative results regardless of the scale: (i) proteases degrade some essential enzymes such as

hydrolases which are important in the hydrolytic phase or enzymes related to acidogenesis and methanogenesis; (ii) they favor accumulation of intermediates such as phenyl acetate and propionate leading to inhibition of microorganisms; (iii) proteases can attack microorganisms at their surfaces. Overall, it seems that positive effects are canceled by negative effects of protease addition in the case of agricultural residues. Therefore, proteases might be appropriate only for the pretreatment of protein-rich material.

Enzymes targeting lignin, such as laccases and peroxidases, have been recently explored as pretreatment before BMP tests of agricultural lignocellulose rich substrates. Frigon et al. (Frigon et al., 2012) applied lignin peroxidases and manganese peroxidases (LiP and MnP) to switch grass and obtained a significant increase in methane yield of 29% and 41%, respectively. Similarly, laccase pretreatment of corn stover by Schroyen et al. (Schroyen et al., 2014) showed a 17% increase in methane yield. However, more recent trials on various agricultural feedstocks with laccases and versatile peroxidases (VP) have shown that the higher the lignin concentration of the substrate, the lower the efficiency of this pretreatment (Schroyen et al., 2015). Indeed, large release of phenolic compounds from lignin degradation can strongly inhibit the anaerobic digestion as can be seen for the miscanthus case. From these results, the use of LiP, MnP, VP and laccases seems to be promising for some types of lignocellulosic agricultural feedstocks. However, at the moment, this kind of enzymes is only tested at lab scale and not yet used for AD industrial purposes. Indeed, their production costs remain high and limit their application to high added value fields such as food and pharmaceutical industries (Brijwani et al., 2010). One potential advantage of AD in comparison to the controlled environment of industrial fermentation is that expensive cofactors/redox mediators may naturally be found in the complex AD environment. Because lignin and links between lignin and carbohydrates have been shown to be the main bottleneck to lignocellulosic biomass AD (Monlau et al., 2013), further research may focus on how to improve the use of lignin-modifying enzymes in AD systems.

Finally, cellulases, hemicellulases, amylases and pectinases which target carbohydrates have been extensively tested on agricultural feedstocks. If Romano et al. (Romano et al., 2009) did not show any significant increase in methane yield of wheat grass using Novozymes cellulase solutions whatever the way they were applied, recent studies have shown more promising results at lab scale. Sutayro et al. (Sutayro et al., 2014) worked on dairy cattle manure on which, pectate lyases and cellulases were added in equal proportion (w/w). Three days pretreatment at 50°C followed by AD permitted a 4.5% increase in methane yield whereas a direct addition did not have any positive impact. Using Novozymes products, Wang et al. (Wang et al., 2016) showed that pretreatment of a mix of cow manure and corn straw by a cellulase blend at 55°C for 18 hours permitted a 103% increase in the methane yield. Furthermore, in this work, direct addition of amylase to AD permitted an increase of the methane yield of 111%. The use of Ultraflo® L, another cellulolytic enzymatic cocktail from Novozymes, in pretreatment of corn cobs (3 hours at 40°C and pH 6) also displayed positive results on subsequent AD as methane yield was increased by 14% (Pérez-Rodríguez et al.,

2017). Finally, by directly adding a mix of endoglucanase, xylanase and pectinase (CeluStar XL) formerly proposed by Dyadic (Jupiter, the USA), Ziemiński et al. (Ziemiński et al., 2015) succeeded in increasing by 28% the methane yield during co-digestion of sugar beet pulp silage and vinasse.

From these lab scale results, it can be suggested that the application of carbohydrates degrading enzymes can enhance both production rate and yield of methane but careful attention has to be paid to process optimization such as enzyme selection for a given substrate, type of application, incubation time, pH, temperature; otherwise results can be negative (Romano et al., 2009). Cellulases, hemicellulases, amylases and pectinases can be considered as promising enzymes due to their positive impact on biogas yield if well applied and no antagonist interference with anaerobic microorganisms or endogenous enzymes. Currently, carbohydrases are the only enzymes commercialized at full-scale for biogas applications. One hypothesis might be that carbohydrases are easier and cheaper to produce at industrial scale than lignin-modifying enzymes. Furthermore, due to its higher simplicity for implementation, direct addition can be seen as the most promising way to apply enzymes even though during AD operations, acidic pH that is optimum to certain enzyme activities cannot be applied. Thus, commercial products currently used are based on direct addition of carbohydrases.

On the market, several enzyme products to boost biogas yield are proposed by Novozymes, DSM, Dupont. It can be observed that other smaller enzyme producers or plant constructors are offering dedicated solutions for biogas production such as for instance Metzyme® Forci™ by Metgen (Kaarina, Finland) or Enzymaxx by Agrikomp (Merkendorf, Germany) but these products will not be further described as existing literature on their use is scarce. In 2019 Novozymes, is commercializing a range of enzymatic solution under the trademark BG Max® that is mainly dedicated to wastewater treatment market. Finally, DSM and Dupont appear to be more active in developing enzyme products dedicated to agricultural feedstocks.

DSM Methaplus® L 100 was formerly a product of Biopract GmbH before the company was taken over by DSM in 2009. In 2007, a full-scale study carried out by Biopract on 30 agricultural plants showed that the direct addition of this enzyme blend had an average positive effect of 18% on the biogas yield without any effect on the biogas quality. Thus, Methaplus® L 100 addition was proved to be financially beneficial to all the 30 plants (Gerhardt et al., 2007). A precise composition of the DSM Methaplus® L 100 was given; it is composed of xylanase, endo-glucanase, exo-glucanase and β -glucosidase (Sambusiti et al., 2015). More recently, since the purchase by Dupont of the solid advanced fungal fermentation platform technology, called "C1", from Dyadic in 2016, a new product named Optimash® AD-100 was announced mid-2016. This enzyme blend from *Trichoderma reesei* and *Myceliophthora thermophila* has not been used in laboratory studies yet. Only Dupont data are available, showing that a direct addition in a 2000 kW full-scale plant led to an 8% increase in the methane yield and a 10% decrease in costs for digester operators (Biofuels, 2016). However, more data will become

available thanks to a new European funded project called Demeter, started in October 2016. This public-private project aims to demonstrate economic benefits of using Dupont C1-enzyme product in agricultural biogas industry (H2020, 2016). Results of this project were recently presented during IWA international conference on Anaerobic digestion (AD16) (Debeer et al., 2019). It was shown that at lab scale, addition of these enzymes (various doses) did not significantly enhance methane yield of tested agricultural feedstocks (e.g. straw, rye silage...). Nevertheless, large-scale trials are still ongoing, to evaluate potential impact of enzyme addition on digestate viscosity.

Concerning these available products, two points can be highlighted. Firstly, it can be noticed that studies not provided by the supplier on these particular products are very seldom. That is why both lab and full scale academic studies would be very valuable to evaluate and compare the efficiency of these different products. The Demeter project, by its public-private partnership, is a promising example. Secondly, we mainly focused on methane yield improvement but enzyme products can offer other advantages. For instance, DSM Axiasé™ 100 can lower viscosity of substrates mix rich in whole crop cereal silage (Schimpf et al., 2013). Similarly, the addition of Fibrezyme® G4 of Dupont to MiaMethan® ProCut, a biogas powder optimizer from MIAVIT (Essen, Germany) is presented as a viscosity reducer. From that, it is important to understand that enzyme application to agricultural feedstocks actually has a wider range of applications than biogas yield improvement such as: reduction of digester viscosity, improved digester mixing, increase of AD rate which implies shorter hydraulic retention times, better use of feedstocks and a wider range of possible feedstocks. Nevertheless, if these features can be observed at full-scale, they are less commonly studied at lab scale as they are more complicated to carry out than methane potential tests at this scale. Thus, pilot research and full-scale trials would facilitate better evaluation of the technology (Parawira, 2012).

Table 1: Effect of enzymatic pretreatment on biogas and methane yield as a function of feedstock

Feedstock	Enzyme/Product and/or suppliers	Type of application	Enzymes application conditions	AD scale / AD process conditions	Methane yield (L/g VS)		References
					Control	Treatment	
Maize silage, chicken dung and cow manure	Alkaline, serine and aspartic Protease / not available	Direct	Lab scale 2L BMP / triplicate - 39°C - 43 days Lab scale 12L semi-continuous / 39°C - 100 days (among which 17 days of enzyme daily addition)		0.265	0.29 – 0.405	(Müller et al., 2016)
Cow manure (95%) and corn straw (5%)	Cellulase blend / Novozymes	Pretreat	30 mL - 55°C - 18h at pH 5-6	Lab scale 600 mL BMP / Triplicate - 37°C - 30 days - pH 7	0.179	0.364	(Wang et al., 2016)
	Alpha-amylase / Novozymes	Direct	Lab scale 600 mL BMP / Triplicate - 37°C - 30 days - pH 7		0.179	0.377	
	Protease/Novozymes	Direct	Lab scale 600 mL BMP / Triplicate - 37°C - 30 days - pH 7		0.179	0.181	
Switch grass	Lignin Peroxidase + cofactors / Sigma Aldrich	Pretreat	40 mL - 22°C - 8 hours - pH 7.4	Lab scale 500 mL BMP / 35°C - 37 days - pH neutral	0.157 ± 18%	0.202 ± 9%	(Frigon et al., 2012)
	Manganese Peroxidase + cofactors / Jena Bioscience GmbH	Pretreat	40 mL - 37°C - 8 hours - pH 7.4	Lab scale 500 mL BMP / 35°C - 37 days - pH neutral	0.157 ± 18%	0.222 ± 22.5%	
Corn Stover	Laccase + cofactors / not available	Pretreat	180 mL - 30°C - 24 hours - pH 4.7	Lab scale 200 mL BMP / 30 days	0.293	0.344	(Schroyen et al., 2014)
Corn Stover Miscanthus	Laccase and Versatile Peroxidase + cofactors / not given	Pretreat	180 mL - 30°C - 6 hours - pH 4.5	Lab scale 200 mL BMP / 30 days	0.192	0.238	(Schroyen et al., 2015)
			0.139	0.138			
Wheat grass	Endoglucanase - Xylanase and β -glucanase/Novozyme 342	Pretreat	150 mL - 50°C - 7 days - pH 7	Lab scale 1 L batch / Triplicate - 50°C - 14 days - pH 7	0.15 ± 7%	0.16 ± 10%	(Romano et al., 2009)
		Direct	Lab scale 1 L batch / Triplicate - 50°C - 14 days - pH 7		0.17 ± 4%	0.16 ± 9%	
	Cellulase and β -glucosidase / 85% Celluclast 1,5L - 15% Novozyme 188	Direct2st	Lab scale 1 L batch / Triplicate - 50°C – Hydrolysis: 7 days at pH 5 – methanogenesis: 14 days at pH 7		0.22 ± 3%	0.29 ± 13%	
Dairy Cattle manure	Mix of Pectate lyase - Cellulase in equal proportions (w/w) / Novozymes	Pretreat	50°C - 3 days - pH 7.8	Lab scale 10L semi-continuous / 50°C - 42 days (HRT 14 days) - pH 7.8	0.135	0.146	(Sutaryo et al., 2014)
		Direct	Lab scale 10L semi-continuous / 50°C - 42 days - pH 7.8		0.135	0.136	
Corn cob	Endoglucanase - Xylanase - Cellulase - Cellobiase - Feruloyl esterase / Ultraflo® L - Novozymes	Pretreat	30 mL - 40°C - 3 hours - pH 6	Lab scale 500 mL BMP / 35°C - 32 days - pH neutral	0.25	0.29	(Pérez-Rodríguez et al., 2017)
Sugar beet pulp silage (75%) vinasse (25%)	Endoglucanase - Xylanase and pectinase / CeluStar XL - Dyadic	Pretreat	50°C - 7 days	Lab scale 1L batch / 37°C-30 days-pH 7.2	0.35	0.465	(Ziemiński and Kowalska-Wentel, 2015)

Various agricultural feedstocks (not given)	Xylanase - Endo-glucanase - exo-glucanase and β -glucosidase / Methaplus® L 100 - DSM	Direct	Full-scale tests in 30 agricultural plants from 55 kW to 1022 kW / Various conditions of operation HRT is varying from 35 to 221 days and OLR ranged from 1.5 to 15.1 kg(m ³ d) ⁻¹	Biogas yield increased in average of 18%*. Biogas quality not affected by the enzyme addition	(Gerhardt et al., 2007)
Various (manure, corn, sugar beet, oat meal, shea nut meal)	Probably mix of cellulase / Optimash 100-AD - Dupont	Direct	Full-scale - Biogas plant type plug-flow 2000 kW / 40-44 °C – 60 days HRT - loading of 110 tons/day of fresh matter	Methane production increased by 8%	(Biofuels, 2016)
Co digestion of whole plant Rye silage, maize silage	Pectinase - cellulase and hemicellulase / Axiase™ 100 - DSM	Direct	Full-scale - Test in a 2000 m ³ digester / 63 days of HRT - other information not given	Energy** increased by 4.7%	(Schimpf et al., 2013)

Type of application: Direct: Direct addition in the digester – Pretreat: Addition in a specific vessel before digestion – Direct2st: Addition in the hydrolysis digester of a two-stage process – Recircu: Addition in recirculated leachate.

Methane yield: * Biogas yield (L/g VS) is given instead of methane yield - ** Energy coming from the cogeneration (kWh/t FM) of the plant is given instead of methane yield.

Finally, at higher scale, main issues hindering widespread use appear to be the enzyme cost and the enzyme activity lifetime after addition. To solve these issues, several tracks appear: (i) advance in enzyme engineering using recent molecular tools may permit to obtain more robust enzymes having a higher activity and less sensitivity to specific inhibitions (phenol for instance in the case of lignocellulose degradation). For instance Speda et al. (Speda et al., 2017) recently improved lab scale AD of ensiled forage grass ley by using an enzymatic cocktail (previously obtained from an AD environment) that displays long-lasting activities and stability during the digestion but full characterization of this enzyme cocktail for its production in recombinant systems remains challenging; (ii) development and use of highly productive genetically modified microorganisms by companies should permit to reduce the enzyme production cost; (iii) on-site production of enzymes for large biogas plant can be a solution to reduce enzyme cost. A company called Greenmove technologies (Leeuwarden, the Netherlands) is developing this kind of solution in plug-in containers; (iv) the addition, in complement of enzymes, of highly productive enzyme microorganisms under solid (fungal mash for instance) or liquid form could permit both to reduce the cost of downstream process for enzyme purification and to increase enzyme activity as they are produced *in situ*; (v) finally, enzyme fixation on a three-dimensional support is a promising way to enhance the activity lifetime of the enzyme and thus reduce dosage.

Besides, it is important to underline that enzymatic pretreatment is a versatile technology that should not only be economically evaluated through the prism of biogas yield increase. For instance, a cheaper substrate use, a reduced HRT or a lower mixing cost would be very profitable in certain full-scale plants and should be considered in calculations. For these reasons, enzymatic pretreatments remain a very promising tool for AD enhancement that may continue to develop.

3.3.2. Anaerobic pretreatments

3.3.2.1. Two-stage digestion

Two-stage digestion is based on the separation in time and space of the hydrolysis and acidogenesis stages from the acetogenesis and methanogenesis stages (Montgomery and Bochmann, 2014). This is generally made by using two separate vessels: the first step can thus be considered as a pretreatment of the methanogenesis step. Based on this primary principle, several technologies can be distinguished as a function of the temperature applied in these vessels: thermophilic two-stage digestion, mesophilic two-stage digestion and temperature Phased Anaerobic Digestion (TPAD), where the first vessel is thermophilic (or hyper-thermophilic) and the second vessel is mesophilic. Ariunbaatar et al. (Ariunbaatar et al., 2014) listed both the advantages and drawbacks of these technologies, given in **figure 17**.

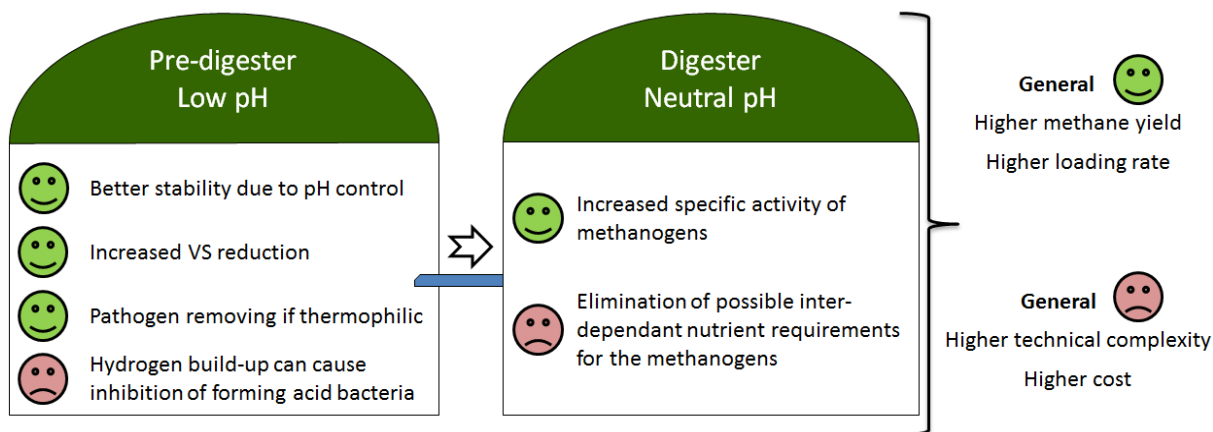


Figure 17: Advantages and drawbacks of two-stage digestion

It is commonly accepted that two-stage digestion technology is valuable for easily biodegradable waste that can generate acidification issues (Carrere et al., 2016). Agricultural feedstocks, often rich in lignocellulose, are rarely easily biodegradable and application of two-stage digestion is in this case very questionable. Indeed, if pH does not drop and hydrolysis is a long process, methanogenic activities will appear and a single vessel will be sufficient. To support this theory, Lindner et al. (Lindner et al., 2016) recently showed that two-stage AD is better adapted to sugar rich feedstocks. They evaluated the most suitable feedstocks for a continuous two-stage process at pilot scale, among maize silage, sugar beet and a mix of hay/straw. After 50-day experiments, it appears that organic loading rate (OLR) and substrate degradation were respectively 5 times and 4 times higher for sugar beet than hay/straw. Besides, in comparison to their respective BMP, methane yield was 70% lower for hay/straw whereas sugar beet was only 8% lower.

Besides, a distinction must be made with the other type of two-stage AD that is commonly found in agricultural biogas plants, which consists in a digester and a post-digester. Indeed, in this particular set-up, hydrolysis and methanogenesis are taking place in both digesters and the pH of the first digester is neutral. In this case, it is not a hydrolytic pretreatment but rather a way to increase HRT and to recover more biogas from a given feedstock by having a secondary digestion step.

Some biogas plant manufacturers are selling two-stage digestion systems for agricultural feedstocks such as Bioplex Ltd (Stockbridge, the UK), Snow leopard projects GmbH (Reisbach, Germany) or Evalor (Plérin, France). However, regarding the two previous conclusions, full-scale applications at farm scale of a “hydrolytic” two-stage digestion remain very seldom as it generates both higher complexity and costs without clear benefits compared to a single stage digester. To reduce costs at farm scale, innovative compartmented vessels can be used such as Linear Vortex™ by DVO Inc. (Chilton, the USA) that displays a very specific “U” design or Arkometha™, by Arkolia Energies (Mudaison, France), a highly productive, small size and multi-step vessel digester dedicated to solid digestion. However, these innovations can be considered as process configuration or bioreactor design improvement and therefore are

outside the scope of the current pretreatment study. Another option is to enhance the first step hydrolysis/acidogenesis using specific consortia as it will be developed herein below.

3.3.2.2. Enhanced two-stage digestion

To manage lignocellulose rich substrates in a two-stage digestion set-up, several ways to enhance the first hydrolytic step were explored. The common objective is to add to the system anaerobic hydrolytic microorganisms that would degrade lignocellulose more efficiently and faster. In other words, bioaugmentation is applied to the first hydrolytic step. These exogenous microorganisms can come from different origins that permit to define several ways to enhance a two-stage digestion process:

- (i) They can derive from rumen. It is one of the most efficient microbial ecosystems in nature to carry out degradation of lignocellulosic biomass and is found in guts of ruminant species. This ecosystem is made of bacteria, fungi, protozoa and archaea that can grow rapidly on lignocellulose and can secrete high quantities of hydrolytic enzymes. Thus, in this environment lignocellulose is quickly degraded into VFAs that can be further metabolized by ruminants (Sauer et al., 2012). The production of methane is not the aim of this ecosystem therefore; methanogens content is naturally low in rumen fluid. To make it short, rumen ecosystem is foremost a super hydrolytic consortium and secondarily a methane producer.

Pretreatments using rumen ecosystem has been widely tested at lab scale on several lignocellulosic feedstocks to increase their methane production. One of the underlying aims is to recreate an efficient artificial rumen system. This kind of pretreatment can be gathered under the name rumen derived anaerobic digestion (Deng et al., 2017). Here, focus is made only on two-stage AD but it has to be noted that several other experimental set-ups using rumen fluid have been tested up to now such as continuous-flow culture reactor, dual-flow continuous culture reactor, anaerobic sequencing batch reactor and modified UASB reactor (Yue et al., 2013). Zhang et al. (Zhang et al., 2016) pretreated rice straw with rumen fluid at 39°C, for 24 hours under anaerobic conditions and then digested it with sludge inoculum at 35°C for 30 days. At the end, biogas production from AD was increased by 66.5 %, its methane yield was improved by 82.6%, and the digestion time was 40% shorter than the control. This better digestion can be explained by biomass structural changes during pretreatment such as an increase of the specific surface area, the removal of the hemicelluloses, a decrease in cellulose crystallinity and in lignin content (Li et al., 2016; Yue et al., 2013).

Rumen fluid addition pretreatment displays very interesting results showing that lignocellulosic biomass can be efficiently hydrolyzed. However, several points strongly hinder its application at full-scale for methane production: (i) the understanding of the rumen ecosystem is still limited; (ii) the reproduction of an efficient and long-lasting artificial rumen system is for the moment kept out of research hands; (iii) promising lab scale results are based on the application of a high ratio of rumen fluid on solid

biomass (ratio of 10 to 20) that indicates the need to use a large amount of rumen fluid in case of a scale up; (4) rumen fluid is produced in limited quantity from slaughterhouses, its extraction has a high cost and it is better to use it fresh. Therefore, to our knowledge, there is no example of industrial two-stage AD plant using rumen fluid as pretreatment in the first stage. Research still needs to advance on rumen understanding and on ways to maintain in a hydrolytic digester an efficient rumen ecosystem over time, at low costs. From these advances, promising potential pretreatment scale-up for biogas production might emerge.

- (ii) Exogenous microorganisms can also come from pure culture or designed anaerobic hydrolytic consortia. Microorganisms isolated from other environments than rumen can be used to enhance the hydrolytic first stage of a two-stage digestion. Pure culture of hydrolytic microorganisms was explored with contrasting results. A first study using *Caldicellulosiruptor lactoaceticus*, a thermophilic anaerobic bacterium isolated from hot springs, was carried out on a laboratory two-stage continuous thermophilic (68°C/55°C) digestion treating fiber rich cattle manure. Bioaugmentation of the first stage with *C. lactoaceticus*, led to a 10% higher methane yield than the control (Nielsen et al., 2007). However, results can be very different from one strain to another and it is needed to carefully select the strain to obtain positive results. For instance, Nkemka et al. (Nkemka et al., 2015) used an anaerobic hydrolytic fungus, *Piromyces rhizinflata* YM600, to pretreat corn silage and cattail in a laboratory two-stage digestion system. In this study, bioaugmentation of the first stage did not increase the methane yield from both corn silage and cattail due to the competition for growth between the fungus and methanogens microorganisms.

Consortia are another option that can be more efficient and robust than pure culture for bioaugmentation of the hydrolytic first stage. Martin-Ryals et al. (Martin-ryals et al., 2015) tested at lab scale a commercialized cellulolytic culture of anaerobic bacteria (mainly from the genus *Clostridium*) on corn lignocellulosic residues in a two-stage continuous digestion. Daily bioaugmentation increased hydrolysis and VFAs concentrations that led to a final methane yield 56% greater than the control. Furthermore, economic analysis showed that daily bioaugmentation was economically feasible and could improve the economics of AD by 27-34 dollars/dry ton for this specific feedstock. Similarly, Poszytek et al. (Poszytek et al., 2016) designed a Microbial Consortium with High Cellulolytic Activity (MCHCA), made of 16 selected strains, that was used to treat maize silage in a two-stage digestion system. MCHCA addition succeeded in significantly enhancing the biogas yield by 38% and increased the methane content by 14%.

From these results, it seems clear that bioaugmentation using anaerobic hydrolytic consortia would be preferred to pure culture as they adapt better to environmental changes (pH, T°) and tend to show higher resistance to the presence of toxic organic

compounds, heavy metals or contamination by other strains (Poszytek et al., 2016). Several hydrolytic anaerobic consortia have been developed and it would be interesting to test them in full-scale applications (Nzila, 2016). One of the impediments for a technology scale up is probably lying in the lack of a market. Indeed, nowadays full-scale “hydrolytic” two-stage digesters dedicated to lignocellulosic substrate are very seldom. Bioaugmentation scale-up will be part and concomitant with the development of a reliable hydrolytic two-stage digestion solution at full-scale dedicated to lignocellulosic feedstocks.

3.3.2.3. Ensiling

Ensiling is a very conventional and widely applied method for efficient storage of crops at farm scale. In biogas plants, silage are generally carried out in large silage silo (concrete structure), under the form of covered pit, as it can be shown in **figure 18**. Ensiling is based on the application of anaerobic and wet conditions that favor lactic and acetic acid fermentations, transforming free sugars, hemicelluloses and cellulose mainly into organic acids. It results in an environment acidification that inhibits most of the activities of microorganisms that would otherwise degrade the biomass. An important feature of ensiling is that it does not degrade lignin efficiently (Rouches et al., 2016). Montgomery et al. (Montgomery and Bochmann, 2014) underlined that ensiling cannot be considered as a pretreatment to enhance biogas production, due to its minimal effect reported on methane yield. However, this statement can be discussed.



Figure 18: (A) Silage silo in Pot-au-Pin (©Constant Formé-Bécherat); (B) Close view on rye silage

Indeed, ensiling is a complex biological process that needs a careful tuning. To ensure its success, the following parameters need to be optimized: particle size, moisture, storage conditions, storage duration, temperature and additives (Teixeira Franco et al., 2016). Each of these parameters has an impact on the storage quality but also on the future methane yield of the feedstock. Only a few studies that took into account organic matter loss succeeded in showing an increase in methane yield using ensiling. For instance, Pakarinen et al. (Pakarinen

et al., 2011) reported a 50% increase in methane yield of hemp after ensiling. More recently, ensiling of giant reeds permitted to obtain an increase in methane yield ranging from 4 to 14% (Liu et al., 2016) and ensiling of switchgrass also improved subsequent AD despite a 6% mass loss (Zhao et al., 2017). According to Teixeira Franco et al. (Teixeira Franco et al., 2016), these results may be explained by gains in biochemical accessibility that overcame the potential organic matter loss during storage, by microorganisms respiration. In this regard, if anaerobic conditions are well applied, the most critical parameter to ensure an increase in methane yield appears to be the feedstock biochemical characteristics. Therefore, results are strongly feedstock dependent and that might explain why ensiling of some feedstocks (mainly energy crops) have shown no effect or negative effects on the methane yield (Herrmann et al., 2011; Kreuger et al., 2011).

Even if it remains very challenging, future research may find ways to enhance biogas production for promising feedstocks and with optimized ensiling conditions. Over time, ensiling may not only be a storage process but also become a potential low-cost pretreatment to enhance subsequent digestion of specific feedstocks.

3.3.3. Aerobic pretreatments

In the case of aerobic pretreatments, the presence of oxygen allows to take full advantage of decomposition capacities of facultative anaerobic and aerobic microorganisms. These microorganisms degrade organic matter using oxygen as a final electron acceptor in order to ensure their growth. Through their metabolism, they mainly produce CO₂, water, nitrate and sulfate (Merlin Christy et al., 2014). These types of pretreatments can be very interesting in the case of lignocellulosic substrates. Indeed, they can offer better subsequent accessibility to organic matter during AD by specifically degrading lignin polymers (Rouches et al., 2016).

Aerobic pretreatments can be divided into three categories. The first one is simple aeration where substrate is only subject to aerobic conditions. The second one is aerobic pure culture pretreatment, where pre-aeration is complemented by the inoculation of a given aerobic microorganism featuring interesting degrading properties. The last category gathers aerobic consortium pretreatments, where aerobic consortia under liquid or solid forms are inoculated instead of a pure culture. Micro-aeration during AD and aeration of the digestate will not be discussed here but it can be noticed that several studies displaying interesting results have been published on these topics (Giroto et al., 2016; Tsapekos et al., 2016).

3.3.3.1. Simple aeration

Simple aeration can be operated under solid state. In this case, it is important to make a distinction between simple aeration and composting. Indeed, in the first case, feedstock is shortly exposed to aerobic conditions, it may eventually reach the thermophilic phase but never the maturation phase. While in the case of composting, according to the classical definition, feedstock is subject to a long aeration time, including mesophilic, thermophilic, cooling and maturation steps (Tuomela et al., 2000). Duration, temperature and organic matter stabilization are the main criteria to distinguish between both processes. Besides

(micro-)aeration prior to AD can also be applied. It generally consists in injecting air or oxygen in the system for a given period before to shift to anaerobic conditions. Simple aeration is taking advantage of endogenous aerobic or facultative anaerobic communities' hydrolytic activities.

Simple aeration is particularly interesting in the case of lignin rich substrates as oxygen favors ligninolytic activities of endogenous fungi and bacteria populations. Recent and/or highly interesting literature results using this type of pretreatment on agricultural feedstock are gathered in **Table 2**. Some studies have recently evaluated at lab scale the impact of micro-aeration prior to AD or aeration on lignocellulosic agricultural feedstocks and their subsequent biogas production. Fu et al. (Fu et al., 2015) injected a small amount of oxygen (5 mL/g VS) in bottles flushed beforehand with nitrogen and containing corn straw as well as anaerobic inoculum. After total depletion of oxygen, substrate received additional inoculum and water, and then AD was carried out. The pretreatment had a notable disruptive effect on the structure of corn straw. Besides, methane yield was 16% higher than the untreated group due to improved hydrolysis efficiency (11% higher VS removal). For aeration under solid state, Zhou et al. (Zhou et al., 2012), applied a 20 days pile pretreatment to corn stover that was beneficial to hemicellulose, cellulose and lignin depolymerization (up to 5.7% lignin degradation for the pile middle layer). Consecutive 5 L anaerobic co-digestion of the pretreated corn stover with cow dung had a biogas yield enhanced up to 29% (for the middle part of the pile) in comparison to the control. This improvement was due to a higher cellulose and hemicelluloses degradation during AD, that can be explained by a higher accessibility to these polymers by anaerobic microorganisms thanks to lignin removal. Similarly, the aeration of rice straw showed that cellulose, hemicelluloses and lignin were decreased by 7.5%, 64.5% and 13.6% respectively (Yan et al., 2015). Besides, total solids also showed a significant decrease of 63.6% after pretreatment. No biogas measurement was carried out to measure the efficiency of the pretreatment but it can be assumed that such mass loss during pretreatment was detrimental for methane production.

The question of mass loss during aerobic pretreatments is of the utmost importance in order to fully evaluate their impacts on methane yield. Indeed, during aerobic degradation reactions, carbon can be relocated under different forms that are soluble carbonated molecules, aerobic biomass and CO₂. If for the former two, they can be potentially used as carbon sources during following AD, it is not the case of CO₂, which is a carbon loss that will directly impact the methane yield. **Figure 19** illustrates this mass loss principle. So, the main challenge for efficient aerobic pretreatment dedicated to agricultural feedstocks can be raised: finding a trade-off between a better accessibility to hemicelluloses and cellulose and the loss of organic matter. Therefore, pretreatment optimization and measurement of the matter loss under the form of CO₂ are critical to ensure and fully validate a gain in methane yield. Until now, the question of mass loss has often been neglected in literature and it is highly recommended to take it into account in future studies (Rouches et al., 2015).

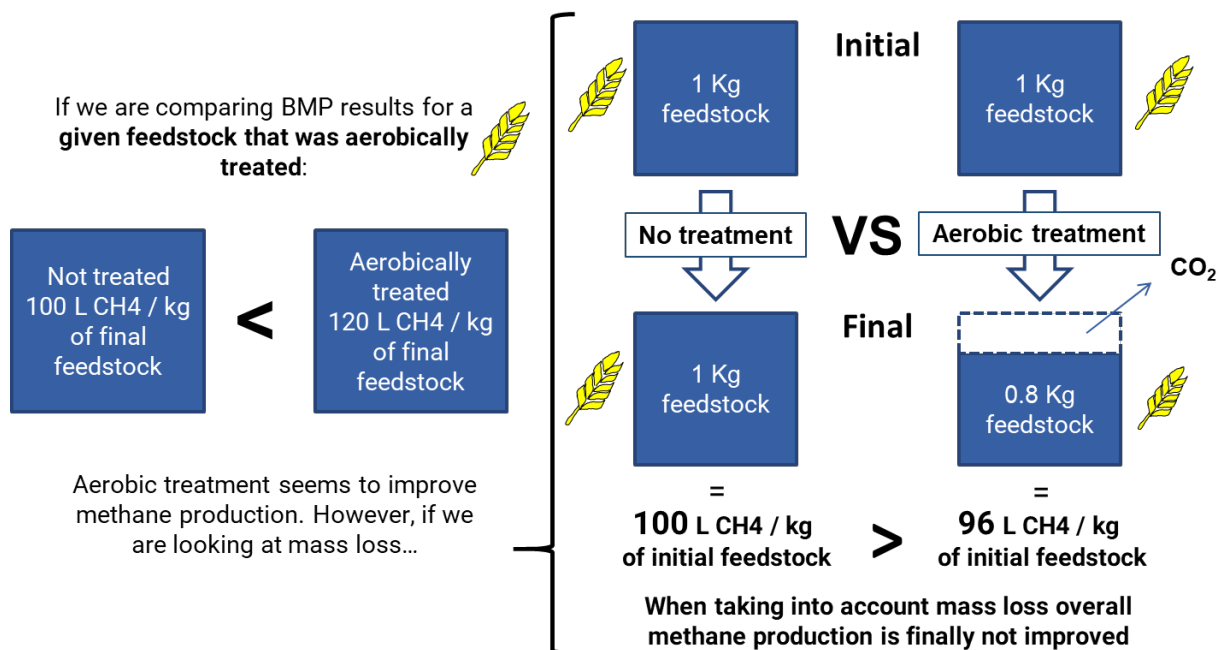


Figure 19: Illustration of mass loss principle for aerobic pretreatments on a specific case

At full-scale, aerobic pretreatment of agricultural feedstocks can be easily carried out. Simple aeration under the form of stacks, piles, pits or bins are commonly carried out in farms. If no results are published on the subject, it is likely that farmers already applied them for specific lignocellulosic feedstocks. Fully detailed full-scale studies would be interesting to evaluate the impact of short aeration on methane production with respects to substrate characteristics and following process optimization. If it proves to be efficient and easily implementable, it may generalize this pretreatment method for lignocellulosic feedstocks similarly to ensiling for crop storage. An important drawback of such pretreatment that can arise is its potential long duration (several days) that may necessitate a more complex substrate management on-site.

Another interesting feature of short aeration that can only be observed at pilot or full-scale is organic material temperature rising similarly to a thermophilic composting phase. This process specificity can be used to avoid a high energy requirement for substrate heating in the case of subsequent thermophilic AD. Historically, such a principle was applied to solid manure, in the Ducelier-Isman process. A demonstration plant of 15 m³ in France, in 1986-1987 showed that pre-aeration of 24-45 hours can increase manure temperature up to 72°C and thus, reduce the lag phase of subsequent AD from 6 to 2 days (Theoleyre, 2014). Nonetheless, this pretreatment application remains confidential on farm plants probably due to the fact that efficient warming of digesters can be ensured by heat surplus from cogeneration or by on-site boiler using biogas excess production in injection plant.

3.3.3.2. Aerobic pretreatment using pure culture of microorganisms

Pure culture of hydrolytic microorganisms can be used in addition to aerobic conditions in order to favor accessibility increase over mass loss. White rot fungi (WRF) in particular have drawn a lot of interest due to their ability to specifically target lignin polymers, consequently an abundant literature exists on the topic (da Silva et al., 2017; Rouches et al., 2016). A few

recent and selected studies using pure cultures and measuring mass loss are given in **Table 2** for agricultural feedstocks.

WRF strains that use lignin for their growth instead of cellulose and hemicelluloses are of high interest to decrease the recalcitrance of lignocellulosic biomass while increasing the hydrolysis of carbohydrates. Therefore, an increase in biogas production of different substrates was found in several lab scale studies following WRF pretreatment. For example, Lalak et al. (Lalak et al., 2016) applied, after sterilization of tall wheat grass, a 4-week pretreatment using the WRF *Flammulina velupites*. This pretreatment increased by 134% the methane yield of subsequent AD. However, mass loss during the pretreatment was around 29% due to degradation of cellulose (20.5%), hemicelluloses (29%) and lignin (35.4%). Despite a higher degradation in lignin than cellulose and hemicelluloses, as mass loss is not taken into account in their calculation, it is unclear if the final methane yield is really improved. Similarly, Mustafa et al. (Mustafa et al., 2016a) obtained a 120% increase in methane yield after 20 days pretreatment using the WRF *Pleurotus ostreatus*. Lignin degradation was still higher (33%) than cellulose (7%) and hemicelluloses (16%), resulting in higher structural decomposition. But mass loss, that was lower in this study (around 11%), was not taken into account leading again to an uncertain improvement. When mass loss was taken into account, results on methane yield were less impressive and even negative. For instance, Rouches et al. (Rouches et al., 2015) after *Basidiomycetes* strain screening, selected *Polyporus brumalis* BRFM 985 to pretreat wheat straw. A non-optimized 21-day pretreatment led to an increase of 21% in methane yield with mass loss (14%) taken into account this time. Alike, a recent study including mass loss (10%) in calculations, displayed a 60% increase in methane yield of hazel branches after a 28-day pretreatment using *Ceriporiopsis subvermispota* ATCC 96608 (Liu et al., 2017). However, identical pretreatment on acacia branches, barley straw and bagasse did not show any effect and even at some point a decrease in methane yield was found. For instance, bagasse had its methane yield lowered by 5%. These results can be explained both by the incapacity of the strain to efficiently degrade lignin for this substrate and by the too high mass loss. Similar results were observed on corn stover, where strains and particle size of the straw had an impact on the pretreatment efficiency (Wyman et al., 2017). Among the three strains tested and two particle size conditions only a treatment with *Pleurotus eryngii* at small size stover (between 0.5 and 4.5 mm) led to a 19% increase in biogas yield, all the others had lower or unchanged yields.

Once mass loss is taken into account, it is clear that positive impact of WRF pretreatment on methane yield is uncertain. Results are highly dependent on feedstock, cultivation parameters (temperature, moisture and duration), nutritional supplementation and fungal strain (Liu et al., 2017; Rouches et al., 2016; Wyman et al., 2017). Therefore, pretreatment design and fine optimization are required in order to obtain an increase in methane yield. Besides, one of the main drawbacks of such pretreatment apart its relative long duration is the necessity to carry out sterilization of the pretreated substrates. It is required both to ensure efficient colonization of the substrate by the selected microorganisms and to avoid consumption by

endogenous microorganisms of the released sugars during pretreatment. At full-scale, sterilization is highly expensive, energy consuming and difficult to put into practice. For this reason, a full-scale application of such pretreatment does not exist yet. A possible solution to this problem would be to practice inoculum propagation, inspired by back-slopping methods of food industry. It consists of the sterilization and colonization of a small amount of substrate that is used to colonize and treat a larger amount of unsterile identical substrate. Then, this colonized material can be used as inoculum for a larger mass of the same unsterile substrate and so on and so forth. This solution was recently explored and promising results were obtained using miscanthus as feedstock (Vasco-correa et al., 2016).

3.3.3.3. Aerobic pretreatment using consortia

The use of consortia or mixed cultures is a promising alternative to pure cultures due to several advantages: (i) greater quantity and variety of enzymes are produced that can act synergistically and thus enhance hydrolysis efficiency; (ii) more tolerance with environment changes (pH, temperature) causing a gain in process robustness; (iii) capacity to thrive and develop in unsterilized environments (Wei, 2016). For these reasons, a large number of studies using consortia were carried out at lab scale. Results from a selection of these studies are given in **Table 2**. Two types of inoculum, added before the aerobic step, can be distinguished:

- (i) Solid inoculum containing aerobic microorganisms can be added to lignocellulosic feedstocks (e.g. rotten straw, compost, fungal mash...). Thus, a method was recently tested on corn straw, where Thermophilic Microaerobic Pretreatment (TMP) was combined with retted and composted corn straw as aerobic inoculum (Fu et al., 2016). A 20h pretreatment at 55°C with 5 mg of O₂ per VS of substrate initially injected led to a 21% increase in methane yield in comparison to the control. However, in this article, inoculum is mixed with corn straw at a high ratio of 700% w/w. Such amount of inoculum would be unrealistic at higher scale. The first feature of a promising aerobic solid inoculum would be to have a positive impact despite a small or reasonable quantity mixed with feedstock.

At full-scale, a solution based on compost addition exists for agricultural feedstocks under the trademark Bacteriometha™ sold by a French company called Sobac (Lioujas, France). It is an additive made of compost rich in hydrolytic microorganisms. It has to be mixed with substrates (such as cow manure) between 3 and 15 days before AD in a ratio of 0.5-1kg/m³ of substrate. Mixture remains in aerobic conditions such as stacks, piles or open tanks. According to Sobac, biogas yield can be increased between 10 and 30% and trials on two full-scale plants displayed higher energy production and an easier mixing in the digester that ensured economic interest despite the additive cost (Sobac, n.d.).

In addition to compost, wood can also be used as inoculum especially for fungi, but this research track remains unexplored. Fungal mash is another option as already seen before (Uçkun Kiran et al., 2015).

- (ii) Aerobic consortia under liquid form can also be used to pretreat lignocellulosic feedstocks. They can be aerobic sludge or a solution containing a designed or isolated consortium. In the former case, Mshandete et al. (Mshandete et al., 2005) used an activated sludge mixed culture as inoculum for an aerobic pretreatment of sisal pulp. Inoculum was brought in large quantity to the system in comparison to sisal pulp with a ratio of 14:1 v/v (unrealistic proportions for a full scale application). After optimization, surface aeration for 9 hours at 37°C appeared to be the most efficient treatment as longer durations did not lead to higher methane yields. After AD, methane yield was enhanced by 26% in comparison to the control. High VFAs concentrations and high activities of hydrolytic enzymes were obtained at the end of the short pretreatment that can explain this positive result. Similarly, Zhou et al. (Zhou et al., 2017) mixed rice straw with aerobic sludge supernatant and applied an optimized 2 days continuous aeration (30 L/h/kg DM) pretreatment. Subsequent BMP showed a 16% increase in methane yield compared to the control.

Several hydrolytic consortia have been constructed and tested at lab scale. It can be noticed that the method, often used to select efficient hydrolytic microorganisms, is based on the speed at which a filter paper is degraded; the faster the degradation, the higher the hydrolytic capacity of the isolated consortium. Zhang et al. (Zhang et al., 2011) designed from soil samples a microbial consortium with high cellulose degradation ability. Then, the consortium was applied to cassava residues for 12 hours in aerobic conditions at 55°C. Subsequent AD of the cassava residues displayed a 97% higher methane yield than the control. Another hydrolytic consortium which is called MC1 was constructed from compost in 2002 and since then, has been regularly used to pretreat agricultural feedstocks. For instance, it was recently used in the aerobic 5-day pretreatment of non-sterile rotten silage maize straw (Hua et al., 2016). Following that, biogas yield was enhanced by 75% compared to the control. Rotten sawdust was used to screen microorganisms displaying both high cellulolytic and ligninolytic activities (Ali et al., 2017). After isolation and identification, selected microorganisms were gathered to form a consortium displaying high lignocellulosic degradation activity. Its application for 10 days as a pretreatment to sawdust improved methane yield of the subsequent AD by 73%. Finally, Zhong et al. (Zhong et al., 2016) designed a microbial hydrolytic consortium from compost and cow manure. It was used to pretreat wheat straw for 3 days in aerobic conditions and led to a methane yield 80% higher than control in BMP tests.

Enhancements of methane yield between 16% and 97% are reported herein above. However, these very positive results have to be put into perspective. First, mass loss was not always taken into account in calculations to obtain these results, even when it was measured. It can be assumed that for short duration (hours), it remains too

low to negatively impact results. But when it comes to several days, it is likely that the calculated increase in methane yield will be lower than described in the studies; for instance, 3 days already led to a 25% mass loss under CO_2 form in the case of Zhong et al. (Zhong et al., 2016). Secondly, mainly BMP or small batches were performed, which limits lab scale evaluation of such pretreatment as it will probably be used in continuous or fed-batch mode at full-scale. Thirdly, to obtain positive results, this pretreatment has to be strongly optimized. CO_2 emission, VFAs concentration, soluble COD and in particular lignin degradation are criteria that can be used to determine the optimum duration beyond which enhancement will be lower or even non-existent.

Finally, at full-scale, a semi-aerobic hydrolytic pretreatment was developed by Bionova Biogas GmbH (Wernsdorf, Germany) for liquid CSTR agricultural biogas plants. It consists in a closed vessel, where air is injected uniformly through feedstocks in a liquid state (**figure 20**). HRT is short (order of days as a function of the plant) and a liquid aerobic inoculum is added once at the operational start. It is dedicated to lignocellulose rich substrates to enhance their degradation before they enter the digester. According to the supplier, up to 20% extra biogas can be obtained. Aeration is performed here using a compressor which is energy consuming. This pretreatment is currently implanted mainly in Germany and France in more than ten plants.



Figure 20: Picture of a semi-aerobic hydrolysis tank – from (Bionova-Biogas GmbH, website)

Table 2: Effect of aerobic pretreatment on biogas and methane yield in function of feedstock

Feedstock	Type of aerobic pretreatment	Pretreatment conditions	AD scale / AD process conditions	Methane yield (L/g VS)		DM loss taken into account in Methane yield	References
				Control	Treatment		
Corn stover (pile middle part)	Simple aeration	Aeration using 1m pile set up – density of 5 kg/m ³ – ambient temperature (25-30°C) – 20 days	Lab scale 5L co-digested with cow dung (1:1) / 60 days	0.315*	0.45*	No	(Zhou et al., 2012)
Corn straw	Simple aeration	AD conditions in bottles and then pure oxygen was added 5 mL/gVS – 55°C – until total oxygen depletion	Lab scale 500 mL batch / 37°C -60 days – 130 rpm	0.28	0.325	No	(Fu et al., 2015)
Wheat straw	Fungal pure culture	Column autoclaved - <i>Polyporus brumalis</i> BRFM 985 (1:166) – 120 mL/min 100% moisture air – 28°C – 21 days	Lab scale 600 mL BMP / 36°C	0.217	0.28	Yes	(Rouches et al., 2015)
Tall wheatgrass	Fungal pure culture	250 mL Erlenmeyer 65% moisture autoclaved – <i>Flammulina velutipes</i> – cotton plugs – 28°C – 4 weeks	Lab scale 2L digester / 37°C – 23 days – pH 7	0.125	0.169	Only calculated (up to 29%)	(Lalak et al., 2016)
Rice straw	Fungal pure culture	1 L Erlenmeyer 75% moisture autoclaved – <i>Pleurotus ostreatus</i> (1:4)– cotton plugs – 28°C – 20 days	Lab scale 500 mL batch / 37°C – 45 days – pH neutral	0.12	0.263	Only calculated (up to 13%)	(Mustafa et al., 2016a)
Hazel branches	Fungal pure culture	250 mL Erlenmeyer autoclaved – <i>Ceriporiopsis</i>	Lab scale 500 mL BMP / 35°C	0.105	0.185	Yes	(Liu et al., 2017)
Sugarcane bagasse		<i>subvermispora</i> ATCC 96608 (1:15) – 28°C – 28 days	0.230	0.220	Yes		
Corn stover (4.5-9 mm)	Fungal pure culture	250 mL Erlenmeyer autoclaved – <i>Pleurotus eryngii</i> (2:1) – 82% moisture air – 30°C – 30 days	Lab scale 250 mL BMP / 35°C – 40 days	0.355*	0.279*	Yes	(Wyman et al., 2017)
Corn stover (0.5-4.5 mm)		250 mL Erlenmeyer autoclaved – <i>Pleurotus eryngii</i> (2:1) – 82% moisture air – 30°C – 30 days	Lab scale 250 mL BMP / 35°C – 40 days	0.302*	0.360*	Yes	
Corn stover (0.5-4.5 mm)		250 mL Erlenmeyer autoclaved – <i>Pleurotus ostreatus</i> (2:1) – 82% moisture air – 30°C – 30 days	Lab scale 250 mL BMP / 35°C – 40 days	0.294*	0.301* ± 2%	Yes	
Corn Straw	Aerobic consortium from retted corn straw composting	1 L bottle – retted and composted corn straw (7:1) – 5 mL of O ₂ /g VS _{substrate} was injected then bottles were closed – 55°C – 20h	Lab scale 200 mL batch / 37°C – 45 days – 130 rpm	0.277	0.343	No	(Fu et al., 2016)
Sisal pulp	Aerobic consortium from WAS	500 mL Erlenmeyer – Aerated WAS (14:1) – open flask permits surface aeration – 37°C – 9 hours – 135 rpm	Lab scale 500 mL BMP / 37°C - 32 days – 70 rpm	0.19	0.24	No	(Mshandete et al., 2005)
Rice straw	Aerobic consortium from sludge	10 L bioreactor – aerobic sludge supernatant (2:1) – moisture 60% – aeration 30L/h/kg DM – 35°C – 2 days	Lab scale BMP type AMPTS / 37°C – 55 days – 160 rpm	0.306	0.355	No	(Zhou et al., 2017)

Cassava residues	Aerobic consortium from soil	250 mL flask – designed hydrolytic consortium (aerobic & anaerobic bacteria) (1:20) – Loose caps – 55°C – 12h	Lab scale 250 mL batch / 55°C – 10 days – pH 7.2	0.132	0.259	No	(Zhang et al., 2011)
Rotted silage maize	Aerobic consortium from compost	300 mL flask – designed consortium (MC1) – static aerobic conditions – 50°C – 5 days	Lab scale 600 mL batch / 35°C – 16 days	0.173*	0.304*	No	(Hua et al., 2016)
Sawdust	Aerobic consortium from rotten sawdust	5 L Erlenmeyer flasks – designed consortium (1:2) – Cotton stoppers – 30°C – 10 days	Lab scale 5L batch / 30°C - 35 days	0.09	0.15	No	(Ali et al., 2017)
Wheat straw	Aerobic consortium from compost and dairy manure	1.5 L bottle autoclaved – designed hydrolytic consortia (mainly facultative anaerobic bacteria) (1:20) – sealed with plastic film open periodically – 37°C – 3 days	Lab scale 600 mL BMP / 37°C – 23 days	0.137	0.246	Only calculated (up to 25%)	(Zhong et al., 2016)


Methane yield: * Biogas yield (L/g VS) is given instead of methane yield



3.4. Biological pretreatments for agricultural feedstocks: a summary

Table 3 is a summary of what has been described above concerning biological pretreatments of agricultural feedstocks. It can be seen that lignocellulose rich feedstocks, due to their recalcitrance, are logically concentrating most of the current research on biological pretreatments. For easily biodegradable substrates such as sequential crops, carbohydrase and two-stage anaerobic digestion process could be interesting solutions to enhance methane yield and AD kinetics. Other types of enzymes as well as aerobic treatments have not been explored and are likely to provide negative results on the methane yield. Finally, enhanced two-stage and ensiling for easily biodegradable substrates were identified as potentially applicable and may lead to positive results on the methane yield. They can be tracks for future research and development projects in this field.

Table 3: Biological pretreatments: Effect on biogas and methane yield and existing full-scale technology

		Agricultural feedstocks	
		Lignocellulose rich	Easily biodegradable
Enzymes	Protease	--	
	Lipase	/	
	Carbohydrase	++ <i>Methaplus® – Optimash®</i>	+ <i>Methaplus® – Optimash®</i>
	Lignin-modifying	++	
Anaerobic	Two-Stage	-/+	+ <i>Bioplex process</i>
	Enhanced two-stage	+	
	Ensiling	+	
Aerobic	Simple aeration	++ <i>Pile composting</i>	
	Pure culture	+++	
	Consortia (solid or liquid)	+++ <i>Methalyse® - Bacteriometha®</i>	

+ / ++ / +++ : Lab or pilot scale positive results - / - : Lab scale negative results  : Positive results with *existing full-scale technologies*

 : Unexplored field with expected positive results  : Unexplored field with expected negative results  : Not applicable

3.5. Combination of biological pretreatments with other types of pretreatments

As each type of pretreatment has its own functioning mode and as pretreatment effects are often complementary, an option to obtain even more efficient pretreatment is to combine them (Zheng et al., 2014). However, it is important to underline that if very interesting results can be obtained at lab scale, additional costs due to combination may be economically unacceptable at full-scale. Therefore, fine economic analysis comparing gain and cost should be carried out before any scale up. Some recent examples of literature on this topic will be given below and gathered in **Table 4**. Additionally, Sindhu et al. (Sindhu et al., 2016) reported earlier examples of biological pretreatment combinations for lignocellulosic feedstocks.

Biological pretreatments can be combined to keep low the energy demand. For example, Thomsen et al. (Thomsen et al., 2016) applied first an ensiling step to wheat straw with

Lactobacillus buchneri followed by a washing step and finally a WRF treatment with *Ceriporiopsis Subvermispora* before AD in BMP tests. Here, ensiling and washing were used as a conditioning method that eliminates waxes, fats and toxic compounds, thus facilitating subsequent feedstock colonization by *C. Subvermispora*. Only a minor 5% mass loss was observed after the combined pretreatment but it was not taken into account in calculations. If methane production rate was faster, methane yield did not significantly increase after this combined pretreatment. Here, the use of ligninolytic consortia instead of pure culture might be an interesting alternative to obtain an increase in methane yield. Another example at lab scale was carried out on agave bagasse (Arreola-Vargas et al., 2016). After an enzymatic treatment, hydrolyzate was anaerobically digested in a two-stage process. In comparison to a single-stage process, energy recovery obtained from the enzymatic hydrolyzate was 3.3 fold higher due to hydrogen production and no inhibition during methanogenesis step (neither VFAs nor MCCAs accumulation). This result is particularly interesting as it shows potential advantages of using two-stage AD for lignocellulosic substrates after their hydrolysis.

Biological pretreatments can also be combined with mechanical, chemical or thermal pretreatments to ensure higher methane yields. Wheat straw grinding followed by a 180 days ensiling showed a 36% increase in methane yield whereas grinding only led to a 26% increase (Gallegos et al., 2017). Nevertheless, this strong increase has to be nuanced as mass loss during ensiling was not taken into account in this study. Mustafa et al. (Mustafa et al., 2016b) applied on rice straw a 30-day WRF pretreatment using *Pleurotus ostreatus* that was followed by a milling step. Despite a 12% mass loss that was not taken into account in the calculations, the methane yield was increased. Indeed, 500 mL SS-AD displayed a methane yield 165% higher in comparison to the control. Alkaline treatments were applied in combination with enzyme or WRF pretreatments. Alexandropoulou et al. (Alexandropoulou et al., 2015) applied the WRF *Abortiporus biennis* for 30 days to willow sawdust and then carried out a 24-hour NaOH alkaline treatment. Here, mass loss during WRF was taken into account and despite a 17% DM loss, subsequent BMP displayed a 115 % increase in methane yield due to high lignin removal during pretreatment. Similarly, NaOH pretreatment was carried out on miscanthus followed this time by an enzymatic pretreatment with cellulase and cellobiase (Michalska et al., 2015). Delignification and higher accessibility to cellulose were observed again, as well as, a 94% methane yield enhancement in comparison to sole enzymatic pretreatment. Finally, thermal pretreatment was applied before two-stage AD at lab scale on sugarcane bagasse (Baêta et al., 2016) or on MSW (Li et al., 2017). Hydrolysis of bagasse was carried out at 182°C for 40 min that released sugars but also inhibitory phenols and furans. Here, the interest of the first AD stage was to biodetoxify the hydrolyzate, as acidogenic microorganisms appeared to be able to decrease the concentration of toxic compounds for methanogenic microorganisms. This led to a higher energy yield in comparison to a one-stage process, as methanogenesis step was less inhibited. Finally, Schroyen et al. recently identified a detoxifying role of laccase following harsh thermal or chemical pretreatments (Schroyen et al., 2017). Indeed, laccase enzymes were used to remove high concentrations of AD inhibiting phenolic compounds. A 24h laccase treatment step was applied to miscanthus and hemp

straw supplemented with different concentrations of p-coumaric acid (up to 2 g/L), that simulated phenolic compounds that could be released following a harsh pretreatment (thermal or chemical). Interestingly, laccases were able to reduce p-coumaric acid concentration and therefore improve initial hydrolysis rate of subsequent AD.

It has been shown that the use of biological pretreatments in combination with other pretreatments displays very interesting and promising results at lab scale. However, to our knowledge, they are not yet applied at full-scale due to potential higher costs and complexity implementation.

Table 4: Combinations of pretreatments - strategies adopted in function of feedstocks

Feedstock	Strategy*	Results	References
Wheat straw	Ensiling/washing + WRF	No significant increase in methane yield / Increase in methane production rate	(Thomsen et al., 2016)
Agave bagasse	Enzyme + 2-stage AD	3.3 fold higher energy recovery vs. Enzyme + 1-step AD	(Arreola-Vargas et al., 2016)
Wheat straw	Grinding + ensiling	36% increase in methane yield vs. untreated	(Gallegos et al., 2017)
Rice Straw	WRF + milling	165% increase in methane yield vs. untreated	(Mustafa et al., 2016b)
Willow Sawdust	WRF + alkaline (NaOH)	115% increase in methane yield vs. untreated	(Alexandropoulou et al., 2015)
Miscanthus	Alkaline (NaOH) + enzyme	94% increase in methane yield vs. enzyme treatment	(Michalska et al., 2015)
Sugarcane bagasse	Thermal + 2-stage AD	14 times higher methane yield vs. thermal + 1 step AD	(Baêta et al., 2016)
Miscanthus/Hemp straw	Thermal/chemical "like" + enzyme (laccase)	No significant increase in methane yield / Increase in methane production rate (reduced inhibition)	(Schroyen et al., 2017)

*: Treatments are given by order of applications

3.6. Final assessment on biological pretreatments

To assess biological pretreatments, the following criteria can be given for a successful pretreatment (Budzianowski, 2016): (i) low energy input; (ii) avoid carbohydrates loss; (iii) use minimal and inexpensive chemicals and/or water; (iv) avoid expensive pretreatment devices; (v) avoid AD inhibitors formation; (vi) avoid the need for waste disposal; (vii) be flexible with respect to the feedstock; (viii) be environmentally friendly; (ix) be cost-effective. These can be confronted with advantages and disadvantages of each biological pretreatment gathered in **Table 5**. All the biological pretreatments meet conditions (i), (iii), (vi) and (viii). Enzymatic pretreatments also meet conditions (ii) and (iv) but they can generate AD inhibitors, especially on lignocellulosic substrates with lignin-modifying enzymes (such as phenols), they have a low flexibility due to the required process optimization and lastly, enzymes high prices are limiting their cost effectiveness. Anaerobic pretreatments with two-stage process meet conditions (ii), (v) and (vii). Nevertheless, they require expensive additional reactor(s) that negatively impacts cost effectiveness. Finally, aerobic pretreatments can meet conditions (v) and (ix). However, the required fine-tuning for a given substrate limits the flexibility of the method. Additionally, carbohydrates loss is difficult to avoid.

Biological pretreatments can also be assessed through the technological readiness level (TRL) scale that goes from 1 (idea) to 9 (full-scale technology widely used). Enzymes and two-stage process are relatively mature technologies, displaying positive results and available at full-scale under the form of several commercialized products. However, their current costs hamper their wide adoption at full-scale. Therefore, their TRL is at 8-9. Aerobic pretreatments, despite existing full-scale technologies, are rather at a development stage. Process optimization is still required for a given substrate to ensure a low mass loss. Besides, in the case of aerobic consortia use, their selection and production are still made at lab scale. Thus, it can be considered that TRL for this pretreatment is comprised between 4 and 6.

From this general assessment, it is clear that biological pretreatments have strong advantages that deserve further work on the reduction of their current drawbacks. By doing so, cost effectiveness of these technologies may be enhanced and full-scale applications may spread.

Table 5: General features of biological pretreatments

Type of biological pretreatment	Enzyme	Anaerobic	Aerobic
Advantages	<ul style="list-style-type: none"> Low energy demand Fast process No matter loss Scalability Lignin breakdown Technology readily available Application versatility 	<ul style="list-style-type: none"> Low energy demand No matter loss Limit acidogenesis risk Potential H₂ production (Pathogens removal) Technology readily available 	<ul style="list-style-type: none"> Low energy demand Potentially low-cost Lignin breakdown Scalability
Disadvantages	<ul style="list-style-type: none"> Current high cost Enzyme lifespan Continuous addition needed Moderate activity/Inhibition 	<ul style="list-style-type: none"> No lignin breakdown Cost for second digester Higher complexity 	<ul style="list-style-type: none"> Matter loss Relatively high exposure time Energy if forced aeration Sterilization can be required Process control Developing technology
Other potential positive effects than methane yield increase	<ul style="list-style-type: none"> Increase AD rate Use of new/local feedstock Reduce energy need (mixing) 	<ul style="list-style-type: none"> Increase AD rate Limit AD inhibitors 	<ul style="list-style-type: none"> Increase AD rate Use of new/local feedstock Reduce energy need (mixing)
Technological readiness level (from 1 to 9)	8-9	7-8 for two-stage	4-6

4. Valorization of agricultural digestates

Besides biogas, digestate is also produced throughout the year on biogas plants. In CSTR biogas plants, digestate is mostly a mix of water, residual undigested feedstocks, microorganisms and inorganic matter (minerals...). Digestate is generally used as a valuable bio-fertilizer (Nkoa, 2014; Wellinger et al., 2013). However, land disposal of digestate in Europe is mainly regulated by the Nitrate directive that aims to reduce ammonia and nitrogen oxide emissions as well as nitrogen brought to the soil (Tambone et al., 2017). Digestate storage is therefore required to allow an adequate land spreading in: (i) time, when digestate is stabilized and crops are in a growth stage that needs nutritional input; (ii) quantity, as it can vary as a function of the soil type and the farm area (Paavola and Rintala, 2008). Digestate land spreading could even be an issue for farmers when pollutants are present in digestate (heavy metals, pathogens, pesticides...) or in high density biogas plant area, where local oversupply of digestate pushes to costly digestate transportation towards nutrient deficient areas. Therefore, new opportunities and ways of valorizations under the form of various post-treatments have been identified to enhance biogas plant profitability and reduce environmental impacts of digestate (Monlau et al., 2015).

Figure 21 gives an overview of the different valorization pathways of digestate, grouped as a function of their final aims that could be either or combined: (i) additional energy production; (ii) biochemicals and other value-added material production; (iii) agricultural use. Thereafter, agricultural digestate features and each type of post-treatment will be detailed.

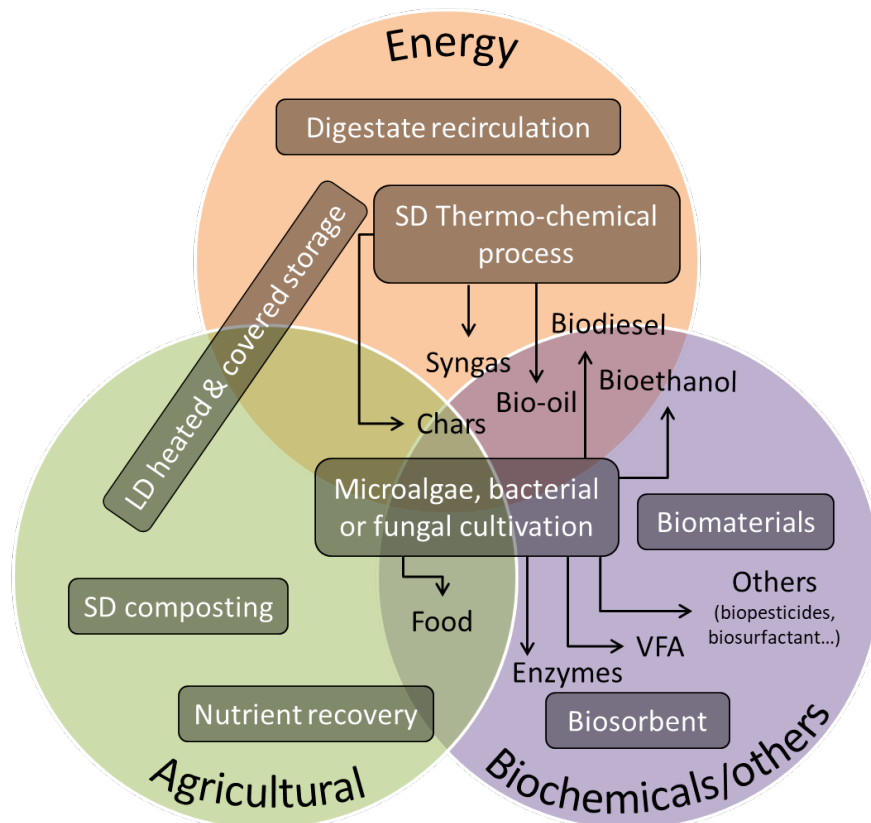


Figure 21: Digestate valorization pathways and their potential end products grouped in function of the three main finalities: Energy – Biochemicals/others – Agricultural

4.1. Phase separation and digestate features

Generally, agricultural CSTR biogas plants with TS above 7-8% (sometimes even lower) apply mechanical separation to raw digestate in order to ease its storage and its handling within the farm or its surroundings. Agricultural raw digestate is separated into a liquid and a solid fraction, using in most cases a screw press (Guilayn et al., 2019b). With this type of phase separator, the total amount of liquid digestate (LD) is higher than solid digestate (SD) (ratio 90/10) and thus most of the organic matter and nutrients are contained in this fraction (Guilayn et al., 2019b). In terms of TS allocation between the two phases, it has been shown that on average 68% of the remaining TS goes to the liquid fraction and 32% into the solid fraction (Guilayn et al., 2019b; Tambone et al., 2017). But, when looking at the concentrations, SD has a higher content in organic matter (mainly fibers) while LD is richer in nitrogen and potassium (Akhiar et al., 2017; Guilayn et al., 2019a). It can be added that LD/SD organic and mineral contents are mainly driven by the type of phase separator as well as by the composition of the raw digestate (itself variable in function type of feedstocks used) (Guilayn et al., 2019b, 2019a).

It has been reported that raw digestate as well as solid and liquid fractions of agricultural digestate can contain, a large part of remaining and biodegradable organic matter despite anaerobic digestion (Akhiar et al., 2017; Guilayn et al., 2019a; Maynaud et al., 2017). As plant efficiency (capacity to recover most methane from feedstocks) is mainly determined by its hydraulic retention time and feedstock type (Ruile et al., 2015), it was identified that this residual biodegradable organic matter was due to too short HRT regarding feedstocks used that are partly lignocellulosic in agricultural biogas plant. Indeed, in the case of a co-digestion process, feedstocks do not need the same duration to be anaerobically digested. For instance, to recover 95% of methane from manure, only around 200 days HRT are necessary while energy crops require around 90 days (Muha et al., 2015). Therefore, tracks to take advantage of this remaining biodegradable organic matter and nutrients have been explored.

4.2. Agricultural valorization of digestate

Agricultural use of digestate is and should remain in the future the main valorization pathway. It implies before its land spreading a stabilization/post-treatment step to: (i) transform its remaining biodegradable fraction towards a more stable fraction with notably a higher degree of humification; (ii) Mineralize nitrogen into ammonium to avoid ammonia or nitrogen oxide emission and potential phytotoxicity; (iii) eliminate potential pathogens that survived to the mesophilic AD process; (iv) control and decrease odor pollution (Nkoa, 2014; Zeng et al., 2016). Composting of the SD and storage in a tank of the LD/raw digestate are the most developed practices in agricultural biogas plants.

Storage in uncovered tanks of LD or raw digestate can lead to high methane emissions into the atmosphere. Such a phenomenon was observed and measured in several agricultural plants, lowering both their environmental footprint and efficiency (Angelidaki et al., 2005; Baldé et al., 2016; Maldaner et al., 2018). Knowing this, coverage and heating of storage tanks

were identified as good management practices for the agricultural biogas sector as it increases HRT of LD/raw digestate, allowing a higher efficiency and a lower environmental footprint of agricultural biogas plants (Bacenetti et al., 2016; Lijó et al., 2017). Besides, it also reduces potential odor emissions. This post-treatment strategy combines additional energy production with a final agricultural valorization. It is now often implemented at full scale.

SD on agricultural biogas plants is mostly stored in a composting pile with sometimes additional bulking agents (wood chips or other type of lignocellulosic bulking agents) before land spreading. The composting process allow to recover a more stable organic matter with low biodegradability, notably containing complex molecules such as lignin-like compounds and stable humic or fulvic acids that are beneficial to the soil (Maynaud et al., 2017; Nkoa, 2014). Again, as a function of the feedstocks and the efficiency of the AD process, composting will have greater or lesser effect on organic matter. The higher the remaining biodegradable organic matter after AD, the higher the composting effect will be (Li et al., 2018; Tambone et al., 2015). Apart from organic matter conversion, a potential advantage of composting comes from natural compost pile heating that can reduce numbers of pathogens in SD (Wéry et al., 2018). However, SD composting has for main drawbacks strong ammonia emissions occurrence as significant amounts of $\text{NH}_3/\text{NH}_4^+$ contained in SD are volatilized, as well as carbon emissions under the form of CO_2 (de Guardia et al., 2010; Rincón et al., 2019).

These two traditional types of post-treatment mainly act on the carbon fraction, while the nutrient fraction and notably volatilization of nitrogen are less efficiently managed. Therefore, additional strategies to efficiently recover and concentrate these nutrients (allow notably a reduction of shipping costs in a case of commercialization) are currently under research and development (Vaneckhaute et al., 2017). Among them, ammonia stripping from digestate storage (or even from SD composting) and recovery via an alternative scrubbing agent (such as citric acid) is one of the most promising ways for farmers to produce a concentrated, easy-to-handle and marketable organic bio-fertilizer as well as reduce biogas plant gaseous nitrogen emissions (Jamaludin et al., 2018). However, additional costs of such post-treatments and a lack of legislation for renewable fertilizers are for the moment hampering their large adoption at full-scale (Bolzonella et al., 2018).

Eventually, it can be noted that alternative agricultural uses for SD exist, notably for fiber rich SD coming from cow manure AD. Indeed, it can be used as cheap stable bedding or as a cover layer for silage (Pelaez-samaniego et al., 2017; Wellinger et al., 2013). In that case SD is re-feed into the digester with the manure or silage.

4.3. Biochemicals and other value product valorization

Digestate valorization towards biochemicals and higher-added-value products is an emerging trend and several tracks have been studied. It is generally based on digestate biological conversion using specific microalgae, bacteria or fungi. It can be noted that all these tracks remain for the moment at research scale and need further optimization.

Microalgae cultivation using LD has been explored and identified as a potential way to efficiently valorize LD nutrients (nitrogen & potassium) into a high-value biomass that can be further transformed into biofuel (biodiesel/bioethanol) or biomolecules (carotenoids, omega-3 fatty acids...) (Monlau et al., 2015; Xia and Murphy, 2016). Most studies used mixotrophic microalgae strains, such as *Chlorella* genus, under indoor batch conditions with filtrated/sterilized/diluted LD in order to reduce potential inhibition due to organic trace elements/heavy metals as well as eliminate/control inherent biological contamination from LD (Xia and Murphy, 2016; Zhu et al., 2019). On this last point, corresponding to the interactions between LD microorganisms and microalgae strains, a better understanding is still needed notably to allow long-term culture stability (Monlau et al., 2015). Additionally, in a fully integrated microalgae-biomethane production system, carbon dioxide from upgraded biogas and VFAs potentially extracted from AD could be used as a carbon source for microalgae growth.

Fungal post-treatment of SD has been recently explored to valorize the remaining carbon into value-added products such as: (i) lignocellulolytic enzymes via submerged fungal fermentation of 21 different strains (Musatti et al., 2017) or via solid-state fermentation (SSF) using *Trichoderma reesei* (Mejias et al., 2018); (ii) volatile fatty acids (VFAs) via SSF using *Pleurotus Sajor Caju* (W. Fang et al., 2018); (iii) edible mushrooms, since *Pleurotus ostreatus* was successfully cultivated on up to 60% w/w SD mixed with traditional substrates (Zhou et al., 2018). Recently, bacterial post-treatments of SD from biowaste have been also explored; SD SSF using *Bacillus thuringiensis* and *Starmella bombicola* succeeded in producing respectively bio-pesticides and bio-surfactants (sophorolipids in low yield) (Cerdeira et al., 2019).

Finally, other value-added products and applications for SD have been explored. SD can be used as an element to produce low-grade building biomaterials or directly as a biosorbent. For the former case, it has been shown that SD fibers from cow manure AD could substitute fibers in medium density fiber board and wood particles in particleboard or be used to produce alternative wood plastic composites with interesting properties (Pelaez-samaniego et al., 2017). For the latter case, SD from corn stover AD has shown to be an effective bio-adsorbent for trapping heavy metals in solutions, such as copper and cadmium (J. Wang et al., 2013).

4.4. Energy Valorization

Bioethanol production has been explored notably via the biological fermentation of SD. This strategy takes advantage of the fact that AD act as a pretreatment on feedstock, modifying fibers and concentrating cell wall polymers such as cellulose and lignin (Sambusiti et al., 2016). Additional dilute alkaline or dry grinding post-treatments have been explored to enhance cellulose accessibility and overcome the lignin barrier in SD. Following that, bioethanol fermentation generally consists in a saccharification and fermentation process where hydrolytic enzymes (cellulase, xylanase...) and yeast (*Saccharomyces cerevisiae* strains) are added simultaneously or sequentially (Monlau et al., 2015). Bioethanol obtained can be used further as a biochemical platform or as an additional energetic vector. It was reported that coupling AD with subsequent bioethanol production can diminish the energy requirement for

grinding and, in the case of CHP valorization, heat produced can be used to dry SD. Considering that ethanol has a heating value of $29.6 \text{ MJ}\cdot\text{kg}^{-1}$, ethanol energetic yield reported, are between 1.1 (37 g bioethanol) to 4.3 MJ (145 g bioethanol) per kg of dry matter of SD (Monlau et al., 2015; Sambusiti et al., 2016). This energy can be added to the one previously obtained from AD; overall it enhances the energy yield of the process. This reported range of energy gain is the lowest in comparison to other energy valorization tracks for SD that will be presented thereafter.

Thermo-chemical processes in order to recover additional energy and co-products from digestate have been extensively studied at lab-scale. They can be divided in four categories that are: (i) hydrothermal/vapothermal carbonization; (ii) combustion; (iii) pyrolysis; (iv) gasification. They allow a higher energy recovery than biological processes (bioethanol or AD recirculation) as both lignin and crystalline cellulose can be converted into energy (Monlau et al., 2015).

Hydrothermal and vapothermal carbonizations can be applied directly to the whole digestate or undried SD, as they occur at temperature comprised between 180 and 260°C, potentially pressurized, in respectively liquid or vapor saturated environments. Under these conditions, initial wet digestate is mainly transformed into a carbon rich product called “hydrochar” very similar to lignite/fossil coals regarding its energy and carbon content (Garlapalli et al., 2016). Reported mass yield are comprised between 45 to 80 g of hydrochar per 100 g of dry digestate (Garlapalli et al., 2016; Monlau et al., 2015). It can be noted that the higher the temperature, the lower the amount of hydrochar produced. Hydrochar can be used as a solid fuel for heat generation with heating value varying between 16.5 and 35.5 MJ per kg of dry digestate, as a function of the operational process and the initial digestate (Funke et al., 2013; Monlau et al., 2015; Mumme et al., 2011).

Combustion, pyrolysis and gasification need dry material to be operated such as dried SD or even hydrochar (Garlapalli et al., 2016; Monlau et al., 2015). For combustion (heat valorization only), dried SD can be pelletized before being put in an air furnace, and reported energetic yields are comprised between 16.4 and 17.3 MJ per kg of dry digestate, very closed to wood (Kratzeisen et al., 2010; Pedrazzi et al., 2015). Concerning pyrolysis of dry SD, it is a thermochemical conversion process taking place in the absence of oxygen and with a temperature comprised between 400 and 700°C that leads to three products: “pyrochar” or “biochar”; bio-oil; syngas. Allocation of the initial matter between these different products will mainly depend on the pyrolysis duration. Fast pyrolysis (high heating rate and few minutes) produces more bio-oil while slow pyrolysis (low heating rate and residence time of up to hours) produce mostly pyrochar and syngas (Monlau et al., 2015). Syngas and bio-oil (containing mainly acids, sugars, ketones, phenols and furans compounds) can be used to produced further energy or be converted into chemicals (Fabbri and Torri, 2016). Additional energy yields from produced syngas and bio-oil are reported to be between 8.6 and 10.8 MJ per kilo of dry digestate (Monlau et al., 2015). Finally, the gasification process, that occurs

under a controlled amount of oxygen and for temperatures above 700°C, transforms dry SD into syngas and residual char (Antoniou et al., 2019). Besides, produced chars from gasification or pyrolysis display very interesting features that allow them to be used as a soil amenders (carbon sequestration, water retention and slow-releasing nutrient reservoir), as a biosorbent (trap heavy metals or furans and if transformed into activated carbon can trap H₂S from biogas) or directly within the AD process to enhance methane yield (eventual AD detoxification and support of microbial biofilm in the digester) (Monlau et al., 2015).

The main drawback of pyrolysis and gasification is that they require high energy for heating. It can be provided from CHP units that do not valorize their heat and efficiently enhanced energy efficiency of the combined AD-pyrolysis/gasification systems can be achieved. Thus for instance, Monlau et al. have shown that the electricity yield of AD-pyrolysis system could be enhanced by 42% in comparison to a sole AD process (F Monlau et al., 2015). However, in cases where heat is already valorized on site or not produced (biomethane upgrading...), drying and heating of SD for pyrolysis or gasification may hinder their energetic/economic sustainability and lower temperature treatment such as hydrothermal carbonization should be preferred (Fabbri and Torri, 2016). For the moment none of these thermo-chemical processes are adopted in full scale agricultural biogas plants due to the additional complexity and additional cost in terms of CAPEX (device such as a pyrolyser) and OPEX (energy need, labor). Further pilot, economic as well as environmental studies need to be performed to fully demonstrate the advantage of such process coupling for additional energy production (Monlau et al., 2015; Pelaez-samaniego et al., 2017). A solution that may possibly emerge in the future will lie in the development of the gasification sector (Gas for Climate, 2019a). SD could be used as a side feedstock to the main feedstocks used in gasification plants (e.g. wood, municipal solid waste...). Thus, SD could be shipped to a gasification plant created nearby, be gasified and remaining chars could be sent back to local farms for land spreading.

Finally, recirculation of digestate has been explored to recover residual methane potential (RMP). On agricultural biogas plants, a common practice is to recirculate digestate from the digester or the post-digester (and sometimes LD from the storage tank) into the hopper to dilute feedstocks and reduce water consumption. Studies on raw digestate coming from full-scale agricultural plants has shown that raw digestate RMP could be comprised between values as low as 3 Nm³/Ton VS up to 126 Nm³/ton VS (Menardo et al., 2011b; Ruile et al., 2015). These values depend mainly on the type of feedstock and the HRT as indicated before. Recirculation of SD is also empirically performed at full scale. However, impact of such practice on the biogas plant yield remains poorly understood and optimal application conditions undefined. Measured SD RMP could range between 60 and 240 Nm³/ton VS (Sambusiti et al., 2015; Thygesen et al., 2014). From SD recirculation, additional energy yield was estimated to be between 1.8 and 6.4 MJ per kilogram of dry digestate (Monlau et al., 2015). One of the main advantages of this practice is that it is relatively simple to apply and it does not need additional investment for a pyrolysis, gasification or hydrothermal reactor. Besides, several studies have tested post-treatment strategies of SD before its recirculation to enhance RMP.

Thermal treatment, wet-explosion treatment (Biswas et al., 2012), milling (Lindner et al., 2015) and enzymatic treatment (Sambusiti et al., 2015) have shown to increase SD RMP between 13-176%. However, in all these studies, techno-economic impacts of recirculation on agricultural biogas plant yield and possible post-treatments costs as well as their feasibilities were rarely discussed.

5. Identified strategies to enhance anaerobic digestion process and PhD objectives

Regarding this literature review it appears that: (i) Future sustainable agricultural feedstock deposit is mainly based on various crop related feedstocks (sequential crop, residues) and manure. These feedstocks can be rich in lignocellulose that is hardly degraded during AD. Therefore, optimisation of the preparation step, the co-digestion process and the final polishing step is essential to recover as much as possible biogas from these various feedstocks; (ii) Concerning the preparation step, biological pretreatments and notably aerobic ones are a promising solution to unlock methane potential of lignocellulosic feedstocks due to their capacity to degrade lignin and their environmental friendliness; (iii) For the polishing step, solid digestate recirculation has been identified as a relatively low-cost and easy to implement way to further anaerobically digest feedstocks and improve biogas plant efficiency. However, biological post-treatments applied to solid digestate as well as recirculation conditions within the biogas plant have been relatively unexplored in literature.

Thus, Com The interest of adding a post-treatment (notably biological) as a way to increase methane recovery will be studied as well. From that, several research questions have been identified and can be gathered in three main research categories:

(A) Strategies and impact of SD recirculation

What are the different identified strategies to apply SD recirculation?

What is the impact of SD direct recirculation on biogas plant methane production?

(B) Interest of additional post-treatment

Is the addition of post-treatment before SD recirculation a realistic option?

Is it possible to increase the efficiency of aerobic post-treatment by:

- (i) reorienting endogenous microbial activities?
- (ii) adding ligninolytic consortia?
- (iii) adding ligninolytic fungal strains?

What are the impact of thermo-alkaline and aerobic post-treatments on organic matter?

Is solid digestate a suitable feedstock for aerobic post-treatment?

(C) Conditions of SD recirculation at full-scale

What is the best conditions for SD recirculation implementation at full-scale?

What could be the impact of SD recirculation on mixing cost?

How to determine if SD recirculation is an interesting practice for a given biogas plant?

Could SD recirculation have additional benefits on anaerobic co-digestion process?

The PhD thesis will provide answers to these questions all along the following chapters. Transitions provided between chapters will be related to these questions.

Chapter II

Materials and methods

In this chapter, materials as well as the main methodological methods used all along the PhD will be presented. More specific set-ups, experiments as well as data analysis will be presented at the beginning of each related chapter. Besides, a summary can be found in section 5, that details chapter by chapter, the aim of the experiment and the associated materials and methods that are presented in detail in this chapter.

1. Materials

1.1. Solid digestate

Solid digestates (SD) were sampled from six full scale agricultural or territorial biogas plants located in France. For a question of confidentiality, a letter was attributed to each plant from A to F. One plant among the six was applying a continuous solid-state anaerobic digestion (SS-AD) process (Plant F), while all the others were CSTR biogas plants. Sampling was carried out similarly on all the plants according to the following procedure. SD was picked up from 5 to 10 different parts of the fresh SD pile (located below the outlet of the phase separator as depicted in **figure 22**) and all SD fractions were gathered in a small pile (generally weighing around 10 kg) that was again mixed and then only, from that pile, sampling was carried out (2 to 5 kg). Such strategy ensures sampling homogeneity and representativeness. Depending on the biogas plant setup, the phase separator was releasing SD either from the post-digester or from the digester (if no existing post-digester). All SD samples were quickly shipped to the lab and frozen in gas-tight containers in order to preserve their methane potential before subsequent experimental handling.



Figure 22: Examples of solid digestate (SD) sampling location. SP states for Screw Press

1.2. Liquid digestate

Liquid digestate (LF) comes from the agricultural CSTR liquid biogas plant noted A. It was sampled directly at the outlet of the screw press, before entering the storage tank. It was stored in a gas-tight container and sent directly to the laboratory. Upon arrival, characterization was performed without any storage at 4°C or freezing.

1.3. Mycelium

Two Basidiomycetes fungus strains were ordered from Mycelia BVBA (Deinze, Belgium), a professional spawn and mycelium culture laboratory. Strain M2191 of *Pleurotus Ostreatus*, also called winter oyster mushroom, and strain M5012 of *Stropharia Rugoso-Annulata*, also called garden giant mushroom, were received under spawn form. For *Pleurotus Ostreatus*, spawn consisted in millet seeds colonized by mycelium while for *Stropharia Rugoso-Annulata*, spawn colonized wheat straw. Characterization was performed upon arrival and then both were stored at 4°C until experiments started (1-month maximum in order to preserve mycelium integrity and activity).

1.4. Initial environments for ligninolytic consortia screening

Six environments were used to screen for ligninolytic consortia. Three of them are forest-originating environments that were sampled in the National Nature Reserve of the Massane Forest (Argelès-sur-mer, France). The particularity of this Reserve is to be particularly rich in dead wood as no forestry operation has been allowed since its classification in 1973. Thus, ground with wood decomposition, deep forest litter and rotten wood were sampled in that forest. Besides, a precise georeferencing of each sampling point was performed to allow for possible new samples to be taken if promising consortia are discovered. Fresh sheep rumen was obtained from the INRAE experimental domain of la Fage (Le Viala-du-Pas-de-Jaux, France), following gut sampling on the sheep livestock according to a standardized method using a gastroesophageal tube and a vacuum pump (Bertide, 2018). Rotten wheat straw was obtained from a field of a farm located in the Gers department (France). Finally, a commercial granulated organic fertilizer coming from a composting process was used. These six environments were conveyed to the lab at room temperature and directly used without freezing in order to conserve endogenous microbial communities. **Figure 23** gives an overview of the six environments used and their origins.

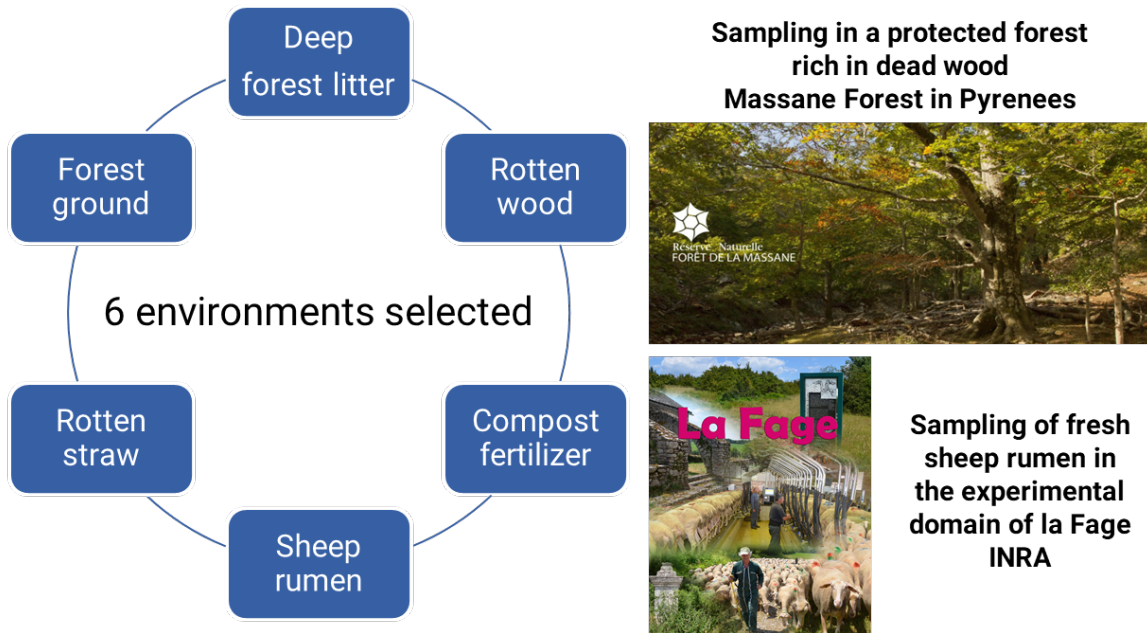


Figure 23: Origin of the six environments used for ligninolytic consortia screening.

1.5. Microbial anaerobic inoculum

All anaerobic digestion experiments were carried out using a microbial anaerobic inoculum. This inoculum has two origins; either a UASB reactor treating waste streams from a sugar factory, either a UASB reactor treating waste streams from a paper mill. In both cases, inoculum was under the form of a granulated sludge that was stored at 35°C. From these granulated sludge, microbial anaerobic inoculum reactors were prepared according to the following procedure. Granules were diluted with osmotic water and milled using a hand-mixer to obtain a homogenous mixture. Then, this mixture was filtered using a 16-mesh sieve, notably to eliminate remaining coarse particles (minerals...) and recover a homogenous liquid microbial anaerobic inoculum. Water was added to obtain a homogenous liquid with an organic concentration close to 40 g VS/L. This liquid was then placed in a 5 L, heated (38°C) and stirred glass reactor. Biogas production was recorded via a Ritter biogas volumetric flow meter (Bochum, Germany) connected to the digester. Ethanol could be added as a feedstock/booster to maintain methanogenic activity for a long duration. Generally, this prepared microbial anaerobic inoculum was used when its endogenous/remaining organic matter was totally depleted, that is translated by a low biogas production. At that point, it was considered that anaerobic microorganisms were starting to starve and this should ensure a strong activity when contacting them with feedstocks to test.

2. Physico-chemical characterization methods

2.1. Total solids and Volatile solids

Total Solids (TS) and Volatile Solids (VS) were determined according to the APHA standard method (APHA, 2005). Samples were weighed in porcelain crucibles, whose mass was obtained after one hour at 105°C and cooling in a desiccator. Then, crucibles filled with sample (1 to 220 g) were left in an oven at 105°C for 24 h in order to dry the samples. After that,

cooling was done in a desiccator. Crucibles were then re-weighted and moisture content corresponded to the mass difference. The total solid content (TS) was the ratio in percent of the weight after 24h drying and the initial sample weight (**Eq. 2**). After this first measurement, dried samples combustion was operated. The crucibles were placed for 3 hours in an oven at 550°C. By weighting the final ash content, the volatile solid (VS) or combusted organic matter was determined by difference with the TS content (**Eq. 3**). Triplicates were always performed to ensure representativeness of the measurement.

$$TS = \frac{\text{Mass of dry sample}}{\text{Initial mass of sample}} \quad (2)$$

$$VS = \frac{\text{Mass of organic matter in the sample}}{\text{Mass of dry sample}} \quad (3)$$

2.2. Total Carbon and Total nitrogen

Total carbon (TC) content of solids was measured on freeze-dried and shredded samples using a Shimadzu TOC-V_{CSN} Analyzer (Kyoto, Japan) coupled to a Shimadzu solid sample module SSM-5000A. For liquids, previously filtered at 0.2 µm, TC content was measured using the same analyzer but with a Shimadzu ASI-V tube rack instead of the solid sample module. TC was measured via catalytic combustion of matter under pure oxygen conditions that converted carbon in matter into CO₂, which was subsequently measured by an infrared sensor.

AutoKjehdahl Unit K-370, Buchi (Flawil, Switzerland) coupled to a Buchi digestion/mineralization unit K-438, were used to determine the total Kjeldahl nitrogen on dry solid samples. Organic nitrogen is first digested in an acid medium (H₂SO₄ + Kjeldahl catalyst), under high temperature and transformed into ammonium sulphate. Then, the solution is alkalinized by the addition of soda and produced ammonia (NH₃) is distilled and collected in a boric acid solution. Ammonia is then dosed by titration with an acid solution of known molarity (HCl). In the case of a liquid sample, filtered previously at 0.45 µm, digestion is not necessary and alkalinization, distillation and titration steps can be performed directly.

2.3. Chemical oxygen demand

The chemical oxygen demand (COD) is a measurement that allows to determine the total amount of chemically oxidizable matter in a sample by heating the sample in strong sulphuric acid containing potassium dichromate and mercury nitrate. COD is expressed by the amount of oxygen required for oxidizing a given amount of matter/volume of liquid. This measurement was performed using an Aqualytic 420721 COD Vario Tubes Test MR 0–1500 mg O₂·L⁻¹ (Dortmund, Germany), based on a colorimetric reaction principle. For liquid samples, 2 mL of filtered sample at 0.45 µm and adequately diluted with MilliQ water can be directly pipetted into each tube. For solid samples, a first step of matter dissolution is required. To do so, solid samples were first freeze-dried and then ground using a Retsch mixer mill MM 200 (Haan, Germany) and associated stainless steel grinding jar. Vibrational frequency was fixed at 25 Hz·min⁻¹·g⁻¹ of material in the jar in order to obtain a homogenous powder. Then, 0.25 g of sample powder were poured into 10 mL of 98% w/w H₂SO₄ and set under strong agitation

overnight to solubilize solid particles. Dilution with MilliQ water up to 250 mL allowed pipetting. Again, 2 mL of sample adequately diluted were then pipetted into each tube.

Once samples were added, oxidation reactions in tubes were performed in a HACH COD reactor at 150°C for 2 hours (Loveland, US). COD concentrations were measured using an Aqualytic MultiDirect spectrophotometer (Dortmund, Germany).

2.4. pH

The pH of liquid and solid feedstocks were measured using a Mettler Toledo Seven S2-meter (Columbus, United States) with an InLab® Expert Pro-ISM sensor. Regular checks were performed, using standard buffer solution (pH 4, 7, 10), to ensure measurement liability.

2.5. Organic matter fractionation method

Complexity of organic matter was characterized via sequential chemical extractions according to the protocol developed by Jimenez et al., 2015. Samples were first freeze-dried and then ground at 1 mm using a Fritsch Pulverisette 19 (Idar-Oberstein, Germany) and associated stainless steel sieve. Subsequent sequential extractions were performed on 0.5 g of sample. Four fractions of decreasing accessibility were then obtained by applying increasingly strong chemical solutions: (i) soluble extractable fraction from particular extractable organic matter (SPOM) using CaCl_2 (10 mM) that corresponds to soluble sugars and proteins; (ii) readily extractable organic matter (REOM) using NaCl (10mM) as well as NaOH (10mM) that allows extraction of exopolymeric substances proteins, nucleic acids, sugars and lipids; (iii) slowly extractable OM (SEOM) using NaOH (0.1 M), which corresponds to remaining proteins and sugars, some humic substances and lipids; (iv) poorly extractable OM (PEOM) using H_2SO_4 (72% w/w) that corresponds to hemicelluloses and celluloses. At each step, the solubilized VS was recovered in the supernatant by centrifugation (18750 g for 20 min at 4 °C) and filtered at 0.45 μm . VS of each fraction was then characterized via COD measurement as described in section 2.3. of this chapter. Finally, the non-extractable OM (NEOM) was calculated by subtracting the four COD fractions extracted from the sample from the total COD. Total COD was obtained by measuring COD on solid sample powder as described in section 2.3. of this section. For solid digestate samples, the readily extractable organic matter (REOM) fraction was not extracted as it corresponds generally only to a small amount of the total VS for SD (Maynaud et al., 2017). REOM was thus in that case comprised in the SEOM fraction. **Figure 24** gives an overview of the different OM fractions, corresponding types of molecule and the type of treatment applied to extract them.

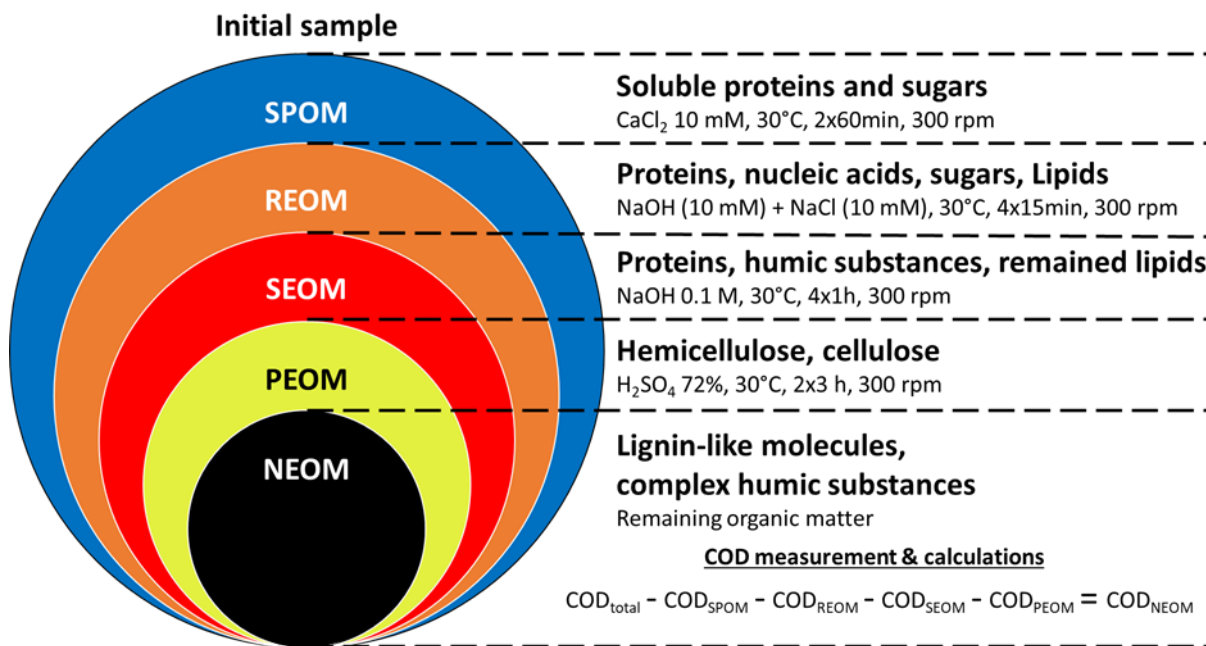


Figure 24: Overview of the organic matter fractionation method

2.6. Fluorescence spectroscopy analysis

Fluorescence spectroscopy analyses were performed on liquid samples coming from the organic matter fractionation method in order to get insight into the complexity of the extracted soluble organic matter. Fluorescence spectra of liquid extracts were recorded on a Perkin Elmer LS55 (Waltham, Massachusetts) and can be divided into seven zones according to Jimenez et al., (2015). Each zone corresponds to a different group of molecules that are gathered in function of their complexity. Zone I to III mainly gather simple molecules (proteins and soluble organic matter), while zone IV to VII gather complex molecules (fulvic acid-like, lignin-like, humic acid-like compounds). From these areas, a complexity ratio can be calculated (Jimenez et al., 2015). This index, calculated according to Eq. 4, is defined as the ratio of the sum of the fluorescence volumes of the most complex molecules (zones IV to VII) over the sum of the fluorescence volumes of the protein-like molecules (Zones I to III). To increase index significance, fluorescence volumes are normalized by a factor calculated from the COD concentration of the liquid fraction (%CODfraction). The higher the value of the complexity ratio, the higher the content in complex molecules in the extracted soluble organic matter.

$$Complexity\ ratio\ fraction = \%COD_{fraction} \times \frac{\sum_{i=4}^7 V_f(i)}{\sum_{i=1}^3 V_f(i)} \quad (4)$$

2.7. Biochemical Methane Potential tests

As previously described (see chapter I section 2.1.4), BMP tests are used to estimate maximal methane potential from a given feedstock, by placing it under optimal anaerobic degradation conditions. Figure 25 gives an overview on how BMPs are performed.

BMP tests are carried out in flasks of 575 mL, with a working volume of 400 mL. Flasks were filled with: (i) 4mL of oligoelements solution (FeCl₂, 2 g.L⁻¹; CoCl₂, 0.5 g.L⁻¹; MnCl₂, 0.1 g.L⁻¹;

NiCl₂, 0.1 g.L⁻¹; ZnCl₂, 0.05 g.L⁻¹; H₃BO₃, 0.05 g.L⁻¹; Na₂SeO₃, 0.05 g.L⁻¹; CuCl₂, 0.04 g.L⁻¹; Na₂MoO₄, 0.01 g.L⁻¹) and 8.6 mL of macroelements (NH₄Cl, 26.6 g.L⁻¹; KH₂PO₄, 10 g.L⁻¹; MgCl₂, 6 g.L⁻¹; CaCl₂, 3 g.L⁻¹) to avoid nutrient limitations; (ii) 20.8 mL of bicarbonate buffer (NaHCO₃, 50 g.L⁻¹) to ensure a stable pH close to 7.5; (iii) microbial anaerobic inoculum at 5 g VS.L⁻¹ to bring anaerobic microorganisms into the system; (iv) sample to be digested at 5 g VS.L⁻¹; (v) distilled water to complete to 400 mL. Then, anaerobic conditions were obtained by flushing the headspace with nitrogen gas and closing flasks with red butyl rubber septum-type stoppers. A control was made by preparing the same mixture with the inoculum but without substrate to evaluate the endogenous methane production. Triplicates were always launched, and all flasks were incubated under agitation at 35°C.

Biogas production was monitored twice a week at the beginning and less frequently as production slowed down. Biogas volume was measured using a Keller LEO 2 digital manometer (Winterthur, Switzerland) and biogas composition was determined using a Perkin Elmer® Clarus 580 gas chromatograph (Waltham, Massachusetts) equipped with two Restek columns (Bellefonte, The United States): the first (RT®-Q-Bond) was used to separate CO₂ from other gases, the second (RT®-Msieve 5A) was used to separate O₂, N₂, CH₄, and H₂. The carrier gas used was argon delivered at 350 kPa and 35 mL.min⁻¹ into the column. Oven, injector and detector temperatures were set respectively at 60 °C, 250 °C and 150 °C. A thermal conductivity detector was used for gas detection. From these measurements the volume of methane produced between each analysis (ΔV_{CH_4}) could be determined according to the following equation:

$$\Delta V_{CH_4} = \left[\frac{(\gamma_{CH_4 i} \cdot P_i - \gamma_{CH_4 i-1} \cdot P_{i-1}) \cdot V_{Headspace}}{T} \right] \cdot \frac{T_0}{P_0} \quad (5)$$

Where $\gamma_{CH_4 i}$ corresponds to the percentage of methane at time i , P_i is the pressure in the flask at time i , $\gamma_{CH_4 i-1}$ corresponds to the percentage of methane at time $i-1$, P_{i-1} is the pressure in the flask at time $i-1$, $V_{Headspace}$ corresponds to the headspace volume of the flask, T the temperature in the incubation room (308 Kelvin) and P_0 as well as T_0 are the standard pressure (1015 hPa) and temperature (0°C) conditions used to normalize the volume of methane produced.

Endogenous methane production was taken into account by subtracting the volume of methane produced in the controls (inoculate only). Total methane production is divided by the initial mass of VS of the sample introduced in the flask at the BMP starting, which gives a methane yield expressed in NmL CH₄/g VS or in Nm³ CH₄/ton VS.

Finally, BMP tests were stopped when daily methane production during three consecutive days was <1% of the accumulated volume of methane according to internationally standardized practice (Holliger et al., 2016).

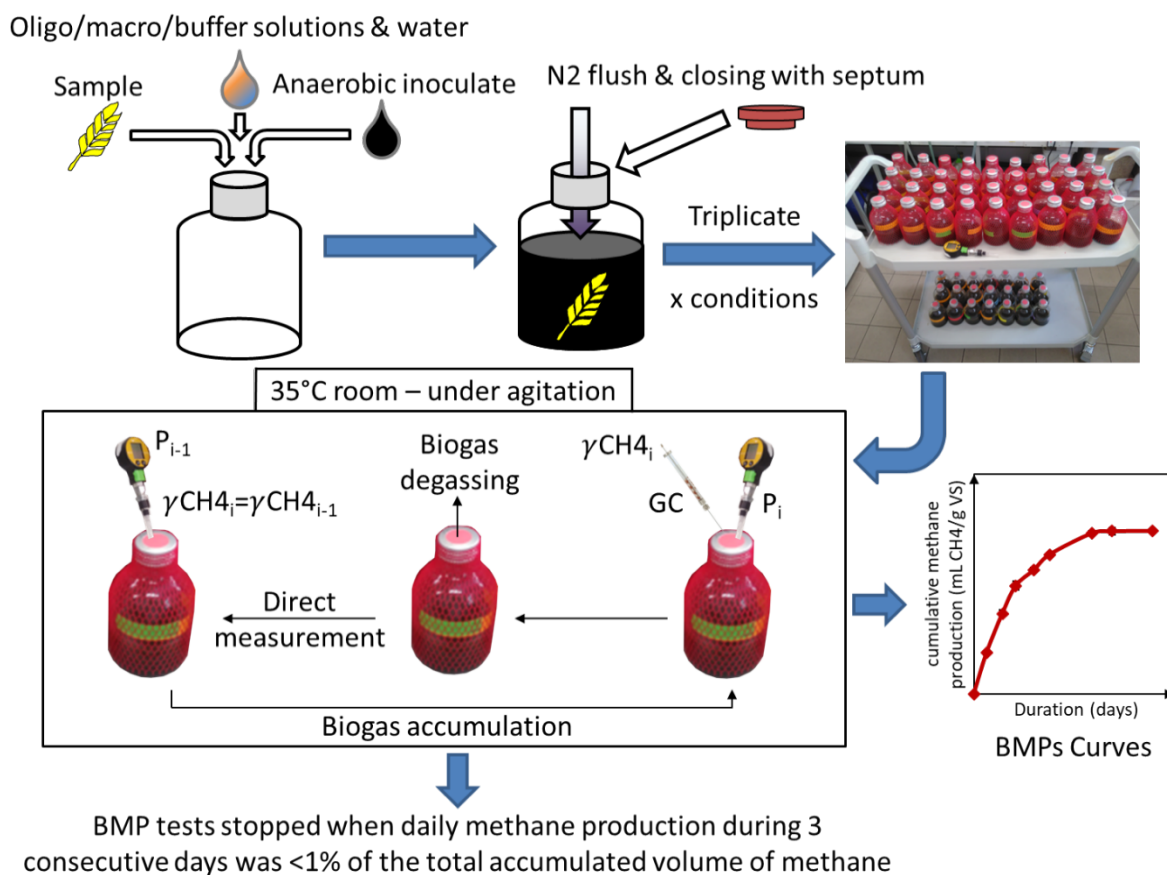


Figure 25: BMP tests procedure overview

3. Microbial analysis

3.1. Sample collection

For microbial analysis, samples were performed according to the following procedures that differ between liquid and solids:

- (i) For liquid sampling, solutions to be sampled were first well mixed to ensure a representative sampling. Then 1.8 mL of these solutions were pipetted into sterile 2 mL Eppendorf tubes. These tubes were centrifuged at 13,400 rpm for 10 minutes using an Eppendorf Mini-Spin centrifuge (Hambourg, Germany). Supernatants were eliminated and remaining pellets were further stored at -20°C before use.
- (ii) For solid sampling, 2 mL Eppendorf tubes used were first weighed. Then sampled solids were homogenized and some were placed into the 2 mL pre-weighted Eppendorf tubes. Eppendorf tubes containing solid samples were weighed again in order to determine the amount of solid sample that will be analyzed (generally between 0.1 and 0.5 grams of solid samples). Finally, Eppendorf tubes were further stored at -20°C before genomic DNA extraction.

3.2. Genomic DNA extraction

DNA from all samples analyzed in this PhD were extracted and purified using a commercial FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, United States). Manufacturer

instructions were followed to recover purified DNA from all samples. The quality and quantity of DNA were verified by spectrophotometry using 2 μ L of DNA extracts with a NanoQuant Plate on a Spark[®] microplate reader (Tecan, Männedorf, Switzerland).

3.3. Real-time quantitative polymerase chain reaction (qPCR)

The qPCR for bacteria and eukaryotes were performed in 96-well Eppendorf PCR plates on a CFX96 thermocycler from BioRad. All samples were run following two dilutions and in triplicate to assess the technical variability associated with the measurement. PCR mixture components and PCR programs vary between bacteria and Eukaryotes qPCR:

- (i) For bacteria, the following components were added: 6.5 μ L Mix Biorad SsoAdvanced Universal Probes Supermix (Bio-rad, Hercules, United States), 0.5 μ L of each primer and probe, 2.5 μ L water and 2 μ L of DNA extracts for a total volume of 12.5 μ L. Primers that were used were F338-354 at 100 nM as well as R805-785 at 250 nM and the probe used was a Taqman Tamra F516-536 at 50 nM (Yu et al., 2005). For sequence details see **table 6**. The bacteria qPCR was performed following a program consisting in 2 minutes at 95°C for enzyme activation followed by 40 cycles of 7 s at 95°C for dissociation and 25 s at 60°C for hybridization and elongation.
- (ii) For eukaryotes, the following components were added: 12.5 μ L of Bio-Rad SsoAdvanced™ Universal SYBR[®] Green Supermix, 1 μ L of each primer, 5.5 μ L water and 5 μ L of DNA extracts for a total volume of 25 μ L. Primers that were used were F574 at 250 nM as well as R952 at 250 nM (Hadziavdic et al., 2014). For sequence details see **table 6**. The eukaryotes qPCR was also performed following a program consisting in an initial incubation of 2 min at 98 °C, 40 cycles (98 °C, 15 s; 52 °C, 30 s; 72°C, 45 s) and a final melting curve.

Finally, standard curves were generated from each assay by using 10-fold dilutions in sterilized water (Gibco, Thermo Fisher Scientific) of a target plasmid (Eurofins Genomics, Ebersberg, Germany). The average number of bacterial cells was estimated by dividing the average number of 16S rRNA gene copies per cell by a factor of 4.1 (Klappenbach et al., 2001).

Table 6: Primers and probe sequences used for the qPCR analysis

Specificity	Name	Sequences 5'-3'
Bacteria	F338-354	ACTCC TACGG GAGGC AG
	R805-785	GACTA CCAGG GTATC TAATC C
	Taqman Tamra F516-536	Yakima Yellow-TGCCA GCAGC CGCGG TAATA C-Tamra
Eukaryotes	F574	GCGGT AATTC CAGCT CCAA
	R952	TTGTC AAATG CTTTC GC

3.4. PCR amplification and sequencing

For bacteria PCR amplification, the V4-V5 regions of 16S rRNA bacterial genes were amplified with primers F515-532 (5'-GTGYC AGCMG CCGCG GTA-3') and R909-928 (5'-CCCCG YCAAT

TCMTT TRAGT-3') as well as their respective linkers (Wang and Qian, 2009). For eukaryotes PCR amplification, the V4 region of 18S rRNA eukaryote genes were amplified with primers F574 and R952 (detailed in **table 6**) as well as their respective linkers (Hadziavdic et al., 2014). The PCR mixtures had a total volume of 60 μ L, containing: 0.6 μ L of MTP™ Taq DNA polymerase (Sigma-Aldrich), 6 μ L of associated Taq buffer, 4.8 μ L of Deoxynucleotide (dNTP) solution mix (GE Healthcare, Boston, United States), 1.2 μ L of each primer at 300 nM, 5 μ L of DNA and 41.2 μ L water. In a Mastercycler thermal cycler (Eppendorf) the following PCR program was followed: after 2 min at 95°C, 30 cycles of denaturation were performed (95 °C, 60 s; hybridization was at 65 °C for bacteria and at 56°C for eukaryotes, 60 s; 72°C, 60 s), concluded by a final extension at 72°C for 10 min.

The obtained PCR products were purified and sequenced using the Illumina MiSeq kit v3 chemistry (San diego, United-States), with read lengths up to 2 × 300 bp, at the GeT PlaGe Sequencing center of the GenoToul Lifescience Network (www.genotoul.fr). Sequences were recovered after demultiplexing, cleaning and affiliating sequences using Mothur version 1.39.0, as described previously (Venkiteshwaran et al., 2016). It can be highlighted that: (i) sequences that appear fewer than three times in the entire data set were removed; (ii) Alignment of the 16S rRNA and 18S rRNA sequences was done using the reference database SILVA version 128 (Schloss, 2009); (iii) sequences in the whole dataset were clustered into operational taxonomic units (OTUs) by an affiliation threshold of 97%.

4. Data analysis

4.1. Principal component analysis

Principal component analysis (PCA) is one of the main factorial analysis methods that allows a synthetic visualization of a data table composed of samples and associated variables. It can be used to study similarities between samples regarding all variables and group them into sample categories characterized by specific variable features. Such a strategy has been applied in section 3.3.1 of chapter III to split solid digestates into 3 groups, each group corresponding to specific SD features. PCA can also be used to carry out an assessment of linear links (notably, correlation) between variables based on sample characteristics. Such a strategy has been applied in section 5.3.4 of chapter V to study the link between methane yield and VS fractions evolution in the case of post-treated samples.

From a mathematical point of view, PCA corresponds to an orthogonal linear transformation of the data table (x observations). A set of linearly uncorrelated principle components is obtained after transformation of the input variables. These principle components are ranked according to the percentage of variability of the data that they represent. In our case, the two first principle components (explaining more than 80% of data variability) were sufficient and relevant to support our analysis. To perform PCA, either SIMCA software from UMETRICS (Umeå, Sweden) was used as it allows biplot representation (variables and samples on the same graph) that clarifies visual analysis. R software was used instead in chapter IV (R Core Team, 2019).

4.2. Principal coordinates analysis (PCoA)

Principal coordinates analysis is an ordination method used to represent any distance or dissimilarity data table in a low-dimensional Euclidean space. It is based on the same mathematical framework as PCA, i.e. an orthogonal linear transformation of distances. Principle components obtained are ranked according to the percentage of variability of the data that they represent. In our case, the first two principle components (explaining more than 70% of data variability) were sufficient and relevant to support analysis. Contrary to NMDS, PCoA ordination does not distort distance between samples. This ordination method is used in section 3.2 of chapter IV and performed via the R software (R Core Team, 2019).

4.3. Non-metric multidimensional scaling (NMDS)

Non-metric multidimensional scaling is an ordination method used to represent pairwise dissimilarity between samples in a low-dimensional space (generally 2 or 3 dimensions). It is a rank-based approach using a dissimilarity or distance matrix. Distances are substituted by ranks (e.g. sample A “first” most distant from sample C...) and then by an iterative process an algorithm is refining placement of samples in the ordination space until to reach minimum stress solution (best visualization solution). The resulted NMDS representation will be then slightly different between two successive run for the same dataset. To evaluate quality of the representation, the following scale was defined: stress < 0.1 provides an excellent representation in reduced dimensions, stress < 0.15 is good, stress > 0.2 provides a poor representation (Clarke and Ainsworth, 1993). This ordination method is used in section 3.2 of chapter IV and performed via the R software (R Core Team, 2019).

4.4. Partial least square regressions

Partial least square (PLS) regression is a statistical analysis that was used to investigate linear correlations between variables. It consists in predicting a variable Y by p quantitative variables X_1, \dots, X_p . It was applied in the section 3.3.1 of chapter III to see correlations between methane recovery at full scale or some VS fractions (PEOM, NEOM) and HRT, SD RMP as well as plant feedstocks. To perform our PLS, SIMCA software from UMETRICS (Umeå, Sweden) was used.

4.5. Wilcoxon rank-sum test

The Wilcoxon rank-sum test is a statistical test that can be applied as an alternative to the t-test when the data are not normally distributed. It allows to determine if two independent sample sets have a significant difference in their median values. It tests the null hypothesis that median values of the two independent sample sets are equalled. A p value below 5% indicates that tested sample sets have significantly different median values. This statistical test is used in section 3.1 of chapter IV and was performed using the hilldiv package in R software (Alberdi and Gilbert, 2019a).

4.6. Envfit analysis

Envfit analysis is provided by the Vegan package in R software (Oksanen et al., 2019). It allows to fit environmental variables onto an ordination plot under the form of arrows. Arrow shows the gradient direction and its length is proportional to the correlation between the variable and the ordination (Oksanen et al., 2019). It is possible to select most significant environmental variables based on their level of correlation with the ordination. This analysis is used in section 3.3 of chapter IV.

5. Summary

Table 7: Overview of experiments performed, their objectives and the associated analysis carried out

Chapter	Sub-section	objective	Type of post-treatment applied	Analysis performed	Objective of the analysis
Chapter III	3.1.	Characterize SD	/	TS/VS	Determine remaining OM
				TC/TN	Determine remaining C and N
				COD	Determine total amount of chemically oxidizable matter
				Fractionation	Characterize OM
				PCA/PLS	Classified digestate among each other
	3.2.	Evaluate impact of post-treatment on methane yield	thermo-alkaline	BMP tests	Measure methane yield
	3.3.	Evaluate impact of post-treatment on carbon loss / methane yield / microbial communities / carbon fractions evolution	aerobic	BMP tests	Measure methane yield
Fractionation*				Characterize organic carbon	
Microbial analysis				Insight into microbial community evolution	
3.4.	Evaluate impact of post-treatment on carbon loss / methane yield	Boosting solution and aerobic	BMP tests	Measure methane yield	
Chapter IV	3.1./3.2. 3.3./3.4.	Evaluate effect of LAMACS and Erlenmeyer on selection of ligninolytic microorganisms	/	Microbial analysis/ NMDS/PCoA/ PCA/ Wilcoxon/Envfit	Insight into microbial community evolution
	3.5	Evaluate impact of post-treatment on methane yield	Aerobic ligninolytic consortia	BMP tests	Measure methane yield
Chapter V	3.1.	Characterize SD and mycelium	/	TS/VS	Determine remaining OM
				TC/TN	Determine remaining C and N
				pH	Measure pH matter
	3.3./3.4. 3.5./3.6.	Evaluate impact of fungal SSF duration on VS loss / methane yield / biodegradability / VS fractions evolution	Fungal	TS/VS	Determine remaining OM
				BMP tests	Measure methane yield
				Fractionation	Characterize OM
PCA	Link CH ₄ yield to VS fractions				

Fractionation* = Similar to fractionation method but instead of COD, carbon was measured to determine its fate in OM (for protocol see chapter 3). 75

Chapter III

Solid digestates characterization and assessment of thermo- alkaline and short aeration post- treatments in a recirculation context

Adapted from Brémond et al., Recirculation of solid digestate at full scale: strategies, conditions and impacts. Submitted to Applied Energy, 2020.

1. Chapter objective

Recirculation of solid digestate (SD) is already performed at full scale in several agricultural biogas plants. As an example, Menardo et al. described biogas plants where SD recirculated corresponded to 4-6% of the mass of the total feedstock mix (Menardo et al., 2011a). However, conditions of SD recirculation implementation as well as its impact on methane production are, to our knowledge, often poorly understood and have never been extensively studied in literature.

Besides, several studies have tested post-treatment strategies of SD before its recirculation to enhance its residual methane potential (RMP). Thermal treatment (Menardo et al., 2011a), milling (Lindner et al., 2015) and enzymatic treatment (Sambusiti et al., 2015) have shown to increase SD RMP by 13-176%. However, in all these studies, the economic impact of recirculation for agricultural biogas plants and possible post-treatment costs were rarely discussed. Sambusiti et al. estimated that direct recirculation of SD could lead to an increase in economic gain of 14% but it was based on very optimistic assumptions: a high solid separation efficiency of the screw press (73%) and a recirculation of the total amount of SD produced (Sambusiti et al., 2015). Solid separation efficiency indexes of a screw press are generally around 30% for total solids (TS) (Guilayn et al., 2019b). Finally, only a partial amount of SD can be recirculated as some solids should exit the system, notably to eliminate the inorganic matter that would otherwise accumulate and cause inhibitions. Realistic hypotheses should be applied to fully evaluate the economic interest of such post-treatment practices in a recirculation scheme.

It should be highlighted that three different implementation strategies exist for SD recirculation:

- (i) SD can be recirculated in addition to the ration, in that case additional biogas can be produced. Such a strategy will be further called: “addition strategy”.
- (ii) SD can also be integrated to the ration and replace some feedstocks that are missing (shortage/voluntary non-produced) or further stored (preservation in the case of a future anticipated shortage). In that second case, SD recirculation allows to offset ration reduction and maintain a steady biogas production over time. Such strategy will be further called: “replacement strategy”.
- (iii) Finally, SD can also be integrated to the ration and replace some feedstocks that are missing but the amount recirculated does not allow to totally offset ration reduction and maintain a steady biogas production over time. In that case it will limit loss in biogas production. Such strategy will be further called: “partial replacement strategy”. This strategy will be studied at full-scale in chapter VI.

Figure 26 gives an overview of the two main strategies studied in that chapter (addition and replacement) and their potential impact on biogas production.

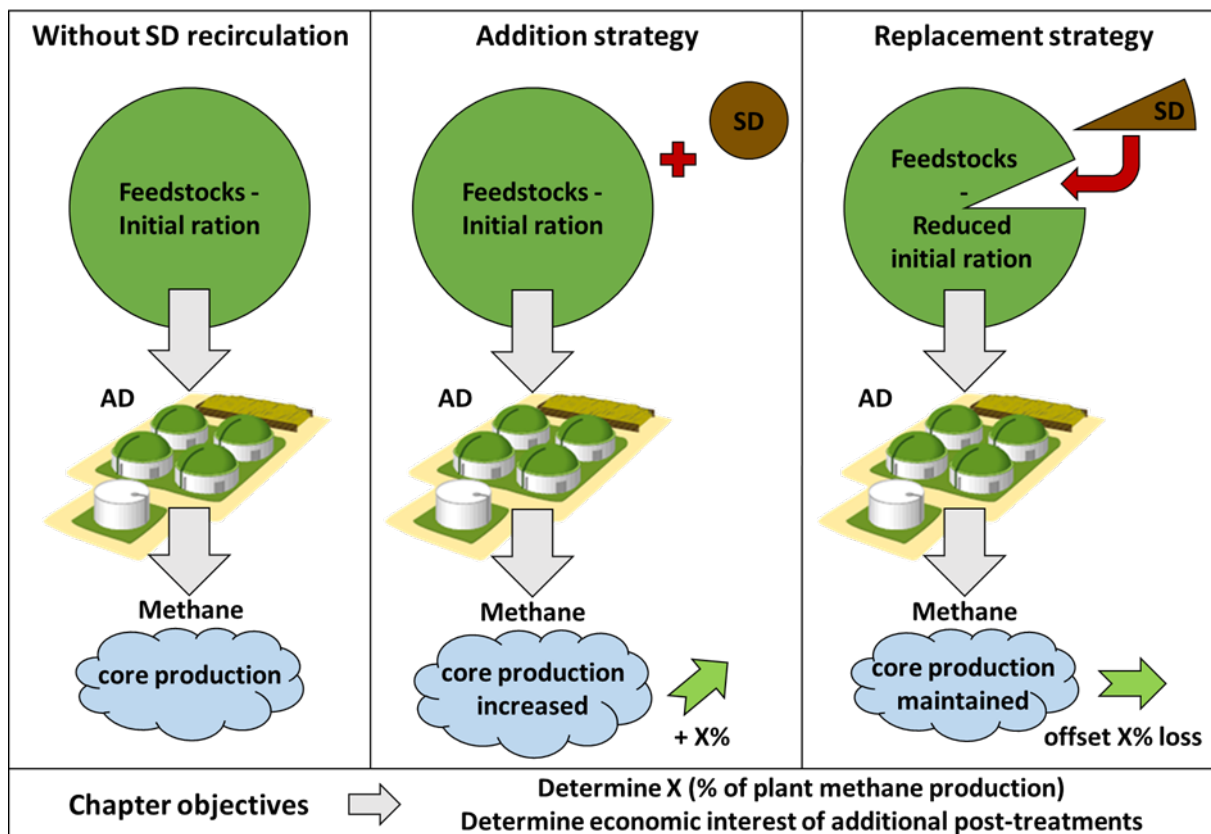


Figure 26: Overview of two possible strategies for SD recirculation & research objectives of this chapter.

This chapter aims to clearly assess the potential of two SD recirculation strategies. First, the impact of recirculation of untreated or post-treated SD on methane production from several biogas plants is evaluated. Thermo-alkaline and short-term aeration post-treatments are the two post-treatments studied. Additional methane production, in the case of an addition strategy, or offset capacity, in the case of a replacement strategy, are quantified. Secondly, technological and economic feasibility of these post-treatment strategies are discussed. A summary of these research objectives is given in **figure 26**.

2. Materials and Methods

2.1. Biogas plant features

SD were sampled from six full-scale agricultural or territorial biogas plants located in France according to the protocol detailed in section 1.1. of Chapter II.

Besides, an interview was carried out with each plant operator in order to obtain details about biogas plant features. Information about the type and reactor volume (RV), the type of phase separator, the plant feedstock mix, the percentage of TS in the digester, the quantity of feedstocks per year ($\dot{m}_{feedstock}$), the HRT before phase separation, the pH, the temperature, the average percentage of methane in biogas (x_{CH_4}), the quantity of SD produced yearly (\dot{m}_{SD}), the type of valorization and the associated flowrate of biomethane injected ($Q_{bioCH_4_hour}$) or combined heat and power valorization (CHP) engine nominal power (CHP_{power}) were provided by the plant operators.

From these data, several additional features were calculated. In all calculations, we considered that the plants were realistically operated 8,200 hours per year (approximately 342 days) due to technical maintenance and possible operational contingencies. Loading rate (LR), expressed in kg feedstock/m³/day, was obtained using the quantity of feedstocks used per year in tons/year ($\dot{m}_{feedstock}$) and digester reactor volume in cubic meter ($RV_{Digester}$) according to the following equation:

$$LR = \frac{(\dot{m}_{feedstock} * 1000)}{(RV_{Digester} * 342)} \quad (6)$$

In absence of biogas flowmeter on plants, calculation of the plant biogas production (V_{biogas_year}), expressed in Nm³/year, was different as a function of the type of valorization. In the case of upgrading and biomethane injection, we assumed that all methane produced is injected and the following equation was used.

$$V_{biogas_year} = \frac{(Q_{bioCH4_hour} * 8200)}{(x_{CH4})} \quad (7)$$

In the case of CHP, several hypotheses were made: (i) CHP engine were sized to have an optimal efficiency, therefore only 85% of the nominal power of the CHP engine is used; (ii) electric efficiency of the CHP system was 40% as usually found in literature (Sambusiti et al., 2015); (iii) 5% of additional energetic loss to avoid sub-alimentation of the CHP system (Couturier, 2009); (iv) Lower heating value (LHV) of 9.94 kWh/Nm³ is used for methane; (v) all biogas produced is sent to the CHP system. Total electrical energy produced per year (E_{elec}), expressed in kWh_{el}/year, and the plant biogas production (V_{biogas_year}) were calculated using these two equations:

$$E_{elec} = CHP_{power} * 0.85 * 8200 * 0.4 \quad (8)$$

$$V_{biogaz_year} = \frac{CHP_{power} * 0.85 * 8200}{(0.95 * 9.94 * x_{CH4})} \quad (9)$$

It can be noted that calculated values are closed to approximate figures given by plant operators. Finally, average methane yield from feedstock (Y_{CH4}), expressed in Nm³ CH₄/ton of feedstock, was calculated from the volume of methane produced by the plant each year (V_{CH4_plant}):

$$Y_{CH4} = \frac{V_{CH4_plant}}{\dot{m}_{feedstock}} \quad (10)$$

For each biogas plant, given and calculated features in this section, are displayed in **table 8**. For confidentiality reasons, a letter was attributed to each plant.

Table 8: Biogas plant feature

Plant Name	Plant A	Plant B	Plant C	Plant D	Plant E	Plant F
Type of process	CSTR	CSTR	CSTR	CSTR	CSTR	SS-AD
Type of plant	Agricultural	Agricultural	Agricultural	Territorial	Agricultural	Territorial
Type - reactors volume (m ³)	Digester – 2,000 Post-dig. – 2,000 Storage tank – 6,000	Digester – 2,200 Post-dig. – 2,200 Storage tank – 5,500	Digester – 2,000 Post-dig. – 2,000 Storage tank – 4,000	Digester – 3,700 Post-dig. – 2,700 Lagoon – 10,000	Digester – 1,200 Post-dig. – 1,200 Lagoon – 16,000	Digester – 1,200 Storage tank – 5,500
Type of phase separator	Screw press	Screw press	Screw press	Screw press	Screw press	Screw press
Feedstocks intake (% of total mass)	Catch crop (30%) Bovine manure (18%) – Beet pulp (11%) – Cereal dust (8%) – Whey (33%)	Bovine manure (60%) – Energy crop (10%) – Grass silage (10%) – Cereal straw (10%) – Cereal dust (10%)	Catch crop (50%) – Beet pulp (40%) – Cereal dust (10%)	Waste from food industry (43%) – Animal manure (30%) – Cereal residues (12%) – Sludge (12%) – Catch crop (3%)	Swine manure (55%) – Beet pulp (15%) – Energy crop (10%) – Horse dung (10%) – Cereal residues (5%) – Waste from food industry (5%)	Green waste (100%)
%Total solids in digester	12	10.5	10.5	14	7.6	23
Total quantity of feedstock (tons/year)	13,600	18,000	10,500	25,000	20,000	17,800
Loading rate (kg feedstock/m ³ /day)	19.9	23.9	15.4	19.8	48.73	43.4
HRT before phase separation (days)	100	84	130	88	40	23
pH	7.6-7.9	7.6-7.9	7.6-7.8	7.6-7.8	7.6-7.8	7.5-7.8
Temperature	Mesophilic	Thermophilic	Mesophilic	Mesophilic	Mesophilic	Thermophilic
Quantity of produced SD (tons/year)	1,800	2,000	1,200	3,300	2,500	4,000
Type of valorization	Upgrading	CHP	Upgrading	Upgrading	CHP	Upgrading
Biomethane injected (Nm ³ /hour)	145	/	140	160	/	125
CHP engine nominal power (kW)	/	1890	/	/	1090	/
Plant biogas production (Nm ³ /year)	2,123,000	2,632,000	2,126,000	2,340,000	1,411,000	1,767,000
Biogas composition (% of methane)	56	53	54	56	57	58
Plant methane production (Nm ³ /year)	1,189,000	1,395,000	1,148,000	1,312,000	805,000	1,025,000
Methane yield (Nm ³ CH ₄ /ton feedstock)	87.4	77.5	109.3	52.5	40.25	57.6

2.2. Solid digestate characterization

To characterize the different SD, several methods presented in Chapter II were used. TS, VS, TC, TN and COD_{tot} were determined. An organic matter fractionation method was also carried out. SD RMP were obtained via a first run of BMP tests that we will further call RMP test n°1. Finally, a principal component analysis and partial least square were performed using some of these characterization data.

Besides, anaerobic biodegradability (BD_{ana}), expressed in percentage of total COD, was calculated as the ratio between the RMP and COD_{tot} of the corresponding SD, following the formula (Maynaud et al., 2017):

$$BD_{ana} = \frac{(RMP * 100)}{(0.35 * COD_{tot})} \quad (11)$$

After SD characterization, it was decided to work further with plant A, B and C SD as they were representative of SD coming from agricultural CSTR plants.

2.3. Thermo-alkaline post-treatment

SD from plants A, B and C were subjected to a thermo-alkaline post-treatment. Sodium hydroxide (NaOH) was added to SD at a dose of 2% w/w of total SD and then alkalized SD was placed in a New Brunswick Scientific™ incubator shaker Innova® 43 at 55°C and 120 rpm for 3 days. After that, RMP tests were launched via a second run of BMP tests. It corresponds to RMP test n°2 that was composed of the post-treated SD, the corresponding untreated SD as well as the control for endogenous production, without SD (only inoculum).

2.4. Short-term aerobic post-treatment

Short-term aerobic post-treatment was applied to SD from plants A and B. 300 grams of SD were placed inside 2.5 L bioreactors and aerated according to the set-up displayed in **figure 27**. SD was placed in a fine polyester net to avoid matter loss. A calibrated peristaltic pump sent air to the system. Airflow went first into a concentrated soda solution (NaOH – 2M) to trap atmospheric CO₂. Then air was moisturized by being bubbled into water at the bottom of the double jacket 2.5 L bioreactor. For all experiments, temperature in the aerobic reactors was set at 30°C. Finally, carbon loss due to SD respiration was evaluated by trapping the emitted CO₂ in a 0.5 M soda solution (NaOH) set at 30°C, of which conductivity was measured and recorded every five minutes using an internally developed acquisition system. Relationship between conductivity (mS.cm⁻¹) and carbon trapped (mg C-CO₂/L) was determined by using a calibration line obtained by measuring conductivity of the soda solution with different quantities of Na₂CO₃ dissolved inside and 0.273 as correction factor to translate the mg CO₂/L trapped into mg C-CO₂/L. Total amount of carbon in the SD placed in the bioreactor was calculated from the mass of SD, its TS and the average TC content. From this set-up, several short durations and low to strong airflows were tested at 30°C. For plant A, 1.75, 3.6 and 6 days with an airflow of 1.5, 10 and 20 L/h/kg of TS of SD were tested. For plant B, 2.5 and 5 days with an airflow of 1.65, 16.5 and 33 L/h/kg of TS of SD were applied.

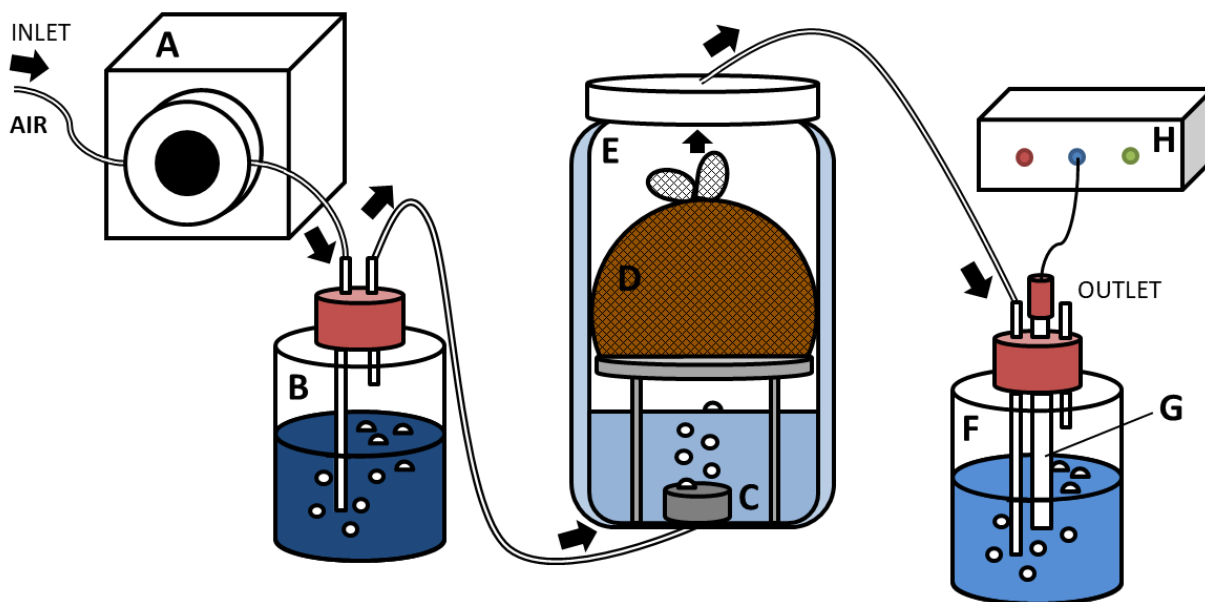


Figure 27: Experimental set-up for short-term aerobic post-treatment of SD. (A) Calibrated peristaltic pump; (B) Air CO₂ trap using 2M NaOH solution; (C) Humidifier system; (D) SD contained in a net; (E) 2.5 liters double jacket aerobic reactor at 30°C; (F) NaOH 0.5M trap for CO₂ emitted from SD respiration; (G) Conductivity probe; (H) Acquisition system.

After these post-treatments, the organic matter fractionation method was performed on aerated SD from plant B following a protocol slightly different from section 2.5. of Chapter II. Indeed, instead of using COD tests to evaluate the distribution of VS in the different fractions, TC content was measured to understand carbon distribution after the post-treatment. TC was measured on the four liquid fractions (SPOM, SEOM, PEOM and NEOM) and on the raw sample. Besides, the fluorescence spectra of liquid extracts were recorded to get insights into fraction complexity.

Samples from aerated SD were also used as substrates for additional BMP tests. This BMP test run, further called RMP test n°3, was composed of the aerated SD, the corresponding untreated SD as well as the control for endogenous production, without SD. For aerated SD, BMP of final post-treated matter (BMP_{FPTM} in mL CH₄.g⁻¹ VS final) is defined as the methane directly measured and produced by the remaining matter without any respiration correction and can be assimilated to biodegradability. To determine RMP values, carbon loss was taken into account. It was obtained from raw BMP values corrected following this hypothesis: all carbon losses under the form of CO₂ during the post-treatment would have otherwise given biogas (ratio 60% CH₄/40% CO₂).

Finally, a bacteria community analysis was carried out on the aerated plant B SD samples using hilldiv package to evaluate species richness and evenness as well as Krona graph to represent taxonomic abundance (Alberdi and Gilbert, 2019a; Ondov et al., 2011).

2.5. Addition of a Kraft solution to enhance short-term aerobic post-treatment

In order to orientate endogenous microbial communities towards aerobic ligninolytic activities, a designed solution was sprayed and mixed with SD from plant A and plant B before performing a short-term aerobic post-treatment. This solution, that will further be called

“Kraft solution”, was based on the following recipe: KH_2PO_4 , 2 g/L; NaCl , 0.5 g/L; Kraft lignin, 1 g/L; CuSO_4 , 0.08 g/L; FeSO_4 , 0.2 g/L; MnCO_3 , 0.2 g/L; CaCO_3 , 0.2 g/L. The pH of the solution was 7.8. Two solution addition ratios were subsequently tested, first 10% w/w of total SD on plant B SD and then 125% w/w of total SD on plant A SD. Besides, a blank was performed with water used in the same proportions as the Kraft solution, to distinguish the effect of water addition from added chemicals. Subsequently, short-term aeration was performed at 30°C, at a low airflow ($1.65 \text{ L}\cdot\text{h}^{-1}\cdot\text{kg}^{-1} \text{ TS}$) and for 3 or 6 days. Carbon loss due to SD respiration was measured as previously. Finally, effect of this treatment on the RMP of post-treated SD was evaluated via a last run of BMP tests (run n°4). As previously, carbon loss was taken into account and RMP values expressed in $\text{Nm}^3 \text{ CH}_4/\text{ton}$ of initial VS. **Figure 28** shows an overview of this experiment.

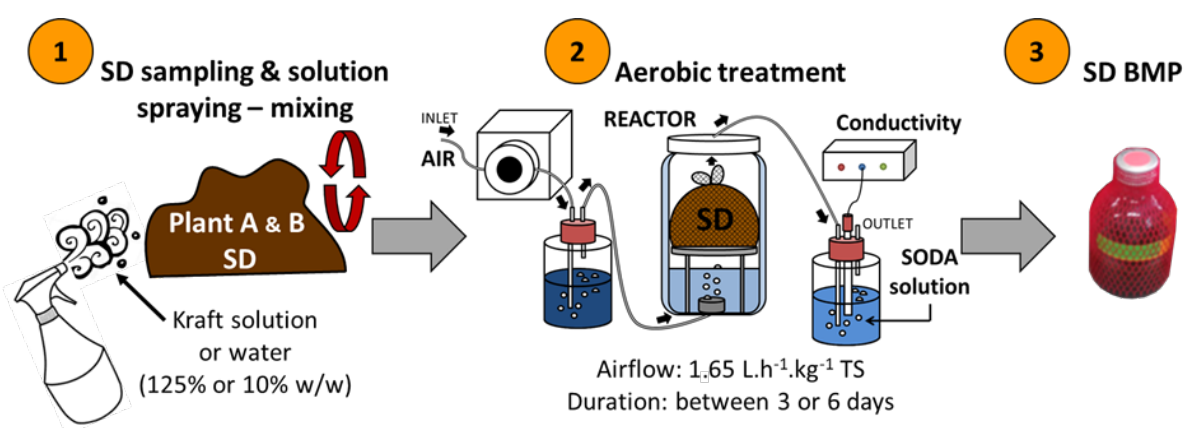


Figure 28: Overview of the experimental protocol for the addition of a Kraft solution to enhance short-term aerobic post-treatment

2.6. Calculations to determine the impact of SD recirculation on plant methane production in the case of an addition or a replacement strategy

Impact of SD recirculation on plant methane production (gain or offset) is evaluated as a percentage of the total plant methane production. This percentage ($\%_{\text{total_plant_CH}_4}$) is obtained by dividing the volume of methane produced from recirculation ($V_{\text{CH}_4_SD}$) by the plant methane production ($V_{\text{CH}_4_plant}$). $V_{\text{CH}_4_SD}$ is calculated using the measured RMP from SD, the quantity of SD recirculated each year ($\dot{m}_{SD_recirculated}$) and the %VS of SD ($[\text{VS}]_{SD}$). For RMP values, in the case of SD from plants A, B and C, the value considered was the average of the values obtained during the three different RMP tests (control values in the case of post-treatment). As an example in the case of plant A, RMP values of run 1, 2 and 3 were 130, 155 and 153 $\text{ml CH}_4\cdot\text{g}^{-1} \text{ VS}$, respectively. Value considered was thus their average: 146 $\text{ml CH}_4\cdot\text{g}^{-1} \text{ VS}$. For SD from plants C, D and E, their RMP values were only coming from RMP test n°1. Finally, a 0.8 correction factor (see **Eq. 1**, section 2.1.4. of chapter I) was applied to all RMP values from plants A, B, C, D, E and F, to simulate a recirculation process at full scale rather than lab scale and to assess realistic values (Holliger et al., 2017). These new values are called corrected SD RMP ($\text{RMP}_{\text{corrected_SD}}$). Besides, it is important to underline that all SD produced cannot be recirculated. Indeed, some recalcitrant organic matter as well as minerals need to exit the system to avoid AD inhibitions and be spread on land in order to bring back carbon and

nutrients. We performed calculations on three SD amounts that can be recirculated within the biogas plant and appear to be realistic in the case of a full-scale application. They correspond to 30%, 50% and 80% of the total amount of SD produced each year. Therefore, $\dot{m}_{SD_recirculated}$ varies according to these percentages. All these values were used to calculate the impact of SD recirculation on plant methane production:

$$\%_{total_plant_CH4} = \frac{V_{CH4_SD}}{V_{CH4_plant}} = \frac{(RMP_{corrected_SD} * \dot{m}_{SD_recirculated} * [VS]_{SD})}{V_{CH4_plant}} \quad (12)$$

In the case of an addition strategy, this percentage of the total plant methane production will represent an additional amount of methane produced. It is assumed that this additional methane is bought at the same purchase price or feed-in tariff (FIT) than contractual production, leading therefore to additional economic gains (AEG).

As all plants are located in France, FIT for injected biomethane and electricity, set by the French government, were obtained by using the calculator provided by the Association Technique Energie Environnement (ATEE) Club biogaz (Atee Club Biogaz, 2019) and are given in **table 9**. For all plants, we assumed that purchase agreement of biomethane or electricity was signed on 01/01/2019. For biomethane injection, higher heating value (HHV) of biomethane was set at 10.8 kWh/Nm³ and bonuses for the presence of catch crops, sludge or municipal waste inside the feedstock mix were taken into account to obtain the feed-in tariff. In the case of electricity, similarly bonuses due to the use of livestock manure were considered.

Table 9: Calculated feed-in tariff of each biogas plant

	Plant A	Plant B	Plant C	Plant D	Plant E	Plant F
Feed-in tariff biomethane (€/MWh HHV)	115.95	/	117.07	102.77	/	92
Feed-in tariff electricity (€/MWh)	/	144.286	/	/	202.211	/

In French biogas legislation, as a standard rule, methane energy equivalence is calculated with the HHV coefficient in the case of biomethane injection, while the lower heating value (LHV – 9.94 kWh/Nm³) coefficient is used for CHP valorization. According to that, additional economic gains were obtained using two different formulas as a function of the type of valorization. For upgrading, HHV value (kWh/Nm³), additional amount of methane produced ($V_{additional_CH4}$ in Nm³ CH₄/year) and corresponding feed-in tariff for the biogas plant (FIT_{biomethane} in €/MWh) were used as displayed in **Eq. 13**. For CHP, the LHV (9.94 kWh/Nm³), the electrical conversion efficiency set at 40%, 5% of additional energetic loss to avoid sub-alimentation of the CHP system and the corresponding feed-in tariff for the biogas plant (FIT_{electrical} in €/MWh) were used as displayed in **Eq. 14**.

$$AEG_{biomethane} = \frac{(V_{CH4_SD} * HHV * FIT_{biomethane})}{1000} \quad (13)$$

$$AEG_{electrical} = \frac{(V_{CH4_SD} * LHV * 0.95 * 0.4 * FIT_{electrical})}{1000} \quad (14)$$

In the case of a recirculation replacement strategy this percentage of the total plant methane production will make possible to calculate the number of tons of feedstocks ($\Delta_{\text{feedstock}}$) that could be replaced by the amount of SD recirculated ($\dot{m}_{\text{SD_recirculated}}$) per year. This amount of feedstock can be calculated using methane yield (γ_{CH_4} – obtained via **Eq. 10**) and according to the following equation:

$$\Delta_{\text{feedstock}} = \frac{V_{\text{CH}_4_SD}}{\gamma_{\text{CH}_4}} \quad (15)$$

3. Results

3.1. Characterization and classification of solid digestates

Table 10 summarizes the physico-chemical and biological properties of the six SD. For SD coming from CSTR biogas plants, their TS were comprised between 22.5% and 25.7% and their VS between 82.5% and 87.3% of TS which is in the range to values found in literature for SD coming from a screw press; generally comprised between 20-30% for TS and 80-90% of TS for VS (Maynaud et al., 2017; Menardo et al., 2011a; Sambusiti et al., 2015; Thygesen et al., 2014). Plant F, which is a SS-AD plant, had a higher TS content (almost 40%) and a lower VS content (74% of TS). This is likely due to the process and feedstocks used in plant F. High TS in the digester leads to higher TS in SD as screw-press efficiency remains similar and green waste are generally rich in woody biomass (branch...) that has a higher mineral content. Similar observations can be drawn from the C/N ratio. SD from Plant F has a C/N ratio 70% higher on average than the rest of SD, that can be explained by a low nitrogen content due to woody biomass. SD from CSTR biogas plants had on average a C/N ratio of 21, slightly lower than the optimal C/N ratio for AD often found between 25 and 30 (Hills, 1979; Wang et al., 2014).

Table 10: Solid digestate physico-chemical and biological properties

SD origin	Plant A	Plant B	Plant C	Plant D	Plant E	Plant F	
[TS] _{SD} (%FM)	22.7±0.3	22.9±0.3	25.7±0.4	22.5±0.3	24.1±0.2	39.3±0.6	
[VS] (%TS)	86±0.2	87.3±0.2	83.6±1	82.5±0.2	84.2±0.4	73.9±0.4	
[VS] _{SD} (%FM)	19.5	20.0	21.5	18.6	20.3	29	
%C/g TS	36.3±0.9	36.0±0.5	33.9±0.4	34.4±0.7	33.3±0.8	36.1±0.1	
%N/g TS	1.8±0.1	1.5±0.1	1.6±0.1	1.5±0.1	1.9±0.1	1.0±0.1	
C/N	20.0	23.8	21.4	23.6	17.2	35.8	
RMP test n°1 (Nm ³ CH ₄ /ton VS)	130±4	145±3	99±6	191±3	155±6	67±6	
COD _{tot} (gO ₂ /kg VS)	1,360±50	1,288±76	1,282±10	1,200±62	1,236±30	1,110±65	
%BD _{ana} (%COD _{tot})	27.3	32.2	22.3	45.5	35.8	17.2	
Chemical sequential extraction (%COD _{tot})	SPOM	5.0±0.1	3.5±0.1	2.7±0.1	2.7±0.1	3.2±0.1	2.0±0.1
	SEOM	10.8±0.1	12.9±1.1	12.7±0.1	12.7±0.3	11.2±0.2	9.0±0.3
	PEOM	39.8±0.1	52.9±0.2	43.6±3.0	50.3±1.9	46.2±1.6	43.1±1.1
	NEOM	44.3±0.3	30.7±1.4	41.0±4.4	34.3±2.4	39.4±2.5	46.0±1.2

RMP values are in accordance with values found in the literature for SD (60 to 240 Nm³ CH₄/ton VS) (Monlau et al., 2015). High RMP (> 145 Nm³ CH₄.ton⁻¹ VS) and anaerobic biodegradability (between 32 and 45% COD_{tot}), that were obtained for plants B, D and E, can be explained by the low to intermediate HRT of these plants (84, 88 and 40 days respectively). Intermediate to low RMP values were obtained for plants A, C and F (67-130 Nm³ CH₄.ton⁻¹ VS) leading to a lower anaerobic biodegradability (< 30% COD_{tot}). For plant A it can be due to the presence of complex feedstocks such as manure or straw that need a longer HRT than the 100 days applied to be degraded (closer to 200 days). For plant C, catch crop and beet pulp are the main feedstocks. They are easy to degrade, and the applied HRT of 130 days is sufficient to recover most of the biogas from the feedstocks lowering thus the RMP value.

Finally, in the case of plant F, low RMP is due to the type of feedstocks used, as green waste generally does not produce much biogas.

Chemical sequential extraction of the six SD, displayed in **table 10**, gave insights into VS accessibility. Plant F has the lowest SPOM and SEOM content, a low PEOM content and the highest NEOM content (46% COD_{tot}). This reflects a high lignin content probably due to the initial feedstock and matches well with our previous hypothesis, explaining such low RMP and low anaerobic biodegradability. For SD coming from CSTR biogas plants, sum of SPOM and SEOM fractions were close to 15% COD_{tot} showing that most of the remaining VS is poorly accessible. Two categories of SD can be distinguished: (i) SD with high PEOM content (higher than 45% COD_{tot}) and low NEOM (lower than 40% COD_{tot}), which corresponds to SD from plants B, D and E; (ii) SD with low PEOM content (lower than 45% COD_{tot}) and high NEOM content (higher than 40% COD_{tot}), which corresponds to SD from plants A and C. Finally, looking at the recent digestate typology made by Guilayn et al. (Guilayn et al., 2019a), all SD of this study can be classified as fibrous material coming from low performance separation regarding the calculated TS, VS and C/N ratio. PLS and PCA analysis described subsequently will allow us to further classify the six SD in three subgroups.

For SD from CSTR biogas plants, to better understand the effect of biogas plant parameters and SD RMP on matter composition and methane recovery, a PLS regression was performed using six explicative variables (X-variables) composed of RMP, HRT, feedstock intake composition and PEOM, NEOM and methane yield at full scale as response variables (Y-variables). Feedstock intakes were divided into 5 classes: manure (horse, swine, bovine), cereal residues (straw and dust), urban waste (sludge, waste from food industry, whey), green waste and silage/pulp (energy crops, beet pulp, grass silage, catch crops). The PLS correlation matrix (**table 11**) shows that RMP of SD are negatively correlated with NEOM content and positively correlated with PEOM content. A similar trend was already observed in a previous study on SD, where a negative correlation was found between SD RMP and Van Soest residual fraction (similar to NEOM) (Bayard et al., 2015). Besides, manure appears to be positively correlated with higher PEOM content and lower NEOM content, while easy to degrade feedstocks (silage/pulp) are strongly correlated with high NEOM content in SD and low PEOM content. This result might be explained by the fact that in our data set, the plants A and C that use more than 40% of easy to degrade feedstocks in their mix have long HRTs, leading to SD with a higher complexity.

Table 11: Partial least square regression correlation matrix between X-Variables and Y-variables - the darker the orange color, the stronger the correlation

X-variables\Y-variables	Poorly Extractable OM (PEOM)	Non Extractable OM (NEOM)	CH ₄ yield at full scale
RMP	0.58	-0.53	-0,85
HRT	-0.31	0.23	0.91
Manure	0.60	-0.56	-0.75
Cereal residues	0.27	-0.35	0.48
Silage/pulp	-0.77	0.74	0.81
Urban waste	-0.04	0.03	-0,32

Data on HRT, methane recovery, feedstock intake and annual quantity of feedstocks, from **table 8**, for the six SD were used to perform a PCA analysis presented in **figure 29**. Three subgroups can be identified and were highlighted in the PCA representation. The two major components were sufficient to describe 73% of the data variability. Component 1 explains 47% of the total variability. High TS, VS, C/N ratio, quantity of green waste feedstock and low RMP, COD_{tot} and SEOM content are significant variables that allow to distinguish Plant F SD from the others. The first subgroup corresponds to SD coming from SS-AD. SD from CSTR biogas plants can then be divided into two subgroups. They are distributed along the second component, which explained 26% of the total variability. Methane recovery at full scale and feedstock quantity are the most significant variables in this component. PLS analysis shows that methane yield at full scale is negatively correlated to high SD RMP and positively correlated with longer HRT. This makes sense as the longer the plant HRT, the higher the methane yield recovered and the lower the RMP. Methane yield is also positively correlated with feedstocks such as silage, beet pulp and cereal dust that have high methane potentials and rapid degradation rates. Finally, methane yield is mainly negatively correlated with manure as this feedstock has generally low methane potential per ton of fresh matter. Thus, SD from plants A and C are close together and form a second subgroup that gathers SD coming from plants with high HRT and high methane potential feedstocks. The third subgroup corresponds to SD from plants B, D and E that have manure in their mix, lower HRT and SD with higher RMP and lower matter complexity.

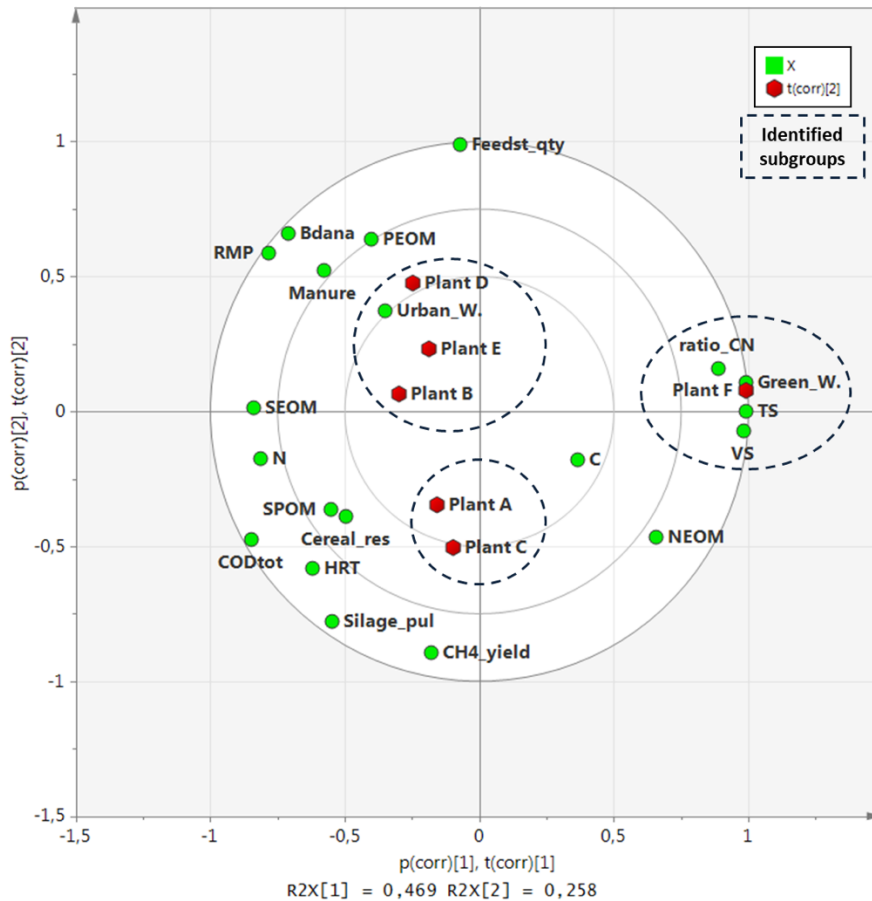


Figure 29: Principal component analysis on SD biodegradability, volatile solids characterization and biogas plant features

Regarding these three subgroups, it was decided to: (i) work further on SD from plants A and C as they are representative of the agricultural CSTR biogas plant sector with long HRT, mainly easy to degrade feedstocks (> 40% total ration) and SD with high NEOM content; (ii) exclude SD from plant F as SS-AD remains for the moment not widely used at full-scale (André et al., 2018); (iii) eliminate among the last subgroup SD from plants D and E as they have too short HRT regarding the amount of feedstock they treat each year and their mix (manure rich); (iv) also select SD from plant B as it displays an interesting profile with intermediate RMP and high PEOM content that is due, despite a long HRT and thermophilic conditions, to the high amount of manure used as feedstock (60%).

3.2. Effect of thermo-alkaline post-treatment on solid digestate residual methane yield

It was observed on separated manure fibers that after 3 days at 22°C soaking in an aqueous ammonia solution (32% w/w), RMP was increased by up to 80% due to strong lignin breakdown (Jurado et al., 2013). Similar effects, using sodium hydroxide (2% w/w) and thermophilic conditions (55°C for 3 days), were targeted on SD from plants A, B and C. RMP and BD_{ana} after this thermo-alkaline post-treatment are given in **table 12**. RMP and BD_{ana} of all treated SD were increased between 30 and 46%. Such an increase is likely to be due to lignin breakdown caused by the combination of sodium hydroxide and prolonged heat. However, in previous studies, rather negative effects on SD RMP were observed following

NaOH treatment at lower temperature. Indeed, it did not increase RMP (NaOH 1% w/w, 40 °C, 24h) (Kaparaju and Rintala, 2005) or even caused a slight decrease of 10% (NaOH 4% w/w, 20 °C, 48h) (Sambusiti et al., 2015). SD lignin content, duration, chemical concentration as well as temperature selected are thus likely to have an effect on the potential enhancement of post-treatment using sodium hydroxide.

Table 12: Effect of thermo-alkaline post-treatment of SD on residual methane potential and anaerobic biodegradability

SD origin	Plant A	Plant B	Plant C
RMP test n°2 – Control (Nm ³ CH ₄ . ton ⁻¹ VS)	155±3	150±4	129±5
RMP thermo-alkaline post-treatment (Nm ³ CH ₄ . ton ⁻¹ VS)	209±7	195±3	188±5
RMP variation	+34.9%	+30.0%	+45.8%
%BDana thermo-alkaline post-treatment (%COD _{tot})	43.9	43.3	41.9

3.3. Effect of short-term aerobic post-treatment on solid digestate residual methane yield, volatile solids repartition and microbial community

Menardo et al. studied the effect of composting storage on RMP SD and showed that composting between two and four weeks led to a decrease in RMP close to 30% (Menardo et al., 2011a). During the composting process, mesophilic and thermophilic phases lead to the removal of easily degradable matter, cellulose and hemicellulose. Lignin components are mainly degraded during the maturation phase (Lin et al., 2018). SD VS evolution was not studied by Menardo et al., but their results seem to indicate that on medium-term, easily biodegradable fractions are more degraded than complex ones, explaining lower RMP.

In this chapter, the effect of shorter and controlled aeration on SD was evaluated as it was previously identified as a potential low-cost biological post-treatment practice at full scale (section 3.6. of Chapter I). It was hypothesized that short-term forced aeration can favor lignin degradation over easily degradable fractions and therefore increase biodegradability and RMP. SD from plants A and B were selected from the two previously described subgroups, as NEOM content was high (44% COD_{tot}) for the former and low (31% COD_{tot}) for the latter. **Table 13** presents anaerobic biodegradation results. It can be seen that BMP of final post-treated matter (BMP_{FPTM}) was not improved in comparison to control for both plant SD. In the case of plant A SD, BMP_{FPTM} was ranging between 126 and 154 Nm³ CH₄/ton VS_{final} either significantly lower or similar to control. For plant B, raw BMP_{FPTM} values were again always significantly lower than control.

Table 13: Effect of short-term aerobic post-treatment on SD on the BMP of final post-treated matter

Sample Name	Airflow	Duration	% C loss	BMP _{FPTM} (Nm ³ CH ₄ /ton VS _{final})
Plant A control	/	/	/	153±8
Plant A SD1	1.65	6	7.1	154±5
Plant A SD2	10	3.6	4.5	126±3
Plant A SD3	20	1.75	2.8	142±4
Plant A SD4	20	3.6	4.8	141±2
Plant A SD5	20	3.6	6.7	140±3
Plant B control	/	/	/	171±4
Plant B SD1	1.65	5	3.1	149±3
Plant B SD2	16.5	5	8.6	159±4
Plant B SD3	33	5	10.5	152±4

RMP curves of test n°3 (obtained after respiration correction of biodegradation curves) as well as carbon loss measured during aeration are given in **figure 30** for plant A and B. It can be noticed that the higher the duration and aeration flow, the higher the carbon loss, which is likely due to longer and higher endogenous microbial activity. Also, SD RMP after various airflows (10 - 16.5 - 20 - 33 L/h/kg TS), whatever the post-treatment duration, were always lower (up to 20% for SD from plant A and 15% for SD from plant B) than the untreated control. In the case of low aeration (1.5 and 1.65 L/h/kg TS) for 5/6 days, RMP was not significantly lower in the case of plant A SD while, for plant B, RMP was 15% lower. A potential explanation is that SD from plant B, being less complex than SD from plant A, has more easily biodegradable matter which was respired. So, this matter is no longer accessible to produce methane in anaerobic digestion.

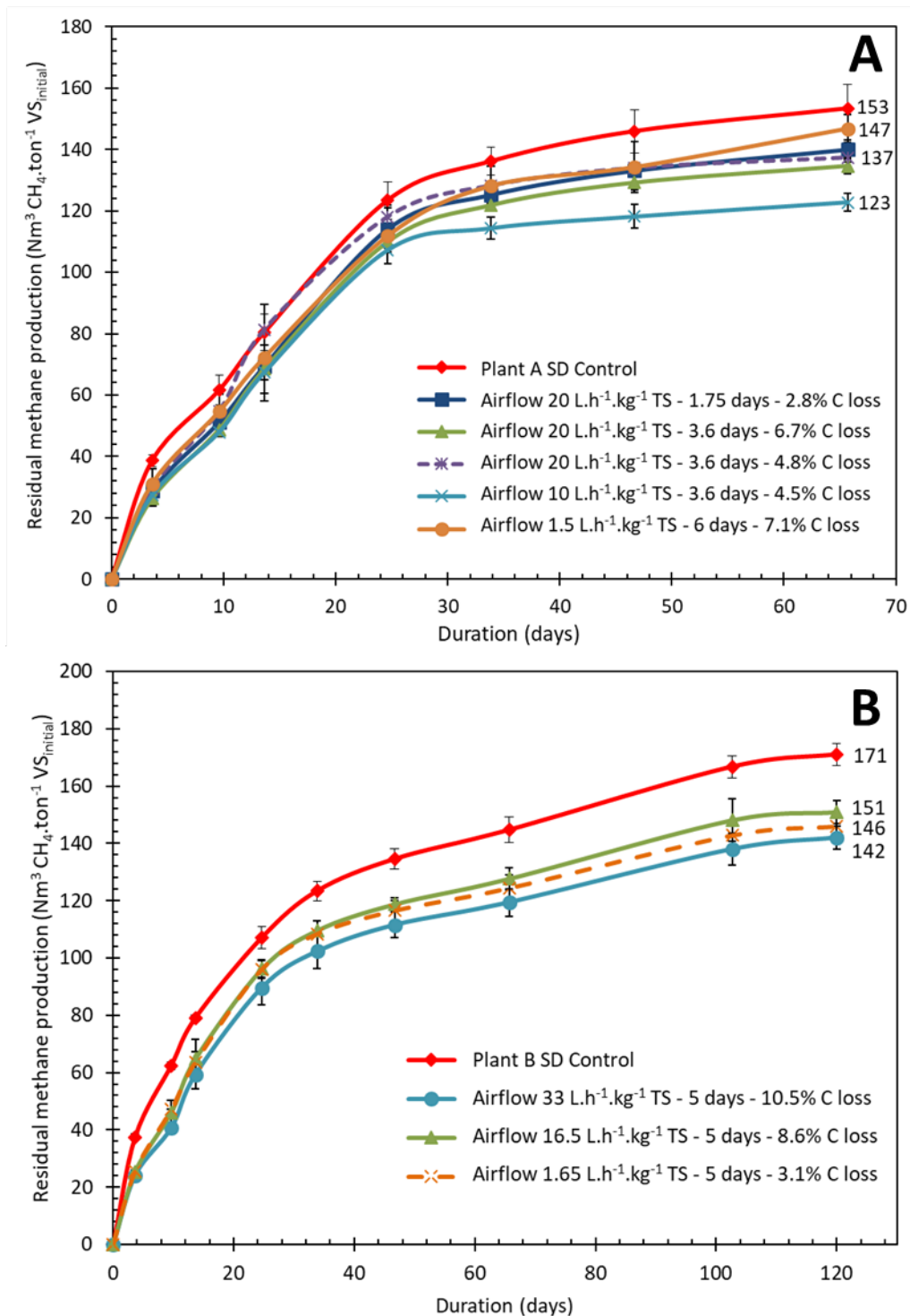


Figure 30: Residual methane potential curves (BMP_{FPTM} tests corrected by carbon losses), from RMP tests n°3, for plant A (A) and plant B (B) solid digestates being subject to short-term aeration post-treatment

For plant A, post-treated SD samples data are not displayed as the analysis of fractionating method was not consistent. Indeed, carbon balance did not close properly (more than 20% missing carbon for unknown reasons). Besides, it was not possible to extract DNA from all plant A microbial samples due to unidentified causes (inhibitors). Therefore, all subsequent analysis, will be based on plant B SD.

Carbon distribution in the different fractions of plant B SD (aerated or not) is given in **table 14**. Distribution of carbon in the different fractions after aeration was significantly different between control SD and SD placed under strong aeration (SD 3). Carbon content in SPOM, PEOM and NEOM was reduced by 18%, 8% and 20%, respectively in comparison to the control. These lower carbon contents can be explained by the carbon respired during the post-treatment (10.5% of total initial carbon). Carbon loss was distributed among the several fractions. It can be assumed that microbial endogenous activities under these aerobic conditions were not only ligninolytic but also proteolytic, cellulolytic and hemicellulolytic. SPOM fraction complexity index was also increased meaning that soluble lignin-like molecules were released. Finally, biodegradability (that is assessed by BMP_{FPTM} values and RMP of SD) were decreased due to lower amount and higher complexity of the most accessible fraction (SPOM). This fraction and its complexity were shown to strongly correlate with biodegradability and RMP in a previous study (Jimenez et al., 2017). For SD placed under intermediate aeration (SD 2) carbon respired was also close to 10% of the total initial carbon. Carbon content in SPOM, SEOM and PEOM were decreased by 25%, 8% and 13.5%, respectively, in comparison to the control. However, NEOM did not decrease significantly. For SD placed under low aeration (SD 1) only PEOM was reduced by 8%. It thus appears that for these airflows, carbon from the lignin-like fraction was not degraded significantly, while carbon from more accessible fractions was, explaining lower RMP. In the case of SD and regarding these results, it is likely that under aerobic conditions, complex fraction may only be degraded after the easily degradable matter has begun to be degraded.

Table 14: Short-term aerobic post-treatment on plant B SD: conditions and carbon distribution

Plant B SD sample	Control SD	SD 1	SD 2	SD 3
Aeration (L air/h/kg TS)	/	1.65	16.5	33
Duration (days)	/	5	5	5
% of total carbon loss/respired	0	3.1	8.6	10.5
% of total carbon in SPOM	2.8±0.2	2.6±0.1	2.1±0.1	2.3±0.1
% of total carbon in SEOM	10.2±0.1	10.2±0.1	9.4±0.3	10.2±0.1
% of total carbon in PEOM	53.2±1.4	46±1.8	46.1±0.1	49.1±0.6
% of total carbon in NEOM	32.6±3	33.6±5.8	28.4±1.6	26±1.4
% of total carbon to close balance	1.2±2	4.5±4.1	5.4±0.4	1.9±0.3
Complexity index of SPOM (in %)	130.5	156.8	161.1	153.6
Complexity index of SEOM (in %)	338.8	296.7	268.9	304

Table 15 displays results of Hill number calculations. It can be seen that aeration has an impact on microbial diversity. Number of rare OTUs (Hill number 0D) increased after aeration (between +40% and +67%). However, it can be seen that the highest aeration flow leads to a lower number of dominant OTUs (Hill number 2D – -46%). Finally, Hill number 1D , that weighs OTUs by their frequency (does not favor rare or abundant OTUs), shows that aeration is slightly increasing diversity of SD. This slight increase is likely due to the growth of aerobic and facultative anaerobic bacteria during the short-term aerobic post-treatment. More details on alpha diversity measurement and hill numbers calculation can be found in section 2.3. of Chapter IV. Taxonomic abundances of bacteria (in post-treated or not SD) are displayed in

Figure 31. *Firmicutes*, dominant species remained between 41%-46% of total abundance. Abundance of *Actinobacteria*, *Gemmatimonadetes* and *Proteobacteria* species was lower after aeration in favor of a strong increase in *Bacteroidetes* species. *Bacteroidetes* from *Flavobacteriia* and *Sphingobacteriia* classes significantly increased. *Bacteroidetes* species are reported using lignocellulosic polysaccharides to subsequently release short-chain fatty acids (Dodd et al., 2011) and were observed in high abundance during the cooling and maturing phases of composting of solid manure (Zhong et al., 2018). Based on these literature observations, degradation of the PEOM fraction (mainly composed of cellulose and xylan) can potentially be explained by a higher activity from the *Bacteroidetes* species. Besides, microbiological studies on SD are scarce and often only focus on pathogens. Ince et al., studied the effect of thermophilic composting on bacterial community in SD coming from an agricultural plant. They also showed that *Firmicutes* were the original dominant species and they are likely to come from manure feedstock (Ince et al., 2018). In this study, thermophilic composting led to a strong increase in *Proteobacteria* species, while *Firmicutes* species were lowered indicating that our treatment is different from a composting process. Finally, as SD microbial communities differ depending on the biogas plant and on the nature of the feedstocks, it can be assumed that microbial community variations observed during this short-term aeration post-treatment may change from an SD to another depending on the initial microbial community.

Table 15: Evaluation of microbial diversity after short-term aerobic post-treatment on plant B SD

Plant B SD sample	Control SD	SD 1	SD 2	SD 3
Aeration (L air.h ⁻¹ .kg ⁻¹ TS)	/	1.65	16.5	33
Duration (days)	/	5	5	5
Hill number ⁰ D (equivalent to richness)	325	543	454	534
Hill number ¹ D (equivalent to Shannon diversity)	49.5	72.8	71.9	60
Hill number ² D (equivalent to Simpson diversity)	28.4	26.6	31.9	18.5

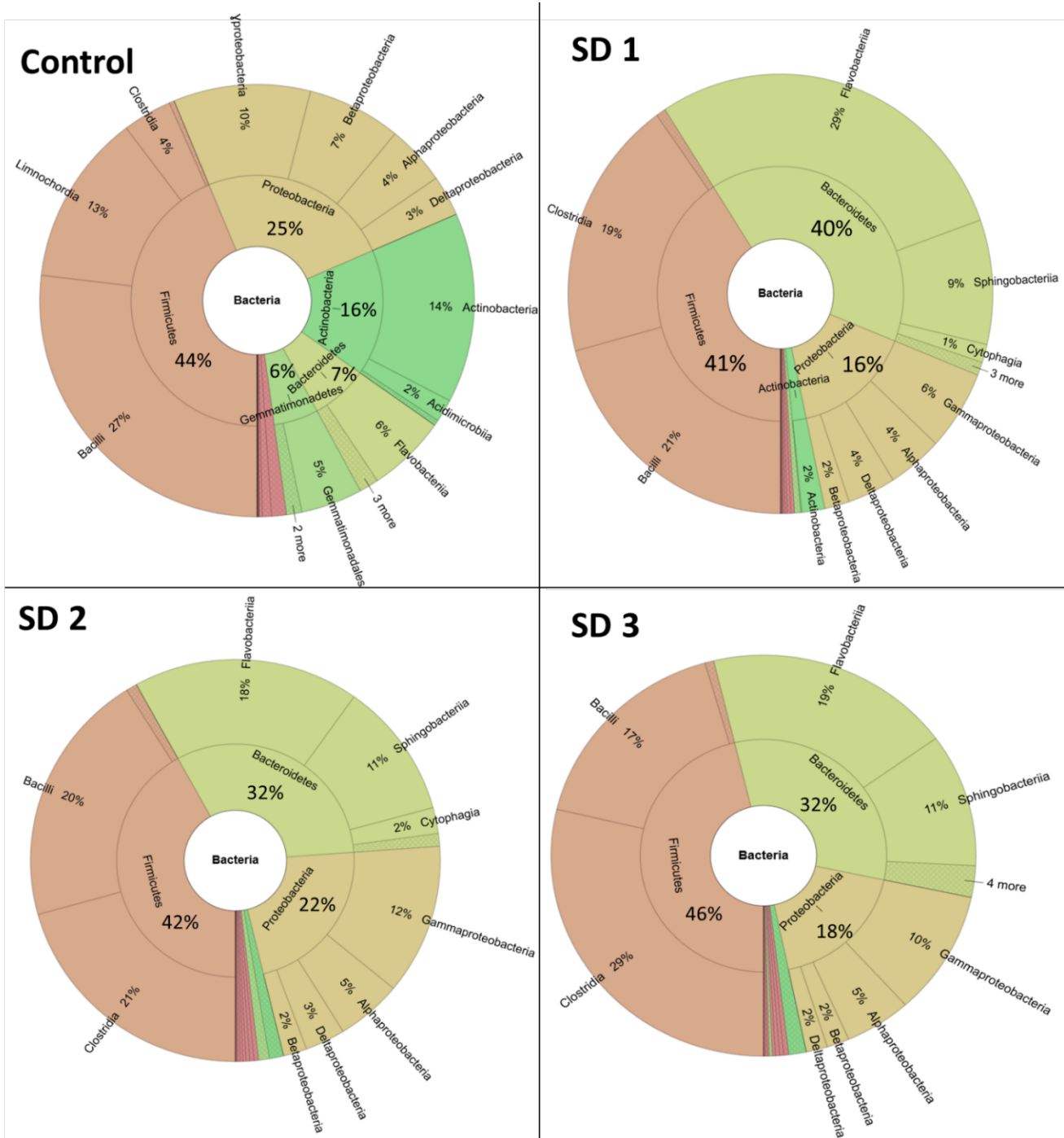


Figure 31: Bacteria community evolution of plant B SD in function of the aeration intensity for 5-day post-treatment: (Control) control SD; (SD 1) Low aeration; (SD 2) Intermediate aeration; (SD 3) Strong aeration.

3.4. Effect of the addition of a Kraft solution on the efficiency of the short-term aerobic post-treatment

It has been shown that under aerobic conditions, SD endogenous microflora did not specifically degrade the lignin-like fraction and preferentially used the most accessible fractions (sugars, proteins, holocelluloses). This leads to lower SD residual methane potentials. It would be interesting to be able to direct the activity of the SD endogenous microflora towards more ligninolytic activities. One track to do so was recently identified by Tian et al. that applied to rape straw, as a pretreatment, a M9 mineral solution complemented with Kraft

lignin followed by a 3-day aeration period (Tian et al., 2017). Addition of this solution induced high aerobic endogenous microbial activity that allows lignocellulosic biomass transformation and increased cellulose accessibility in comparison to the aerated control. It is hypothesized that the solution may have successfully oriented endogenous microorganisms towards more ligninolytic activities. Therefore, the aim of this experience was to evaluate if SD endogenous microflora could also be oriented towards ligninolytic activities by using a similar chemical solution.

KH_2PO_4 , NaCl and Kraft lignin were used and complemented with metals and minerals (copper, iron, manganese, calcium) that can be used by ligninolytic enzymes (laccases, peroxydases...) as structural components or reactional intermediates. Selection of the chemical concentrations was based on avoiding potential chemical toxicity for microorganisms (e.g. copper) and limiting solution costs (here estimated at 5€ per cubic meter). Besides, in addition to their potential effect on endogenous ligninolytic activities, chemicals were selected due to their low toxicity and their ease of handling that render realistic a potential full-scale application.

BMP of final post-treated matter (BMP_{FPTM}) results for plant B SD that was previously subject to Kraft solution or water addition (10% w/w) followed by a short-term aeration were comprised between 152 ± 3 (Kraft solution 6 days) and 167 ± 8 (water) $\text{Nm}^3 \text{CH}_4/\text{ton VS}_{\text{final}}$. These values are not significantly different from control. **Figure 32** displays RMP curves after respiration correction and carbon losses measured during aeration are given as well. It can be seen that Kraft solution did not significantly impact efficiency of short-term aerobic post-treatment as RMP and biodegradability (via BMP_{FPTM}) of SD are not significantly different from control. Besides, addition of Kraft solution did not appear to have more effect on short-term aeration than addition of water as RMP was also not significantly different from the control. Regarding these results, it was decided to increase the solution ratio (from 10% to 125% w/w) to improve the potential impact of such treatment.

Figure 33 presents RMP results for plant A SD that was previously subject to a higher Kraft solution or water addition (125% w/w) followed by a short-term aeration. Carbon losses measured during aeration are given as well. Again, Kraft solution and water addition did not significantly impact efficiency of short-term aerobic post-treatment as RMP or BMP_{FPTM} (almost similar to RMP values due to very low carbon loss) of SD are comparable to control.

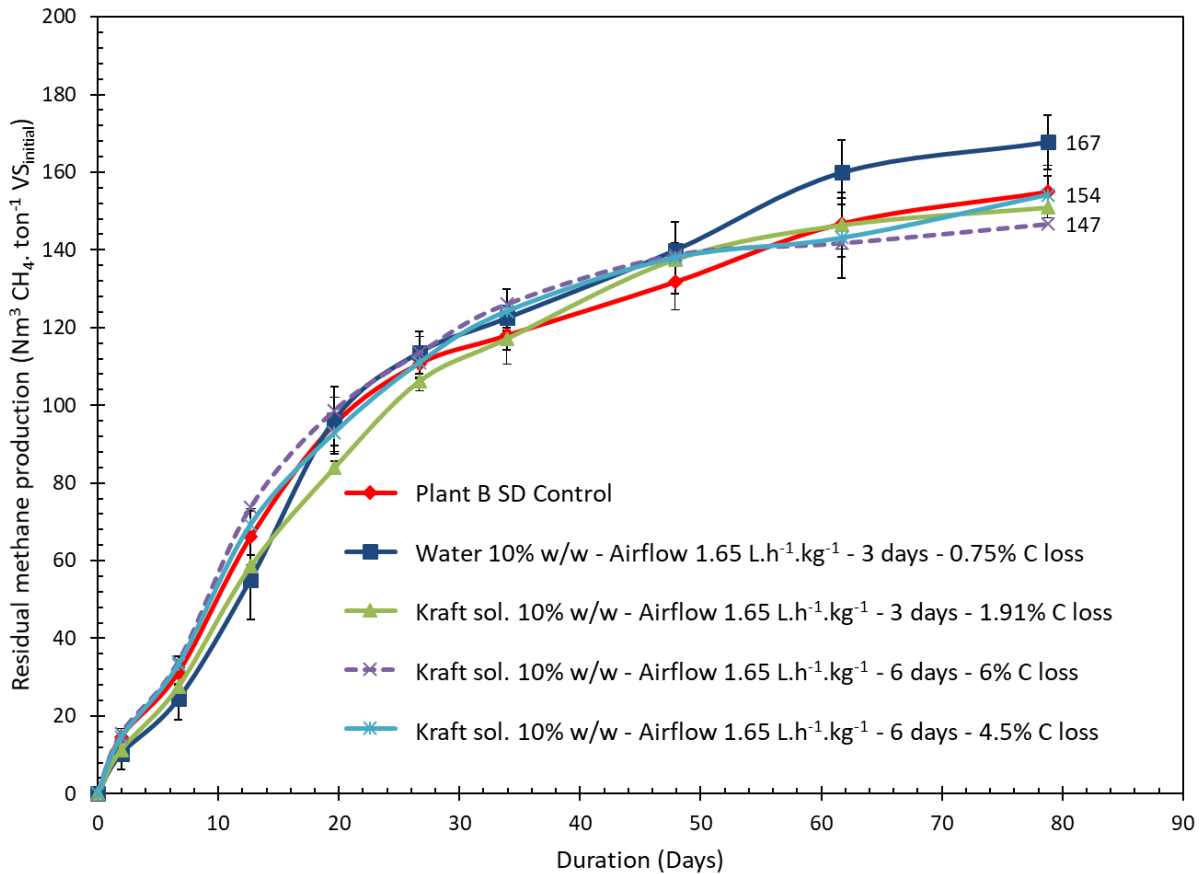


Figure 32: Residual methane potential (BMP_{FPTM} tests corrected by carbon losses) curves plant B SD subject to an addition of water or Kraft solution (10% w/w) followed by a short-term aeration post-treatment.

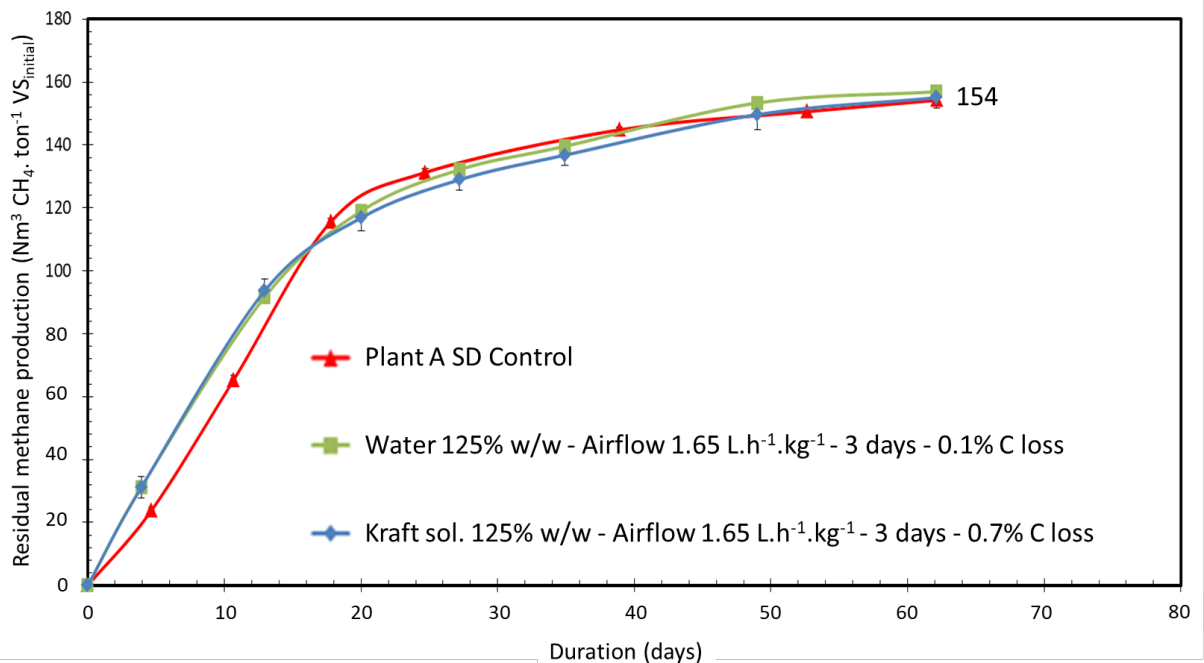


Figure 33: Residual methane potential (BMP_{FPTM} tests corrected by carbon losses) curves of plant A SD subject to an addition of water or Kraft solution (125% w/w) followed by a short-term aeration post-treatment.

It can be concluded that addition of a Kraft solution as well as water, under the tested conditions, have no significant effects on enhancing subsequent SD short-term aerobic post-treatment. As SD RMP was not significantly enhanced, no further studies on the evolution of carbon matter distribution and microbial communities were performed. However, regarding these results, it can be hypothesized that the Kraft solution did not modify the aerobic behavior of the endogenous microflora towards more ligninolytic activities and that the most accessible fractions were again respired. Reasons that could explain why such strategy was efficient for rape straw but did not work for SD are: (i) SD has a higher endogenous bacterial microflora (see chapter V section 3.2.) in comparison to rape straw (10^{11} - 10^{12} cells.g⁻¹ versus 10^9 cells.g⁻¹) that could make it more difficult to orientate its activity (Tian et al., 2017); (ii) rape straw is only made of cellulose, hemicellulose and lignin that should only favor lignocellulolytic activities, while in SD proteolytic activities can also occur.

3.5. Impact of various strategies of solid digestate recirculation on plant methane production and evaluation of potential economic gains or capacity offset.

Table 16 displays calculation results that evaluate the effect of direct recirculation of 30%, 50% and 80% of SD produced each year on plant methane production. Besides, potential additional economic gains (AEG) in an addition strategy case and amount of feedstock that can be offset ($\Delta_{\text{feedstock}}$) in a replacement strategy case are also provided.

First, it is obvious that the higher the amount of SD directly recirculated, the higher the impact on plant methane production. Direct recirculation of 30% of SD represents between 0.6% and 2.3% of total plant methane production and when recirculating 80% of SD reaches between 1.6% and 6.3%. Plants D and E that have a high amount of feedstock, a short to medium HRT and mesophilic conditions display the highest impact of recirculation on plant methane production that can be explained by the relatively high RMP as well as SD amount produced yearly. Interestingly, lowest impact of recirculation on plant methane production (only 1.6% at 80% SD recirculation) is obtained for plant C. It can mainly be explained by the long HRT and the use of easy to degrade feedstocks that lead to low SD RMP.

Secondly, as a function of the recirculation strategy applied, impact on plant methane production differs:

- (i) If recirculation is performed following an addition strategy, the percentage of plant methane production calculated corresponds to a potential increase of the plant methane production. In that case, additional economic gains (AEG) were calculated and ranges between 8k€ and 83.2k€ per year as a function of the plant, the FIT and the percentage of SD recirculated. Such economic gains are not negligible for farmers. Besides, direct recirculation strategy does not need a high cost expenditure to be implemented as part of the produced SD is just sent back into the hopper. It required only a low operational expenditure (OPEX) composed of a short daily labor time (few minutes) and associated tractor operations (gasoline...).

- (ii) If recirculation is performed following a replacement strategy, the percentage of plant methane production calculated corresponds to an offset capacity of the plant methane production in the event, for instance, of a feedstock shortage. It does not generate additional incomes from biogas but it can allow to maintain steady methane production as well as the associated revenues. If we assume that TS and methane yield from feedstock is evenly distributed, such a strategy can replace between 64 and 1431 tons of feedstock as a function of the plant (plant size, SD RMP) and the percentage of SD recirculated. As SD is a free resource produced on site, direct recirculation appears to be a low-cost alternative to buying feedstock when the plant ration is diminished.

Finally, it should be highlighted that for both recirculation strategies, quantities incorporated daily in the digester (SD + feedstock) are increased. Indeed, even in the case of a replacement strategy, for the 6 studied biogas plants, on average 3.3 times more quantities of SD than feedstock are needed to offset methane production loss. This is due to the fact that SD has a lower methane potential than feedstocks in average as it has already been digested once. Therefore, conditions of the implementation of these strategies at full-scale will need to be further discussed in chapter VI and notably their impact on plant TS.

Table 17 presents for plant A, B and C the effect of recirculation of post-treated SD on plant methane production. Additional thermo-alkaline post-treatment increases SD RMP between 30 and 46%. In comparison to direct recirculation, this allows an additional increase of total plant methane production between 0.2% (plant B – 30% SD recirculated) and 1.2% (plant A – 80% SD recirculated). Consequences vary as a function of the type of recirculation but conclusions are similar:

- (i) In an addition strategy, recirculation of post-treated SD leads to AEG between 10.1k€ and 59k€ per year. In comparison to direct recirculation, AEG after a thermo-alkaline post-treatment are increased (+2.1k€ to +17.7k€). However, these economic gains will hardly compensate additional post-treatment expenditures. Purchase of soda (540€ per ton at 2% w/w), is on average already counting for 88% of the additional gains of plant A, 300% of the additional gains of plant B and 67% of the additional gains of plant C. Besides, other expenditures can be added to the soda cost. OPEX has to be increased due to additional labor and heating (thermophilic conditions) and potential capital expenditure (CAPEX), such as the cost for a post-treatment tank (at least 30k€), has to be taken into account. Therefore, such a strategy is not economically viable.
- (ii) Similar conclusions can be drawn in the case of a replacement strategy. Post-treated SD allow to offset an additional amount of feedstock comprised between 49 tons (plant B – 30% SD recirculated) and 163 tons (plant A – 80% SD recirculated) per year (in that specific case, regarding the loading rate, it represents 8 days of feedstocks). However, investment costs are again too high regarding the additional

offset capacity and buying feedstocks will be a less expensive strategy. As an example, in the case of plant A with 80% SD recirculation, cost for soda (15,552€ per year) could be allocated instead to buy at least 346 tons of catch crops at a high price of 45€ per ton (cost ranges normally between 15 and 45€/ton) (Chambre d'agriculture des Landes, 2018). This amount is more than two times higher than the additional 163 tons of feedstocks offset by recirculated post-treated SD. Thus, in that case, buying feedstock largely outcompetes thermo-alkaline post-treatment of SD.

Short-term aeration post-treatments lead to lower SD RMP for plant A and B SD. Calculations where 50% of the total SD produced are recirculated, show that the recirculation impact on total plant methane production is decreased in comparison to direct recirculation. Thus, in the case of an addition strategy, slightly lower AEG are obtained in comparison to direct recirculation (-800€ to -1,800€). Similarly, for a replacement strategy case, the amount of feedstock that can be offset are slightly lower (-16 to -20 tons per year). Regarding these results, it appears that short-term aeration is also not an interesting strategy, despite its low-cost. Besides, it indicates that SD should not be aerated or stored in the open air too long before being recirculated to avoid methane losses under the form of CO₂ respiration.

Finally, it can be observed that due to the small impact of SD direct recirculation on the total plant methane production (mainly coming from low SD RMP), a post-treatment, how effective it may be in the tested scenarios, is unlikely to be profitable as additional CAPEX and OPEX may always be greater than the income or offset capacity from the additional methane.

Table 16: Impact of 30%-50% and 80% SD direct recirculation on plant methane production and its associated additional economic gain in an addition strategy case or the amount of feedstock that can be offset in a replacement strategy case

Ratio of total SD produced that is recirculated		Plant A	Plant B	Plant C	Plant D	Plant E	Plant F
	RMP at lab scale (Nm³ CH₄.ton⁻¹ VS)	146	155	114	191	155	67
	RMP_{corrected_SD} (Nm³ CH₄.ton⁻¹ VS)	117	124	91	153	124	54
30%	$\dot{m}_{SD_recirculated}$ (tons/year)	540	600	360	990	750	1,200
	Mass of organic solids of $\dot{m}_{SD_recirculated}$ (tons VS/year)	106	118	77	184	152	348
	V_{CH₄_SD} (Nm³ CH₄/year)	12,402	14,632	7,007	28,152	18,848	18,792
	%_{total_plant_CH4} (%)	1.04	1.05	0.61	2.15	2.34	1.83
	AEG – addition strategy case (k€/year)	15.5	8	8.9	31.2	14.4	18.7
	$\Delta_{feedstock}$ – replacement strategy case (tons/year)	141	189	64	537	468	326
50%	$\dot{m}_{SD_recirculated}$ (tons/year)	900	1,000	600	1,650	1,250	2,000
	Mass of organic solids of $\dot{m}_{SD_recirculated}$ (tons VS/year)	176	197	129	306	254	580
	V_{CH₄_SD} (Nm³ CH₄/year)	20,592	24,428	11,739	46,818	31,496	31,320
	%_{total_plant_CH4} (%)	1.73	1.75	1.02	3.56	3.91	3.06
	AEG – addition strategy case (k€/year)	25.8	13.3	14.8	52.0	24	31.1
	$\Delta_{feedstock}$ – replacement strategy case (tons/year)	235	315	107	894	783	544
80%	$\dot{m}_{SD_recirculated}$ (tons/year)	1,440	1,600	960	2,640	2,000	3,200
	Mass of organic solids of $\dot{m}_{SD_recirculated}$ (tons VS/year)	282	315	206	490	406	928
	V_{CH₄_SD} (Nm³ CH₄/year)	32,994	39,060	18,746	74,970	50,344	50,112
	%_{total_plant_CH4} (%)	2.77	2.8	1.63	5.71	6.25	4.89
	AEG – addition strategy case (k€/year)	41.3	21.3	23.7	83.2	38.5	49.8
	$\Delta_{feedstock}$ – replacement strategy case (tons/year)	376	504	172	1,431	1251	870
Similar for 30%-50%-80%	Ratio $\dot{m}_{SD_recirculated}/\Delta_{feedstock}$	3.83	3.17	5.59	1.84	1.60	3.68

Table 17: Impact of various SD post-treatments followed by recirculation on biogas plant methane production and its economic impact in comparison to direct SD recirculation

Post-treatment type	Ratio of total SD produced that is recirculated		Plant A	Plant B	Plant C
Thermo-alkaline	30%	RMP_{corrected_SD} (Nm³ CH₄.ton⁻¹ VS)	167	156	150
		V_{CH4_SD} (Nm³ CH₄/year)	17,702	18,408	11,550
		%_{total_plant_CH4} (%)	1.49	1.32	1.00
		AEG – addition strategy case (k€/year)	22.2	10.1	14.6
		AEG variation compared to direct recirculation (k€/year)	+6.7	+2.1	+5.7
		Δ_{feedstock} – replacement strategy case (tons/year)	203	238	106
		Δ_{feedstock} variation compared to direct recirculation (tons/year)	+62	+49	+42
	50%	V_{CH4_SD} (Nm³ CH₄/year)	29,392	30,732	19,350
		%_{total_plant_CH4} (%)	2.47	2.20	1.69
		AEG – addition strategy case (k€/year)	36.8	16.7	24.5
		AEG variation compared to direct recirculation (k€/year)	+11	+3.4	+9.7
		Δ_{feedstock} – replacement strategy case (tons/year)	336	397	177
			Δ_{feedstock} variation compared to direct recirculation (tons/year)	+101	+82
	80%	V_{CH4_SD} (Nm³ CH₄/year)	47,094	49,140	30,900
		%_{total_plant_CH4} (%)	3.96	3.52	2.69
		AEG – addition strategy case (k€/year)	59	26.8	39.1
		AEG variation compared to direct recirculation (k€/year)	+17.7	+5.5	+15.4
		Δ_{feedstock} – replacement strategy case (tons/year)	539	635	283
		Δ_{feedstock} variation compared to direct recirculation (tons/year)	+163	+131	+112
Short-term aeration	50%	RMP_{corrected_SD} (Nm³ CH₄.ton⁻¹ VS)	109	116	/
		V_{CH4_SD} (Nm³ CH₄/year)	19,184	22,852	/
		%_{total_plant_CH4} (%)	1.61	1.63	/
		AEG – addition strategy case (k€/year)	24.0	12.5	/
		AEG variation compared to direct recirculation (k€/year)	-1.8	-0.8	/
		Δ_{feedstock} – replacement strategy case (tons/year)	219	295	/
			Δ_{feedstock} variation compared to direct recirculation (tons/year)	-16	-20

4. Conclusions

In this chapter, we provided initial answers to several research questions of this PhD:

1) What are the different identified strategies to apply SD recirculation?

Three different strategies for SD recirculation were identified and described in this chapter. The addition case (SD is added to the ration) that allows additional biogas production and the replacement case (SD replaces missing or unproduced feedstocks) that, as a function of the quantity of SD recirculated, either allows to maintain a steady biogas production over time (“replacement strategy) or to limit loss in biogas production (“partial replacement strategy”). For the rest of the PhD, these strategies will be distinguished.

2) What is the impact of SD direct recirculation on biogas plant methane production?

Based on SD characterization and biogas plant features, we quantified the potential impact of recirculating SD on plant methane production. Methane produced from the direct recirculation of SD was estimated to range between 0.6% and 5.7% of the total plant methane production, as a function of the plant studied (plant size, SD RMP) and the percentage of total SD recirculated. It corresponds in the case of an addition strategy to additional economic gains between 8k€ and 83k€ per year, which are not negligible for farmers regarding the ease and low-cost of SD direct recirculation. In a replacement strategy, it could offset between 64 and 1,431 tons of feedstocks that can be an interesting alternative for farmers to buying feedstocks as SD is produced for a very low cost (phase separator OPEX and damping).

However, by performing SD recirculation it is likely that at full-scale operational parameters such as viscosity will be modified. Taking these changes into account will refine the assessment of the impact of SD direct recirculation on biogas plant methane production. This will be further discussed in chapter VI.

3) Is the addition of post-treatment before SD recirculation a realistic option?

Thermo-alkaline post-treatment increased between 30% and 46% the additional economic gains or offset capacity in comparison to direct SD recirculation. However, such increases do not compensate the additional expenditure generated by this post-treatment (e.g. cost for soda). Therefore, addition of a thermo-alkaline post-treatment before SD recirculation does not appear as economically viable under the tested conditions.

Short-term aerobic post-treatment is less expensive to implement than thermo-alkaline. However, under the tested conditions, short-term aeration of SD has led to a lower RMP (on average between -15% and -20% when taking into account the carbon respiration). It results, from unspecific aerobic microbial activities towards the lignin-like fraction of SD, that was thus not degraded significantly, while more accessible fractions were (e.g. proteins, holocelluloses). Thus, carbon losses due to respiration are not compensated by a gain in SD biodegradability (BMP_{FPTM}). Lower SD RMP hamper the interest of this post-treatment as

economic gains or offset capacity from SD recirculation are reduced. Thus, either SD should be recirculated rapidly into the digester to avoid RMP loss, either efficiency of the short-term aerobic post-treatment has to be increased. On the last point, a track that has been explored in this chapter and will be further explored in chapters IV and V, is to enhance the ligninolytic activity during this short-term aerobic post-treatment.

4) Is it possible to increase the efficiency of short-term aerobic post-treatment by reorienting endogenous microbial community towards specific ligninolytic activities?

Impact of the addition of a Kraft solution as a way to orientate endogenous aerobic microbial flora towards more ligninolytic activities was studied. It appears that under the tested conditions, addition of this solution did not have positive impact on short-term aerobic post-treatment. Indeed, when taking into account carbon losses, RMP were still lower or similar than the untreated control. It was therefore hypothesized that ligninolytic activities were not stimulated as SD biodegradability was not increased. It is likely that endogenous SD flora will be difficult to orientate, therefore addition of exogenous ligninolytic microorganisms on SD will be further explored in chapters IV and V.

A summary of chapter III results is given in **Figure 34**.

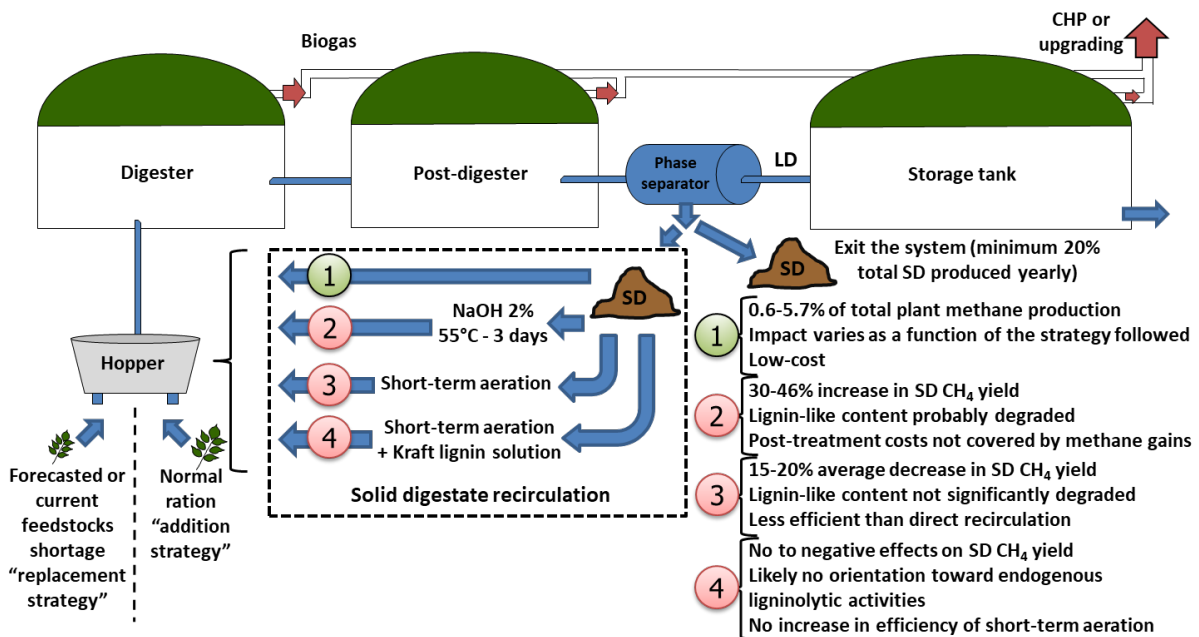


Figure 34: Graphical overview of chapter III results

Transition

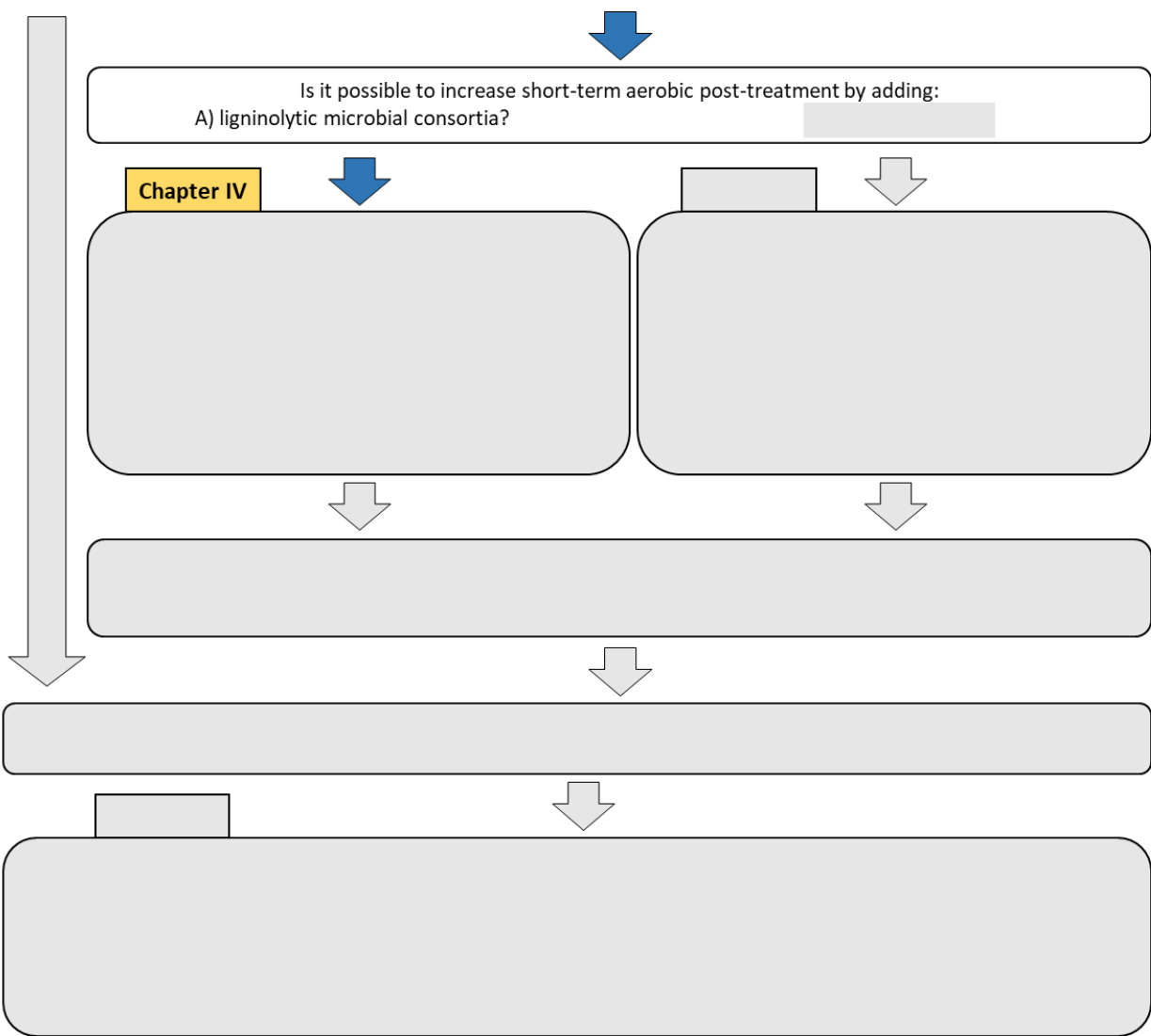
PhD research workflow

PhD objective: Gain insights into the impact of solid digestate (SD) recirculation on agricultural biogas plants.
 Three main research axis*: (A) Strategies and impact
 (B) Post-treatment
 (C) Full-scale
 *(Associated questions → Chapter 1 section 5)

Chapter III

Answer to research axis Strategies and impact and partly to research axis Post-treatment

- ✓ Three strategies of SD recirculation have been defined: addition, replacement and partial replacement.
- ✓ For the six biogas plant evaluated:
 - A) SD direct recirculation following an addition strategy can increase plant methane production between 0,6 to 5,7%.
 - B) SD direct recirculation following a replacement strategy can offset between 64 to 1431 tons of feedstocks.
- ✓ Thermo-alkaline post-treatment costs are not compensated by potential additional methane gains/capacity offset.
- ✓ SD residual methane potential (RMP) decreased after short-term aerobic post-treatment due to unspecific endogenous activities towards the lignin-like fraction (activity reorientation using Kraft solution was not successful).



Chapter IV

Screening and bioaugmentation
of ligninolytic microbial consortia
to enhance aerobic degradation
of solid digestate before its
recirculation

To be submitted

1. Chapter objective

Aerobic pretreatment of agricultural feedstocks can be enhanced by the addition of liquid hydrolytic microbial consortia (see chapter I, section 3.3.3.3.). These microbial consortia were isolated from natural environments (compost, manure, rotten sawdust...) and displayed high lignocellulosic activities under aerobic conditions (Ali et al., 2017; Hua et al., 2016; Zhang et al., 2011; Zhong et al., 2016). Therefore, the idea behind this chapter is to obtain a liquid consortium of ligninolytic microorganisms that would be spread on the solid digestate and then able to degrade specifically the lignin-like fraction of SD during the subsequent short-term aerobic post-treatment. It was hypothesized that efficiency of short-term aerobic post-treatment will be increased and that SD residual methane potential will be enhanced. To test this hypothesis, we performed an experiment in four steps that are represented in **figure 35**.

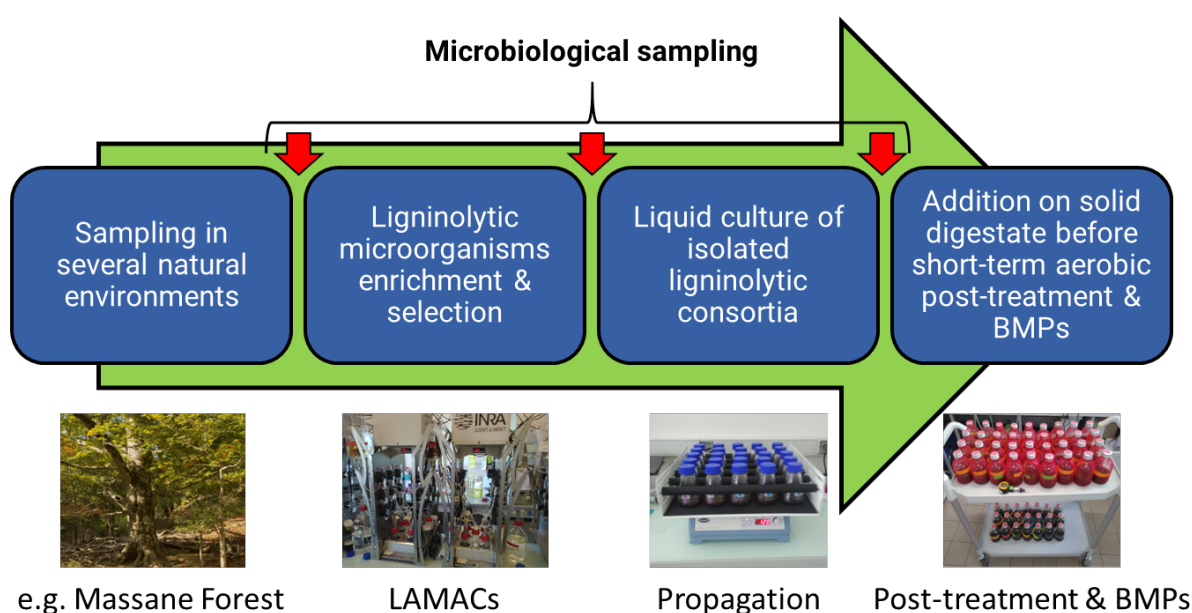


Figure 35: Chapter IV experiment overview

First, six initial natural environments that might potentially contain ligninolytic microorganisms were sampled. From these environments (5 solids and 1 liquid), continuous enrichment and selection of aerobic ligninolytic microorganisms were performed using a modified set-up of the Lab-scale Automated and Multiplexed Anaerobic Chemostat system (LAMACs) (Plouchart et al., 2018). In this modified system with interconnected bioreactors and under aerobic conditions, samples were mixed with a solution containing Kraft lignin as the sole source of carbon to enrich initial environments with microorganisms able to use Kraft lignin. These microorganisms are likely to possess ligninolytic activities (Bandounas et al., 2011; Chandra et al., 2007; Wang et al., 2013). Then, these enriched solutions were transferred, still within the LAMACs system, towards sterile wood and straw substrates as the sole source of carbon. Here, microorganisms were selected according to their capacity to quickly settle on these lignin-rich substrates and use them for their growth. After several months, colonized wood and straw were washed to recover selected consortia into solutions that were subsequently propagated in erlenmeyers. In these erlenmeyers, carbon source was

a “complex” SD to start getting the consortia used to utilize the complex fraction of the digestate. It has to be underlined that microbial sampling and analysis were carried out all along this experiment to understand the evolution of these consortia at each step. Finally, liquid consortia coming from the erlenmeyers were added to solid digestate before a short-term aerobic post-treatment and subsequently its effect on SD RMP was measured via BMP tests. Each of these steps is described in details in the section Materials and Methods of this chapter.

The aim of this chapter is twofold: (i) to better understand the effect of the different selection steps on initial consortia community; (ii) to evaluate if the selection process is successful, leading to consortia that are able to increase efficiency of short-term aerobic post-treatment of SD. The first part of this chapter (section 3.1. to 3.3.) will focus on microbial community analysis over time with the objective to answer the following questions:

- (i) Is a pressure of selection really carried out via the designed strategy?
- (ii) Are the initial microbial communities getting more similar after such treatment?
- (iii) What is the main driver for microbial community evolution: the system (LAMACs, erlenmeyers with complex sterile SD) or the initial carbon sources used for selection (wood & straw)?
- (iv) Do we obtain at the end of this selection strategy consortia enriched in microorganisms that could potentially display ligninolytic activities?

The second part of this chapter (section 3.4.) will focus mainly on evaluating the capacity of these consortia to increase efficiency of short-term aerobic post-treatment of SD. From these two parts, the interest of this strategy for SD post-treatment will be discussed (section 3.5).

2. Materials and Methods

2.1. LAMACs – enrichment and selection

The six initial environments were sampled as described in the section 1.4. of chapter II. The following nomenclature has been developed to distinguish initial environments from each other: F1 for Forest 1 - ground with wood decomposition; F2 for Forest 2 - deep forest litter; F3 for Forest 3 - rotten wood; CF for Compost fertilizer; RU for sheep rumen and RS for rotten straw. Wheat straw that was used as a carbon source and a selection vector in this experiment is described in section 1.2. of chapter II. Size of wheat straw was comprised between 3 and 8 cm following shredding. Untreated chestnut wood pieces, used for a similar purpose than wheat straw, were bought directly from a local sawmill (Ets Guille, Narbonne, France). They were crushed into woodchips of 3 to 5 cm using a BliK® rotary shear shredder type BB 230 (Milly-la-Forêt, France).

In the LAMACs experiment stage, two different solutions were used. The first one is called “enrichment solution”. It contains only as a carbon source Kraft lignin at 1 g/L. Indeed, Kraft lignin was previously successfully used to enrich liquid medium containing soil with lignin-degrading microorganisms (Bandounas et al., 2011). Besides, solution is based on a salt M9 media recipe made of Na_2HPO_4 at 6 g.L⁻¹, KH_2PO_4 at 3 g.L⁻¹, NaCl at 0.5 g.L⁻¹, at NH_4Cl 1 g.L⁻¹ and 1 mL.L⁻¹ of a solution of oligoelements made of FeCl_2 at 2 g.L⁻¹; CoCl_2 at 0.5 g.L⁻¹; MnCl_2 at

0.1 g.L⁻¹; NiCl₂ at 0.1 g.L⁻¹; ZnCl₂ at 0.05 g.L⁻¹; H₃BO₃ at 0.05 g.L⁻¹; Na₂SeO₃ at 0.05 g.L⁻¹; CuCl₂ at 0.04 g.L⁻¹ and Na₂MoO₄ at 0.01 g.L⁻¹. Finally, this solution was complemented with yeast nitrogen base extract at 0.1 g.L⁻¹ and 0.01 mL.L⁻¹ of vitamin solutions (RPMI-1640, Sigma-Aldrich, Darmstadt, Germany). Addition of oligoelements and vitamins were performed to avoid any growth limitations due to nutrients depletion. The second solution used was a simple M9 media that followed the recipe given herein (salts and oligoelements only). Sterilization of these solutions was performed via filtration through 0.2 µm cellulose acetate filter or autoclaving (30 min - 121°C).

LAMACs is a system that was originally designed and used to perform continuous AD and get insights into AD biodiversity and ecosystem functioning over time (Plouchart et al., 2018). However, a great advantage of this system is its modularity and flexibility. It allows for instance to operate up to 30 chemostats under aerobic conditions or to connect reactors to each other to run them in series. These features were used for the experiment. Pictures of the LAMACs system and a detailed scheme of the dedicated operating set-up for the experiment are given in **figure 36**. A LAMACs module is made of six 250 mL glass reactors, eighteen peristaltic pumps (FZ10, A2V, Gazernan, France) with the associated controller module (TMCM 6110, Trinamic, Hamburg, Germany), a heating block (Garaud, Carcassonne, France) and a magnetic stirring plate (Variomag Multipoint 6, Thermo Scientific, Waltham, United States). Three 250 mL reactors were allocated to each environment (in total three LAMACs modules were used and eighteen reactors run continuously in parallel). In the first reactor, called R1, enrichment was performed, while in the reactor 2 (R2) and reactor 3 (R3) selection on straw and wood occurred, respectively. These set-ups made of three reactors, containing a magnetic stirrer in R1, 5 g of wheat straw in R2 as well as 15 g of chestnut wood in R3 and already connected to each other were sterilized (121°C – 30 min). Then, 20 grams in the case of solids (F1, F2, F3, CF and RS) or 20 mL for liquid (RU) of initial environment were added, next to a Bunsen burner flame, in R1. Then, initial environments were diluted as all R1 reactors were completed with sterile “enrichment solution” until 200 mL. After that, set-ups were placed in the LAMACs system (reactors set in the heating block and tubes set inside the associated peristaltic pumps). Sterile bags of 250 mL (Easyflex+, Macopharma, Mouvaux, France) filled in advance with sterile “enrichment media” and 5 L bottle containing sterile M9 media were connected to each set-up. Heating blocs were switched on and set at 30°C. Stirring plate was also switched on and stirring speed was set at 150 rpm to mix R1 reactors. Aquarium pumps were also connected and used to inject continuously filtered air (via 0.45 µm cellulose acetate filter) in each reactor to avoid any oxygen shortage. Finally, program controlling all peristaltic pumps (calibrated beforehand) was launched using a free software (TMCL-IDE, Trinamic, Hamburg, Germany). It is the starting point of the enrichment and selection step.

To perform enrichment followed by selection, the operation of R1 reactors differed from R2 and R3 reactors:

- (i) In R1 reactors, aerobic enrichment was carried out under liquid state. Initial environments were diluted with the “enrichment solution” (ratio 1/10 w/w) in order to favor the development of lignin-degrading microorganisms that would be able to thrive in liquid (Bandounas et al., 2011). Moreover, a long HRT of 10 days was set to avoid any preselection in ligninolytic microorganisms based on their

growth rate. Every day, 20 mL of fresh “enrichment solution” was pumped into R1 and 20 mL of “enriched solution” was pumped out. Of this volume, 10 mL were pumped out the system and discarded, while the remaining volume was split in two (5 mL) and sent towards R2 and R3 reactors.

- (ii) In R2 and R3 reactors, aerobic selection of lignin-degrading bacteria was carried out under solid state. Wheat straw and chestnut wood were only partially emerged as liquid content was set at 50 mL. Enriched liquids, from R1 reactors, before being injected in R2 and R3 were ten times diluted with M9 media (45 mL brought by another peristaltic pump). Besides, liquid HRT in this vessel was shortened to 1 day, as 50 mL of liquid were pumped out and discarded every day. All these conditions aim to select microorganisms, coming from a liquid media, that are able to fix themselves quickly and solidly (to avoid washout due to the short HRT) to wood and straw and used them as main carbon source (dilution aims to reduce Kraft lignin concentration). Finally, these reactors were shaken manually, once a week to ensure that all wood and straw were in contact with pumped in liquid.

Duration of this experiment step was 130 days for F1, F2 and F3 forest environments and 96 days for the other RS, RU and CF environments since all initial environments were not sampled at the same moment. It was assumed that after three-month continuous operation (corresponding to 9 HRT in R1 and 90 HRT in R2 and R3), consortia obtained on wood and straw were stable and this experiment step was stopped.

2.2. Erlenmeyer – consortia selection and culturing

Following microbial consortia solid-state selection on wheat straw and chestnut wood, the aim of this subsequent step was to recover and propagate these consortia in a liquid phase. This liquid phase could be then further used as inoculum for SD before short-term aeration post-treatment. This step can be divided in three parts that are mainly the preparation of the carbon source and solutions that were used in these erlenmeyers, the transfer procedure from LAMACs to the erlenmeyers, the cultivation and sub-culturing steps.

2.2.1. Preparation of the carbon source and solutions

In this step, it was decided to use as a carbon source a complex sterilized SD from plant A to already accustom consortia to use the most complex fractions of SD. SD was complexified using a chemical method based on a simplification of the organic matter fractionation method described in section 2.5. of chapter II. Frozen SD was first dried at 45°C. Then SD was mixed with 0.1 M sodium hydroxide solution for 1 hour at room temperature according to a similar proportion than the organic matter fractionation method (100 grams of dry SD in 5 L solution). Then, supernatant was removed via centrifugation. SD pellets were recovered and put back in a fresh 0.1 M sodium hydroxide solution. This step was repeated 5 times in order to completely remove the most easily degradable fractions from the SD that are SPOM, REOM and SEOM. Finally, the remaining SD pellets obtained were dried at 45°C and aliquoted in hermetic plastic containers (3 grams per container). All plastic containers filled with SD were then sterilized by gamma irradiation by Ionisos (Dagneux, France). Complex SD was subject to a cycle of successive gamma beam sterilization with on average a radiation dose applied of 73.6 kGy. Such a dose was estimated to be sufficient regarding the amount of bacteria in SD (up to 10^{12} cells.g⁻¹; see section 3.2. of Chapter V). Gamma beam sterilization was preferred to autoclaving

to keep organic matter structure undisturbed. After that, sterilized complex SD was used as a carbon source for propagation of selected consortia by erlenmeyer cultivation.

The solution used in erlenmeyers was based on a mineral medium recipe (Atlas, 2010). This solution contains all necessary elements for microorganisms to grow (except carbon). Demineralized water was completed with: $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ at 3.5 g.L^{-1} ; KH_2PO_4 at 1 g.L^{-1} ; $(\text{NH}_4)_2\text{SO}_4$ at 0.5 g.L^{-1} ; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ at 0.1 g.L^{-1} ; CaCl_2 at 0.1 g.L^{-1} , and 1 mL.L^{-1} of a solution of oligoelements made of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at 0.2 g.L^{-1} ; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ at 20 mg.L^{-1} ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at 10 mg.L^{-1} ; MnCl_2 at 5 mg.L^{-1} ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ at 3 mg.L^{-1} ; Na_2SeO_3 at 2 mg.L^{-1} ; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ at 2 mg.L^{-1} and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ at 1 mg.L^{-1} . This solution was autoclaved (30 min – 121°C). Measured pH of the solution was 7.2.

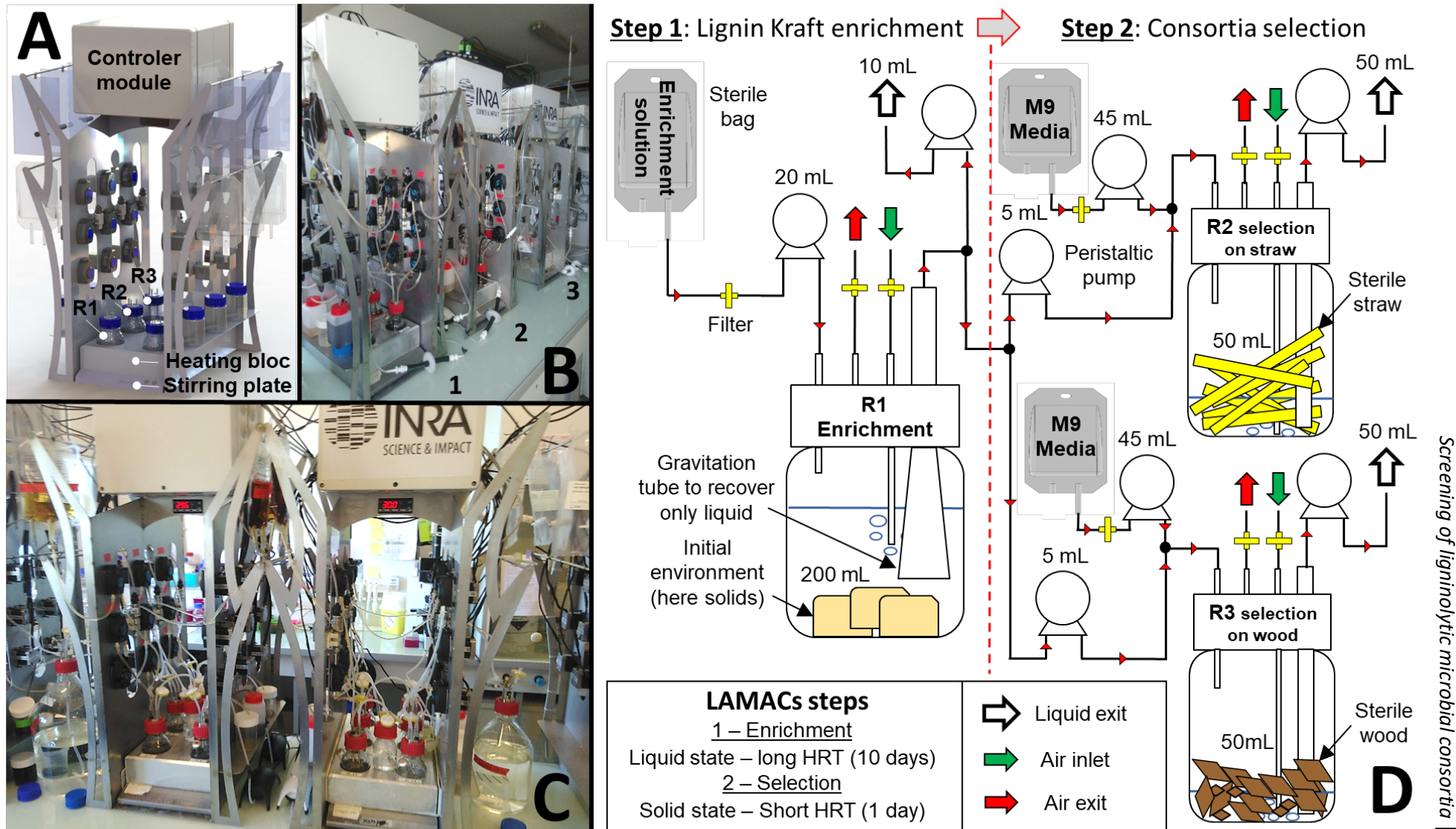


Figure 36: (A) General view of a LAMACs module with reactor localization for one initial environment, adapted from (Plouchart et al., 2018); (B) Picture of the three modules used; (C) Picture of two modules, displaying 12 running reactors (4 initial environments); (D) Components and functioning of one set-up made of three reactors.

2.2.2. Transfer procedure

LAMACS set-ups were disassembled and liquid phases in R2 and R3 reactors were totally purged via pumping. Subsequently remaining wheat straw (R2 reactor) and chestnut wood (R3 reactor) were washed using 60 mL of a sterile phosphate buffer solution (PBS) and Zirconium Oxide ceramic beads of 6.35 mm diameter (MP Biomedicals, Santa Ana, United States) were used to detach and recover into solution as many microorganisms as possible that had grown on the surface. PBS was made of demineralized water completed with NaCl at 22.8 g.L⁻¹ and NaPO₄ at 3.54 g.L⁻¹. Sterilization was performed by autoclaving (30 min – 121°C). 60 mL of this solution and ceramic beads were added in each R2 and R3 reactors that were subsequently agitated one time manually for two minutes. In total twelve solutions (six from straw and six from wood) were obtained. These solutions were further used as microbial inocula for erlenmeyer propagation.

2.2.3. Propagation in liquid phase

Baffled erlenmeyer flasks (Duran Schott, Mainz, Germany) of 250 mL, supplied with a screw cap composed of an autoclavable 0.2 µm PTFE membrane (allows air circulation) were used for aerobic consortia propagation. These flasks were autoclaved, then filled under sterile conditions with 100 mL of sterile mineral medium, then 25 mL of enriched PBS solution (source of microorganisms) was poured in and finally 3 grams of sterile complex SD (source of carbon) were added into the solution. Then caps were closed and flasks were placed on a Stuart SSL1 orbital shaker (Cole-Parmer, Vernon Hills, United States). Room temperature was regulated at 24°C and continuous agitation was set at 120 rpm, to ensure proper mixing of solutions and SD. Only one erlenmeyer per environment was prepared.

On that step, four additional types of erlenmeyers were prepared and placed under the same conditions (agitation, temperature), to answer several hypotheses/research questions:

- (i) Two erlenmeyer flasks where only sterile complex SD was used and no source of microorganisms was added. They are the negative controls and aim to check the sterility of the complex SD used as carbon source in this propagation experiment. They will be further called as “Sterility-check” samples.
- (ii) Erlenmeyer flasks where the source of microorganisms is coming from fresh plant A SD. Practically, fresh SD was washed with sterile PBS (2 grams of TS in 50 mL of PBS). Then this liquid was poured in the same proportion (25 mL) than previously in three flasks containing sterile complex SD and minimal mineral media. They are positive controls that will allow to measure the endogenous activity of microorganisms of SD during this propagation experiment. They will be further called as “Dig” samples.
- (iii) Erlenmeyer flasks where the source of microorganisms is coming from fresh dry fertilizer (CF). Practically, fresh CF was washed with sterile PBS (2 grams of TS in 50 mL of PBS). Then, this liquid was poured in the same proportion (25 mL) than previously in three flasks containing sterile complex SD and minimal mineral media. They are controls that will allow to measure the activity of microorganisms from CF without any enrichment and selection steps using LAMACs. They will be further called as “Direct-CF” samples.

- (iv) Erlenmeyer flasks where the source of microorganisms is coming from a quick selection of endogenous SD microorganisms able to degrade Kraft lignin. Practically, fresh SD was washed with sterile PBS (2 grams of TS in 50 mL of PBS). Then, this liquid was poured under sterile conditions in a dozen Petri dishes containing M9 medium complemented with Kraft lignin at 1 g.L^{-1} and Agar at 15 g.L^{-1} . After one week at 30°C , the biggest colonies were harvested and mixed directly into the minimal mineral medium. It was then supplemented in three flasks with sterile complex SD. They are positive controls that will allow us to measure the activity of endogenous microorganisms of SD, quickly selected on Petri dishes (instead of LAMACs), after their culture in erlenmeyer flasks. They will be further called as “Petri” samples.

Figure 37 gives an overview of that experiment step and the five different types of erlenmeyer flasks that were prepared.

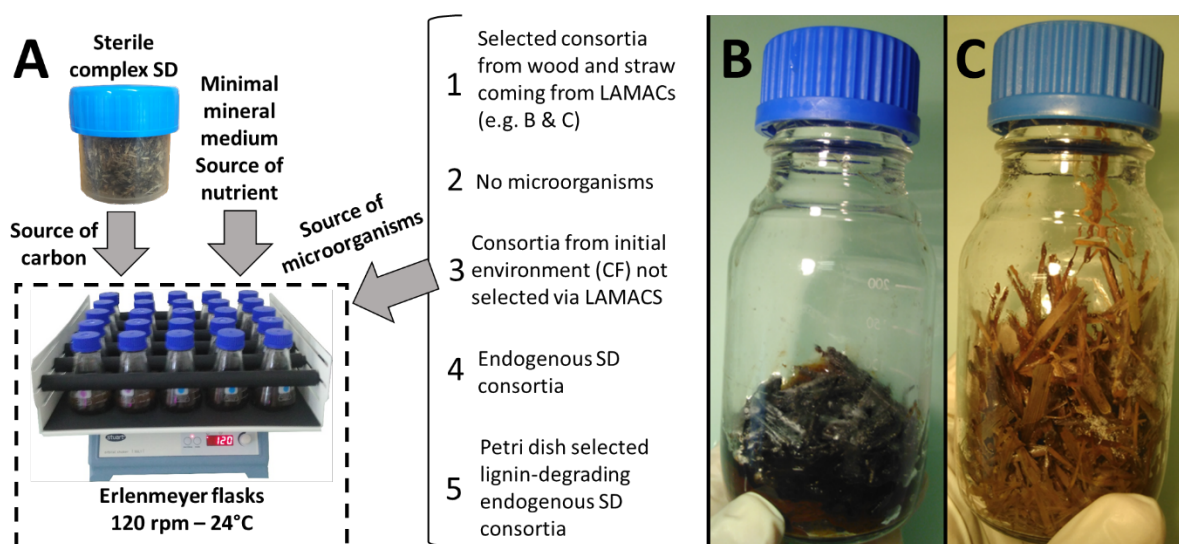


Figure 37: (A) Overview of the preparation of the erlenmeyer flasks and the five different sources of microorganisms that were used for the propagation step; (B) Reactor R3 coming from CF before the PBS washing step; (C) Reactor R2 coming from F1 before the PBS washing step.

2.3. Microbial sampling and analysis

Microbial sampling was performed according to protocol described in section 3.1. of Chapter II. As it can be seen in **figure 35**, microbial sampling was carried out at three different moments of the experiment:

- (i) The six initial environments were sampled before their use in R1 reactors of LAMACs. For F1, F2, F3, CF and RS, it corresponds to a solid sampling, while for RU it was a liquid sampling.
- (ii) At the end of the LAMACs step, liquid sampling was performed on the twelve solutions obtained after PBS solid washing of wheat straw and chestnut wood, for each of the six initial environments. Liquid solutions were also sampled following PBS washing step of solids used for the additional conditions tested (Dig, Direct-CF, Petri). Besides, sterility-check sample consisted of a solid sample of sterilized SD.

- (iii) Finally, at the end of the erlenmeyer step, liquid sampling was carried out on all erlenmeyer flasks that were used as inoculate for the short-term aerobic post-treatment.

Subsequently, DNA extraction, purification, amplification and sequencing were performed on all these samples for bacteria and eukaryotes according to protocols describe in sections 3.2. and 3.3. of chapter II. Besides, qPCR for bacteria and eukaryotes, following protocols of section 3.4. of chapter II, were performed on all samples.

2.3.1. Alpha diversity measurement

Alpha diversity measurement aims to answer the question: how diverse is a microbial sample? It can be defined as the average diversity in species composition for an individual microbial sample (Legendre and Legendre, 2012). In this study, species correspond to the determined operational taxonomic units (OTUs) following Mothur bioinformatics pipeline (see section 3.3 of chapter II). Besides, the amount of DNA sequences (also called abundance) assigned to each OTU is used to give them an importance value, that allows to distinguish rare OTUs from dominant OTUs. Construction of a phylogenetic tree, that would allow finer diversity analysis (use of phylogenetic distance between OTUs), was not performed due to technical limitations (computer calculation performance) regarding the large amount of OTUs obtained from all samples (more than a thousand) (Lozupone and Knight, 2005).

The three most commonly used alpha diversity measurements are: richness, Shannon and Simpson.

- (i) Richness corresponds to the total number of OTUs present in the sample (similar weight for all OTUs);
- (ii) Shannon index measures entropy, that corresponds to the incertitude in the OTU identity of a randomly selected sequence in the sample;
- (iii) Simpson index yields the probability that two DNA sequences selected in a random way effectively belong to two different OTUs (Chao et al., 2014).

It has been reported that a robust estimation of microbial diversity was only possible with Shannon and Simpson measurements. Indeed, richness measurement is highly uncertain due to the difficulty to determine total number of rare species in an environment via traditional sampling (Haegeman et al., 2013). However, values obtained from Shannon and Simpson diversity measurements are difficult to interpret and to compare without any mathematical transformation. Thus, Hill numbers, that gather directly these measurements (as well as richness) in a single statistical framework, has been preferred for this study. Hill numbers are always expressed in “effective number of OTUs”, which can be defined as the number of equally abundant OTUs that would be required to provide a similar value of diversity (Jost, 2007). This unique expression way is a major advantage of Hill numbers that greatly simplify interpretation and comparison between samples.

In **table 18**, from the study of Alberdi et al., formulas and correspondence between the different alpha diversity measurements are presented (Alberdi and Gilbert, 2019b). The parameter q allows to modulate sensitivity towards rare and abundant OTUs. The higher the parameter q value, the lower the sensitivity towards rare OTUs. Besides, it can be noticed that relative abundance (i.e. a percentage of the total amount of DNA sequences measured in the

sample) is used for alpha diversity measurement in general. Finally, the R package hillDiv was used to plot continuous alpha diversity curves that were obtained for q parameter values varying from 0 to 2 (Alberdi and Gilbert, 2019a).

Table 18: The three most common alpha diversity measurements and the associated transformations to calculate Hill numbers of orders 0, 1 and 2; from (Alberdi and Gilbert, 2019b). p_i refers to the relative abundance of OTU i .

	q	Diversity index qH	Hill number in terms of qH	Hill number qD
OTU diversity	0	Richness ${}^0H \equiv \sum_{i=1}^s p_i^0$	Richness ${}^0D = {}^0H$	${}^0D = \left(\sum_{i=1}^s p_i^0 \right)^{1/(1-0)}$
	1	Shannon entropy ${}^1H \equiv - \sum_{i=1}^s p_i^1 \ln p_i^1$	Shannon diversity ${}^1D = \exp({}^1H)$	${}^1D = \left(\sum_{i=1}^s p_i \log p_i \right)$
	2	Simpson index ${}^2H \equiv 1 - \sum_{i=1}^s p_i^2$	Simpson diversity ${}^2D = 1/(1 - {}^2H)$	${}^2D = \left(\sum_{i=1}^s p_i^2 \right)^{1/(1-2)}$

2.3.2. Beta diversity measurement

Beta diversity measurement aims to answer the question: how different are microbial samples from each other? It measures the difference in microbial composition between all samples. In the framework of Hill numbers, beta diversity is obtained by dividing gamma diversity, that can be defined as the diversity of the entire meta-community, combining all the samples, with the averaged basic sums of the samples alpha diversity. Corresponding equations are presented in Table 16. It gives a number comprised between 1 (when all samples are identical) and the total number of samples (when all samples are totally different). Finally, The Hill numbers beta diversity can be further used to measure similarity index between samples.

In this study, only the Sørensen-type overlap will be used. This dissimilarity index, given in **table 19**, quantifies the average proportion of a sample OTUs that are shared across all samples (Alberdi and Gilbert, 2019b). It ranges between 0 (when all compared samples are identical) and 1 (when all compared samples are totally different). Similarly to alpha diversity, q parameter can vary between 0 and 2 and the higher its value, the higher the importance given to abundant species. For q = 0, it corresponds to the well-known Bray-Curtis index, for q = 1 it corresponds to the classical Horn index and for q = 2, it corresponds to the Morisita-Horn index (Chao and Chiu, 2016). As previously, hilldiv R package was used to calculate beta diversity for q=1 and principal ordinations were displayed via NMDS or PCoA methods (Alberdi and Gilbert, 2019a).

Table 19: Formulas for α , β and γ calculations based on Hill numbers and associated similarity measurement derived from beta-diversity; adapted from (Alberdi and Gilbert, 2019b). N refers to the number of samples, S refers to the number of OTUs, w_j refers to the relative weight of sample j (here samples have even weights, it corresponds therefore to $1/N$), p_i refers to the relative abundance of OTU i.

Alpha diversity	${}^qD_\alpha = \frac{1}{N} \left\{ \sum_{i=1}^S \sum_{j=1}^N (w_j p_{ij})^q \right\}^{1/(1-q)}$	Gamma diversity	${}^qD_\gamma = \left\{ \sum_{i=1}^S \left(\sum_{j=1}^N w_j p_{ij} \right)^q \right\}^{1/(1-q)}$
Beta diversity	${}^qD_\beta = {}^qD_\gamma / {}^qD_\alpha$	Sørensen-type Overlap similarity measurement	$C_{qN} = \frac{[(1/{}^qD_\beta)^{q-1} - (1/N)^{q-1}]}{[1 - (1/N)^{q-1}]}$

2.3.3. Dominant OTUs and microbial growth over time

To display evolution of the dominant OTUs over the different experiment steps, relative abundance from sequencing results for bacteria and eukaryotes were used in the Phyloseq R package (McMurdie and Holmes, 2013). Dominant OTUs were defined as those with a relative abundance above 3%, this threshold was arbitrarily selected. PCA plotting and Envfit analysis were performed on OTU relative abundance table (variables: OTUs; samples: microbial sampling) using the Vegan R package. Significant OTUs (p value < 0.01 arbitrarily selected) were blasted using NCBI Blast® platform (Rockville Pike, United States). Finally, qPCR results were used to evaluate microbial growth over the different experiment steps. DNA quantity per vessel was determined by combining qPCR results and the associated amount of solids or liquid present in the vessel. DNA quantity in all vessels used in the different experiments steps were thus measured: R1 at the beginning of the LAMACs step, R2 and R3 at the end of the LAMACs step, erlenmeyers containing blanks at the beginning of the propagation step and erlenmeyers at the end of the propagation step.

2.4. Addition of a consortia solution to enhance short-term aerobic post-treatment

After 40 days in the erlenmeyer flasks, consortium propagation was stopped. To screen obtained consortia on their capacity to enhance efficiency of short-term aeration post-treatment of SD, the following protocol was applied. It is the last experiment step as described in **figure 35**.

First, to only evaluate the action of the liquid consortia and not endogenous SD microorganisms, we used as a substrate a sterile SD coming from plant A. Fresh plant A SD was dried at 45°C and then it was gamma beam sterilized according to the same procedure as for complex SD (see section 2.2.1.). Here, easy to degrade fractions of SD were not chemically removed to fully evaluate capacity of liquid consortia to specifically degrade the most complex fractions. Then, 1.5 g TS of sterile SD were placed in 575 mL BMP flasks and 11 mL of liquid consortia from erlenmeyer flasks were added. Such a ratio allows SD matter to be slightly emerged in liquid. For each liquid consortium, flasks in triplicate were prepared. Besides, a blank was added, where liquid consortia were replaced by water. Finally, flasks remained open and were placed in an incubator at 30°C without agitation for a 6-day duration corresponding to a short-term aerobic post-treatment.

At the end of this period, BMP tests were launched directly using the flasks according to protocol described in section 2.7. of chapter II. A control made of untreated sterilized SD was added to the BMP run. In total, 17 different types of short-term aerobic post-treatment were tested, depending on the type of consortia used (LAMACs x12, Sterility-check, Dig, Direct-CF, Petri and water). Methane production obtained from BMP tests were expressed as a function of the initial amount of SD placed in the BMP flasks. Therefore, carbon losses due to respiration were taken into account despite the fact that they were not measured during the post-treatment. Finally, COD measurements were done on liquid consortia to evaluate additional methane that could be produced during the BMP test due to remaining organic matter in the liquid. **Figure 38** describes this last step.

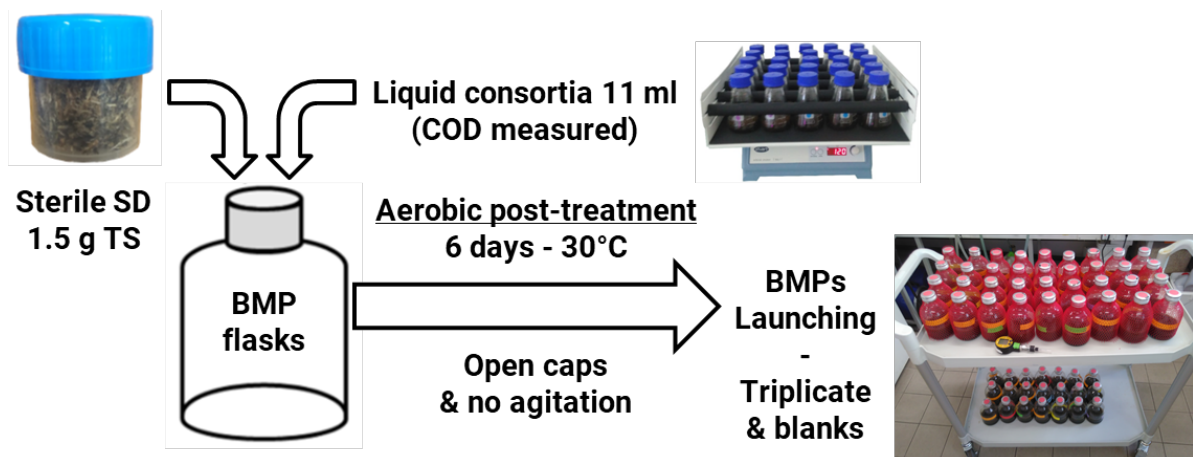


Figure 38: short-term aerobic post-treatment step

3. Results

3.1. Alpha diversity analysis

In this section, the samples that were enriched and selected via the LAMACs process are analyzed. Evolution of alpha diversity for bacteria and eukaryotes over experiment steps is presented in **figure 39**.

For bacteria and eukaryotes, the average alpha diversity profile significantly decreases between initial environments and samples recovered at the end of the LAMACs or propagation step. For bacteria, initial median Shannon diversity (Hill parameter $q=1$) value of 205 effective number or OTUs significantly drops to 50 after LAMACs step and then to 34 (also significant) after propagation step. For Simpson diversity (Hill parameter $q=2$), corresponding to dominant OTUs, initial median value of 66 effective number of OTUs significantly drops to 21 after LAMACs step and to 15 after propagation step. A similar trend was observed for eukaryotes. Initial median Shannon diversity value of 19 significantly drops to 4 after LAMACs step and to 3 after propagation step. For Simpson diversity, initial median diversity value of 8 significantly drops to 2 after LAMACs or propagation steps. Only compost fertilizer (CF) did not show a reduction in its diversity (lowest value of all initial environment box plots). This can be explained by the fact that this sample comes from an industrial composting process that has already applied a selective pressure on microbial flora leading to a low initial diversity in comparison to samples originating from natural environments (forest...).

Regarding these results, it can be concluded that LAMACs steps efficiently applied a selective pressure on five out of six tested environments reducing initial microbial diversity. For the propagation step, its impact on diversity is more contrasted. For the eukaryotes, no significant change was observed indicating that diversity is maintained over time. It is in accordance with our initial strategy as the aim of this step was mainly to propagate selected consortia and not particularly apply an additional selective pressure. However, for bacteria a significant drop was observed for $q = 1$, which can be due to the shift from a solid-state environment in LAMACs to a liquid cultivation in erlenmeyers as well as the use of another source of carbon (complex SD). Additional selective pressure is not particularly a negative fact as in this case it is directed towards what we were looking for: to obtain a liquid consortium able to use complex fraction of SD.

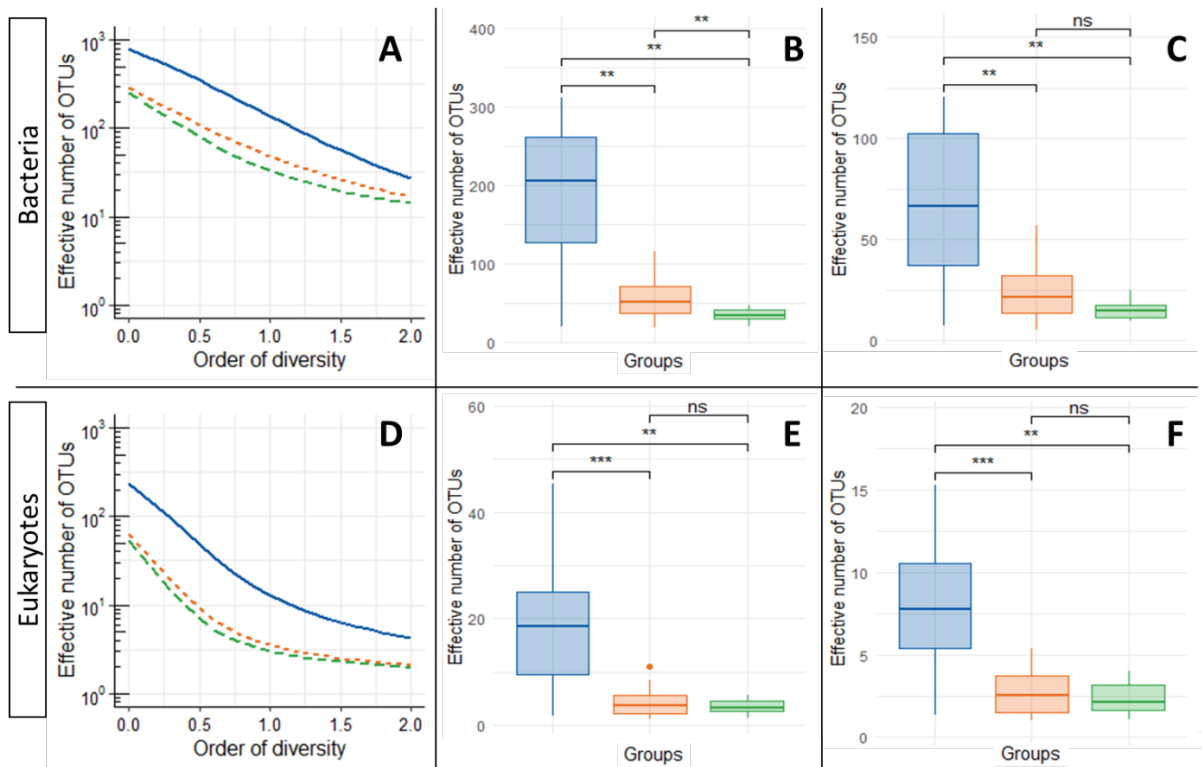


Figure 39: (A) Alpha diversity profile of bacteria population for an order of diversity (q) varying between 0 and 2; (B) Boxplot comparison of bacteria Hill numbers for $q=1$ (Shannon equivalent); (C) Boxplot comparison of bacteria Hill numbers for $q=2$ (Simpson equivalent); (D) Alpha diversity profile of eukaryotes population for a q varying between 0 and 2; (E) Boxplot comparison of eukaryotes Hill numbers for $q=1$ (Shannon equivalent); (F) Boxplot comparison of eukaryotes Hill numbers for $q=2$ (Simpson equivalent). For all sub-figures: (solid blue lines & blue boxplot) correspond to initial samples; (dotted orange lines & orange boxplot) correspond to samples recovered at the end of the LAMACs step; (dashed green lines & green boxplot) correspond to samples recovered at the end of the propagation step. Box plot pairwise median comparison was performed using a Wilcoxon rank-sum test, the following scale was used to indicate significance test result: ns: $p.value > 0.05$; **: $p.value \leq 0.01$; ***: $p.value \leq 0.001$.

3.2. Beta diversity analysis

Similarly to the previous section, samples that were enriched and selected via the LAMACs process are analyzed. It was decided to base this analysis on Horn distance ($q=1$) as it is a trade-off between rare and dominant species and thus gives a fair visualization of beta diversity. First, a NMDS method, presented in **figure 40**, was performed to represent main distance relationships among all the samples.

For bacteria and eukaryotes, NMDS were plotted over 3 dimensions to reach a reliable representation. For bacteria, low ordination stress value (0.097) indicates that representation in three dimensions is excellent. It can be seen that after the LAMACs step, all initial environments get closer and seem to converge to a central point in that representation. It means that initial bacterial communities are getting more similar after LAMACs. Besides, this higher similarity is maintained over the propagation step as samples remain closer to each other. For eukaryotes, relatively low ordination stress value (0.139) indicates that representation in three dimensions is good. However, in that representation, initial eukaryote communities do not seem to get more similar after LAMACs or propagation steps as samples do not display any clear convergence pattern.

Thus, microbial communities from initial environments are getting more similar after LAMACs and propagation step, while it does not appear to be the case for eukaryotes. One potential explanation may lie in the fact that for the six initial environments, effective number of OTUs for bacteria is on average always almost one log above than for eukaryotes (see **figure 40**). Therefore, initial reduced number of eukaryotes species may lower the probability to have shared species between the different environments that would be positively selected by the LAMACs step.

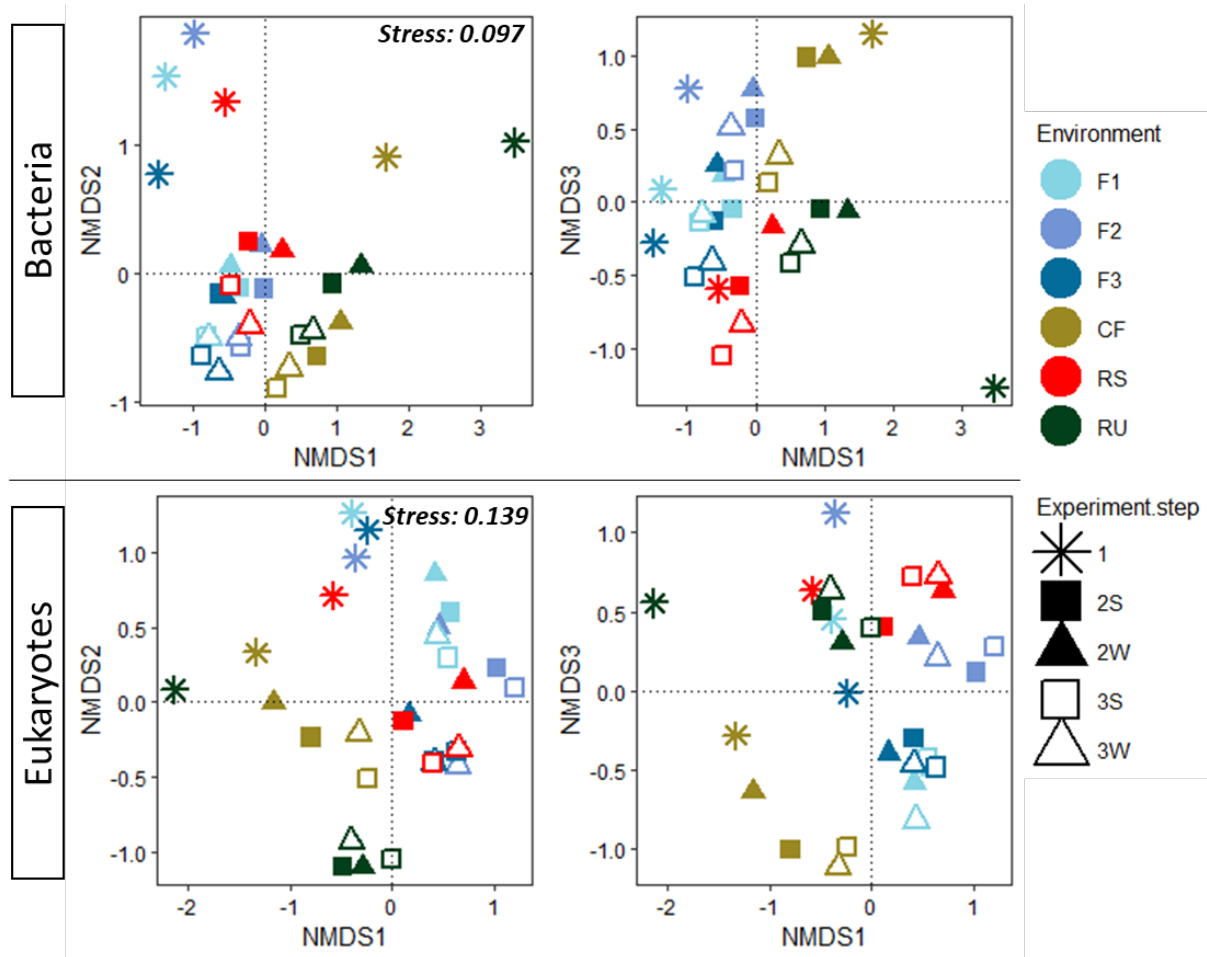


Figure 40: Bacteria and eukaryotes NMDS representation of Horn dissimilarity matrix for all environments and experiment steps.

Figure 41 displays bacteria and eukaryotes beta diversity ordination plots for the six initial environments. PCoA method was performed in the first two dimensions for all the twelve plots. Indeed, variability explained by these ordinations were considered as sufficient to withstand subsequent analysis (cumulated values are comprised between 71.2% up to 100%). Besides, PCoA has the advantage to provide non-distorted distance visualization.

In all cases, the first dimension separates the initial environments from the subsequent steps samples. For bacteria, distance between initial and subsequent samples is generally lower than for eukaryotes, as first dimension explained on average 48% of the total variance for bacteria and 84% for eukaryotes. From these observations, it can be concluded: (i) LAMACs step created an important shift in initial microbial communities due to the selective pressure

applied; (ii) Higher average first dimension value for eukaryotes might be explained by a combination of a potential stronger selective pressure on eukaryotes during LAMACs step as well as a lower to absent selective pressure during propagation step, in comparison to bacteria. On that last point, no significant change in eukaryotes community composition is likely to be due to the already really low number of eukaryotes species present after the LAMACs step (on average 4 effective number of OTUs for $q=1$) that is unlikely to be further reduced in a liquid cultivation step.

The second dimension separates samples from LAMACs and propagation step. For bacteria, shift from LAMACs to propagation step leads to a shift in microbial communities that can be seen by an increase distance between the two groups of samples. Except for F3, distances between wood and straw samples are reduced after propagation step in comparison to LAMACs step. It means that microbial communities are getting more similar. For eukaryotes, plots with dimension 2 explaining more than 10% of total variability display identical trends (for CF plot LAMACs wood sample is located behind the propagation wood sample, explaining why it is not displayed). These distance reductions show that for most environments tested in this study, the system type (LAMACs, erlenmeyers) drives the microbial community evolution more than the carbon sources used for selection (straw and wood).

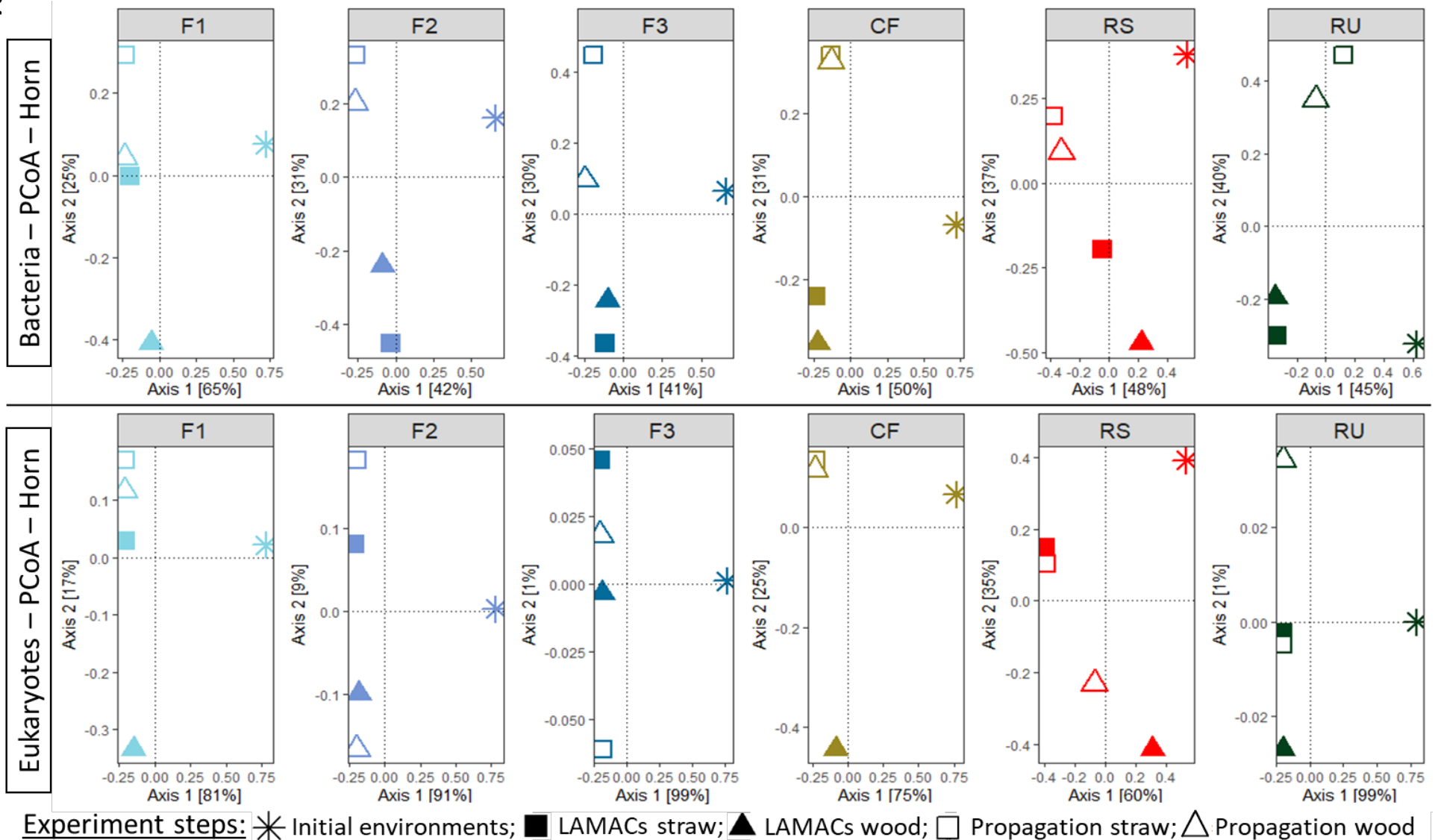


Figure 41: Beta diversity ordination plots resulting from a Principal Coordinates Analysis (PCoA) based on Horn distance (q=1) between samples coming from a similar initial environment. Beta diversity of bacteria corresponds to the upper row and beta diversity of eukaryotes is presented in the lower row. A distinction is made in all these plots between the different experiment steps.

3.3. Evolution of microbial communities and vessel DNA quantity over experiment steps

Relative abundance of microbial communities as a function of initial environments and environment steps are displayed in **figure 42** for bacteria and in **figure 43** for eukaryotes. Blanks microbial communities are also displayed (Direct-CF, Dig, Petri and Sterility-check). It can be noticed that it was not possible to amplify eukaryotes DNA for sequencing in the case of the two sterility check samples. Therefore, sequencing data are not presented for these samples.

Several observations can be made for bacteria when looking at **figure 42**: (i) CF sample is indeed less diverse than the five others. Initial amount of minor OTUs (<3% relative abundance) is around 30%, while others are comprised between 63 and 88%; (ii) For F1, F2, F3, RS and RU initial environments, up to 65% (for RU) reduction in the percentage of minor OTUs through experiment steps can be observed; (iii) Effect of LAMACs is underlined by Direct-CF blank, as its bacterial community after propagation was mainly dominated by Bacilli that is different from CF environment after LAMACs that is dominated by Flavobacteria, Gammaproteobacteria, and Sphingobacteriia; (iv) Petri dish selection on Kraft lignin was efficient to decrease amount of minor OTUs. Besides, Sphingobacteriia was the dominant class after propagation of Petri sample; (v) Finally, sterility-check blank showed a shift in its microbial composition after the propagation step, which means that they were still some remaining living bacteria despite the strong sterilization procedure. Actinobacteria and Alphaproteobacteria were the two major classes that developed during the propagation step. However, the profile of the sterility-check blank is very different from all other propagation samples. Dominant Bacilli, Actinobacteria and Alphaproteobacteria for sterility-check do not exceed 25% of total relative abundance in propagation samples that were supplemented with a source of microorganisms (except for Direct-CF – almost 60%). Therefore, it can be assumed that when an additional source of microorganisms was brought to the propagation system, development of remaining endogenous SD bacteria has a limited impact on the final bacteria composition.

Similarly, **figure 43** allows to draw several observations concerning eukaryotes: (i) Percentage of minor OTUs are really low (7% in average for all samples), even for initial environments in comparison to bacteria; (ii) For the six initial environments, Fungi dominate in forest, compost fertilizer and rotten straw samples while sheep rumen is dominated by Ciliphora. Such difference can be explained by the fact that rumen is a liquid media rich in bacteria favorable to the development of Ciliphora that are mainly bacteria predators. (iii) There is no clear trend in eukaryotes evolution over experiment steps for the six environments. For F1, F2 and RS, initial dominant fungi dropped in favor of Ciliphora, for F3 and CF initial dominant fungi remain and for RU, dominant Ciliphora are replaced by fungi. This could explain why no convergence was observed for eukaryotes in figure 38. (iv) Direct-CF displays after the propagation step a profile very similar to CF (almost 100% of fungi) showing that LAMACs step has no clear effect on eukaryotes for an environment with an already low diversity profile; (v) Finally, selection on a Petri dish of endogenous SD microorganisms favors Ochrophyta and Fonticulea eukaryotes over fungi and Apicomplexa (for Dig). However, in Petri sample after the propagation step, Fonticulea and Ochrophyta were strongly reduced in profit of Fungi. Final

composition is very similar to the Dig sample. Therefore, it appears that the effect of Petri dish is erased by the propagation step.

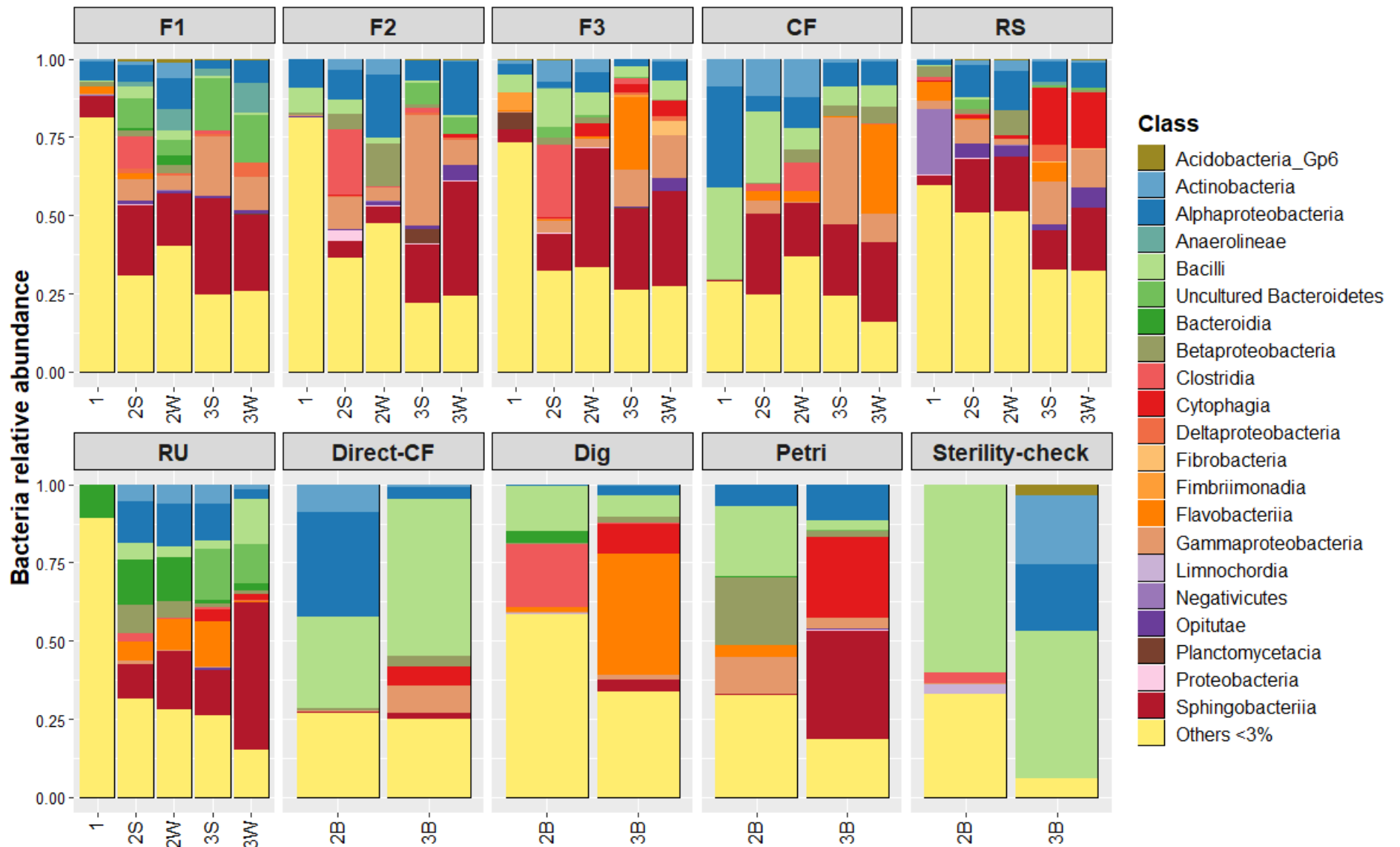


Figure 42: Evolution of bacterial communities over experiment steps as a function of the initial environment. Experiment steps distinction within each initial environment is following this nomenclature: (1) Initial environment; (2B) Blank initial environment; (2S) LAMACs straw; (2W) LAMACs wood; (3B) Propagation blank (3S) Propagation straw; (3W) Propagation wood.

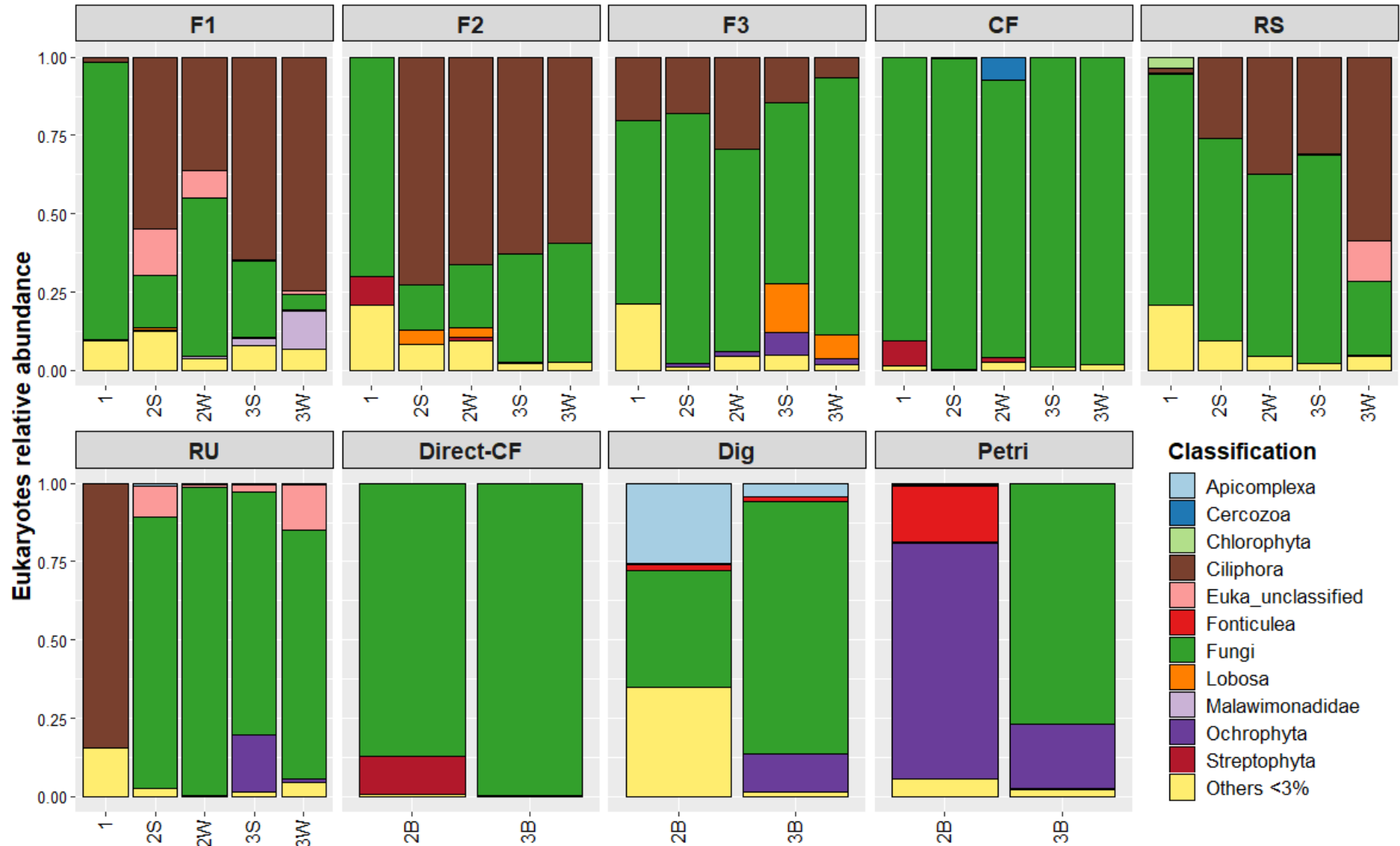


Figure 43: Evolution of eukaryotic communities over experiment steps as a function of the initial environment. Experiment steps distinction within each initial environment is following this nomenclature: (1) Initial environment; (2B) Blank initial environment; (2S) LAMACs straw; (2W) LAMACs wood; (3B) Propagation blank (3S) Propagation straw; (3W) Propagation wood.

One conclusion that emerges from these observations is that there is no clear trend regarding the type of microorganisms that were selected over these experiment steps. Therefore, an additional data visualization was required to get insight into these microorganisms. It corresponds to **figure 44** that was based on PCA representation of the OTUs table and the application of the Envfit analysis.

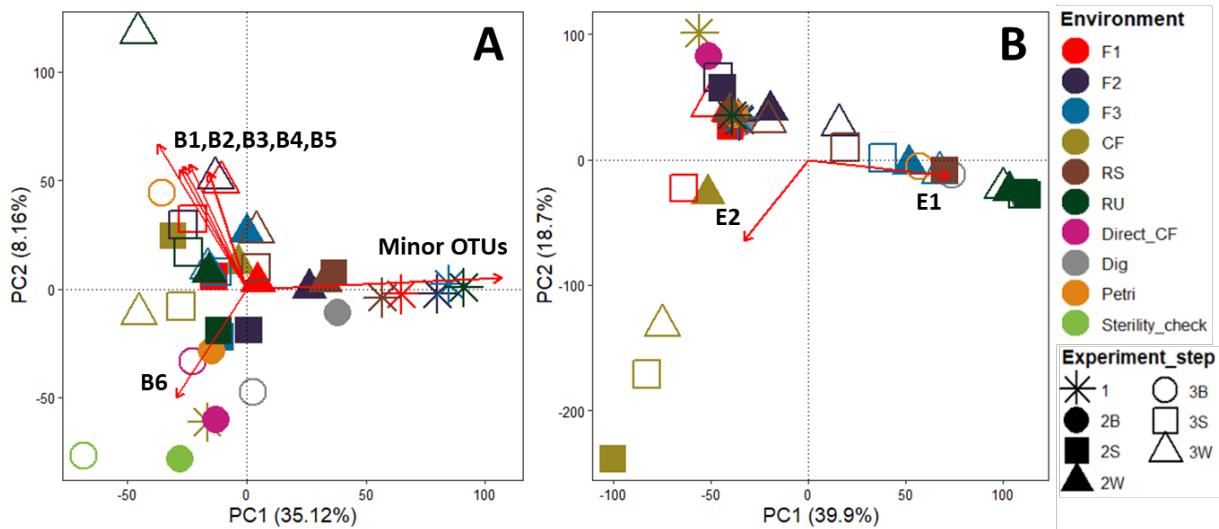


Figure 44: Principal component analysis based on relative abundance of bacteria OTUs (A) and eukaryotes OTUs (B). The red arrows were obtained via Envfit analysis and indicate significant OTUs (p value < 0.01). B1 to B6 and E1 as well as E2 correspond to bacteria or eukaryotes that are further described in table 17. Experiment steps distinction within each initial environment is following this nomenclature: (1) Initial environment; (2B) Blank initial environment; (2S) LAMACs straw; (2W) LAMACs wood; (3B) Propagation blank (3S) Propagation straw; (3W) Propagation wood.

For bacteria, the first dimension represents around 35% of the total variability and allows to distinguish samples rich in minor OTUs. Most initial environments are rich in minor OTUs (except CF as previously stated) and LAMACs and propagation steps successively reduce their amount in favor of dominant species. Second dimension axis explains 8% of the total variability and 6 OTUs (B1 to B6) were determined as significant variables. **Table 20** gives a detailed taxonomy of these bacteria. First, Dig, Petri and sterility-check samples after the propagation step can be distinguished from the other samples and were enriched in *Paenibacillus*. They are facultative anaerobic or strictly aerobic bacteria known to be able to hydrolyze a variety of carbohydrates (Carboxymethyl cellulose, xylan, starch, chitin...) by releasing extracellular carbohydrases (Whitman, 2009). For selected samples via LAMACs or Petri dish, five OTUs appear to have been enriched after the propagation step. Four of them (B1 to B4) are Bacteroidetes and one is a Firmicute of the genus *Paenibacillus* (B5). Bacteroidetes are reported to be in general efficient degraders of complex carbohydrates (Thomas et al., 2011). Three out of the four are from the Chitinophagia class (ex-Sphingobacteriia) and two of them are part of the Chitinophagacea family. In this family, bacteria are reported to be aerobic or facultatively anaerobic and are often found in soils. Besides, some species are reported to be able to degrade chitin polymers or cellulose (Mckee et al., 2019; Rosenberg, 2014).

For eukaryotes, the first two dimensions represent almost 60% of the total variability. Here, for 9 out of 12 samples coming from the six initial tested environments and subjected to LAMACs and propagation steps, contents in *Sordariomycetes* were significantly increased. It was also

the case for Dig and Petri samples after the propagation step. Sordariomycetes are Ascomycota fungi commonly found in soils and decaying wood but also in aquatic environments (Zhang et al., 2006). Most of them are reported to be able to break down lignin and cellulose from plant debris. In this study, liquid state cultivation during the propagation step may have potentially favored Sordariomycetes growth.

Table 20: Detailed taxonomy of significant OTUs obtained from Envfit analysis - % ID corresponds to the % of identity of the closest relatives NCBI using BLAST

	Envfit Pr(>r)	SuperKingdom/ kingdom	Phylum	Class	Genus	Species	% ID
B1	0.001	Bacteria	Bacteroidetes	Chitinophagia (ex-Sphingobacteriia)	<i>Pseudoflavitalea</i>	<i>Pseudoflavitalea</i> <i>sp.</i>	97.85
B2	0.001	Bacteria	Bacteroidetes	Chitinophagia (ex-Sphingobacteriia)	/	<i>Uncultured</i> <i>Chitinophagaceae</i> <i>bacterium</i>	99.46
B3	0.007	Bacteria	Bacteroidetes	Chitinophagia (ex-Sphingobacteriia)	<i>Terrimonas</i>	<i>Uncultured</i> <i>Terrimonas sp.</i>	98.39
B4	0.003	Bacteria	Bacteroidetes	/	/	<i>Uncultured</i> <i>Bacteroidetes</i> <i>bacterium</i>	96.12
B5	0.008	Bacteria	Firmicutes	Bacilli	<i>Paenibacillus</i>	<i>Paenibacillus sp.</i>	97.33
B6	0.009	Bacteria	Firmicutes	Bacilli	<i>Paenibacillus</i>	<i>Enrichment culture</i> <i>clone LDC-5</i>	99.47
E1	0.001	Eukaryota/Fungi	Ascomycota	Sordariomycetes	<i>Scopulariopsis</i>	<i>Scopulariopsis sp.</i>	98.76
E2	0.003	Eukaryota/Fungi	Ascomycota	Sordariomycetes	<i>Acremonium</i>	<i>Acremonium sp.</i>	98.55

Finally, it was checked if LAMACs and propagation steps have led to a microbial growth over time. Calculations were performed, based on qPCR results, to determine the DNA quantity in each vessel. These quantities are presented in **figure 45** as a function of the initial environment and the experiment step.

For bacteria, DNA quantities after the propagation step ranged from 2.9×10^{12} 16S copies (sterility-check) to 1.2×10^{13} 16S copies (CF). DNA increased for all initial environments and blanks. Increase happened to a larger extent for F1, F3, CF, RS, Direct-CF, Dig and Petri (more than a log of difference) than for F2, RU and sterility-check (less than a log of difference). However, for these three latter, initial bacteria DNA quantities were already high (close to 10^{12}) in comparison to the other environments. For F1, F2 and F3, slightly more DNA were obtained at the end, for samples that went through LAMACs selection on straw than samples selected on wood. For CF, RS and RU final amount was identical between samples from straw and wood.

For eukaryotes, DNA quantity after the propagation step ranged from 1.4×10^{10} 18S copies (CF-straw) to 1.6×10^{11} 18S copies (F3-straw). Evolution of DNA quantity seems to converge towards this amount range. Indeed, DNA quantity was increased for environments with initial DNA quantities below 5×10^9 (F1, F3, RS, RS, Dig and Petri). DNA quantities were stable for environments having initial DNA quantities already comprised within the final region (F2, RU

and Direct-CF). Decrease in DNA quantity happened for the CF initial sample that had a really high DNA quantity in comparison to all other samples (4.2×10^{11}).

Thus, except for CF eukaryotes, quantities of DNA in erlenmeyers at the end of the propagation step were increased both for bacteria and eukaryotes species in comparison to initial enrichment reactors (R1). This result is positive as it shows that microbial community were active and have grown throughout experiment steps.

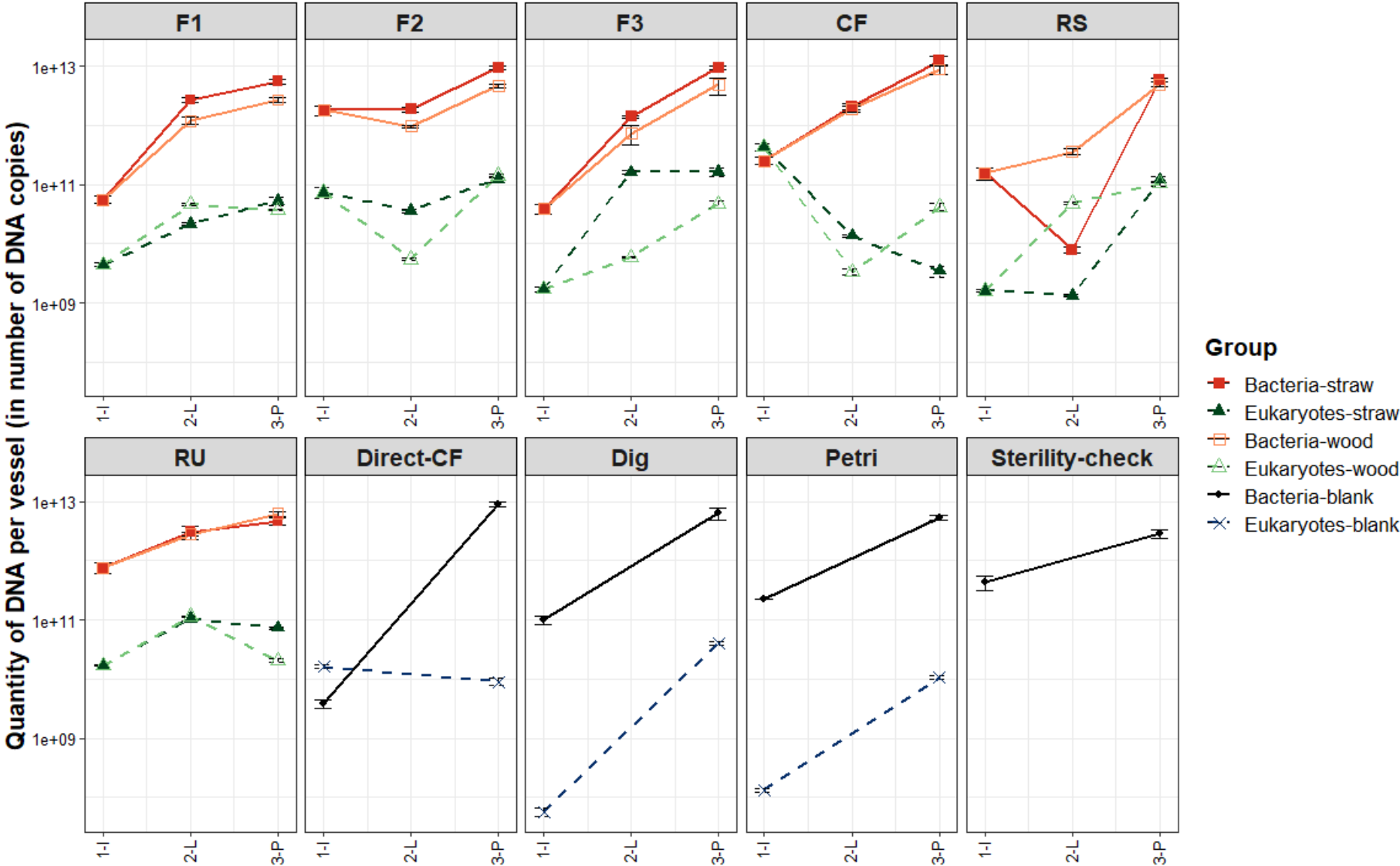


Figure 45: Evolution of vessel DNA quantities as a function of the experiment step and the initial environment. Bacteria and eukaryotes were separated into different lines. Experiment steps distinction within each initial environment is following this nomenclature: (1-I) Initial environment; (2-L) End LAMACs step; (3-P) End propagation step.

3.4. Evaluation of the impact of consortia addition on efficiency of short-term aerobic post-treatment – Ecosystem Function relationship

Figure 46 displays BMP results for plant A sterilized SD that were subject to short-term aeration post-treatment with selected consortia or blank solutions obtained from the propagation step. First, BMP value of control ($164 \pm 11 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1} \text{ VS}$) is in the range of BMPs previously obtained in chapter III ($146 \pm 10 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1} \text{ VS}$), showing that drying and sterilization had no significant effect on methane potential. Water blank (water instead of consortia) is significantly 10% lower than control ($148 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1} \text{ VS}_{\text{initial}}$). It shows that remaining endogenous microorganisms consume organic carbon and still do not display specific ligninolytic activities as previously observed in sections 3.3 and 3.5 of chapter III.

After short-term aerobic treatment with addition of consortia selected on straw, average BMP was at $144 \pm 7 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1} \text{ VS}_{\text{initial}}$. It was significantly lower than the control value, by 14%. For consortia selected on wood, average BMP value was at $143 \pm 12 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1} \text{ VS}_{\text{initial}}$, not significantly different from the control value. Similarly, all 4 blanks (Direct-CF, Dig, Petri, Sterility-check) tested had BMP comprised between 156 and $164 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1} \text{ VS}_{\text{initial}}$, not significantly different from the control.

Here, it is important to underline that the 11 mL of liquid consortia added had a soluble COD that were estimated to give an average maximal theoretical amount of 50 additional mL CH_4 . As it is a liquid solution, we can arbitrarily assume that anaerobic biodegradability is close to 80% (Moletta et al., 2008). Therefore, for inoculated BMP tests, an additional $28 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1} \text{ VS}_{\text{initial_dig}}$ is expected and final value should be close to $190 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1} \text{ VS}_{\text{initial_dig}}$. However, none of the BMPs tests containing liquid consortia reach this value and obtained values were even lower or similar to control.

From these considerations, it can be concluded that selected consortia did not improve the short-term aerobic post-treatment. This is likely to be due to unspecific activities of selected consortia toward the lignin-like fraction of SD. During the aerobic post-treatment, respiration of easy to degrade fractions (e.g. sugars, proteins, amorphous cellulose) has occurred, leading to lower methane yield in comparison to untreated SD.

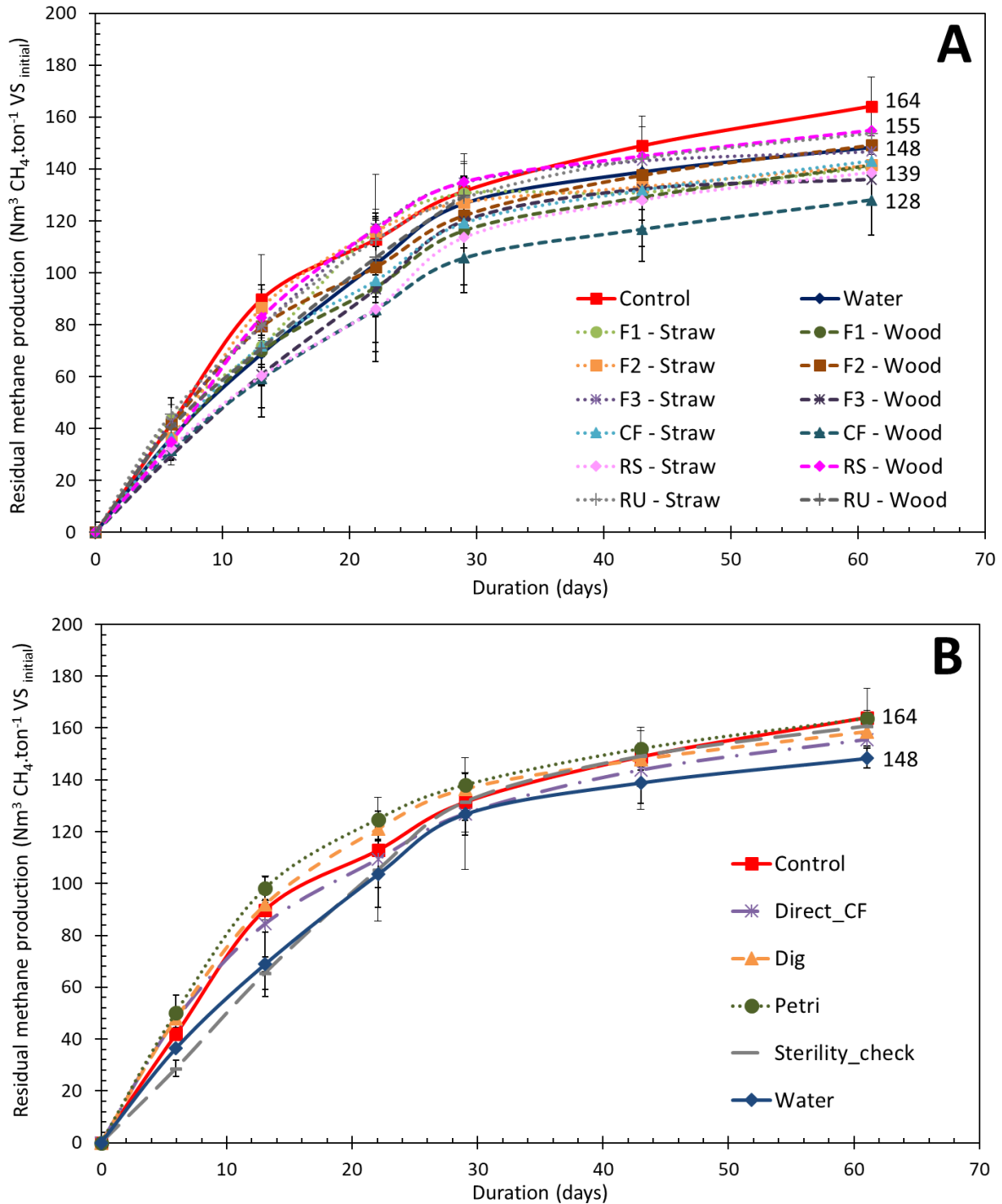


Figure 46: Residual methane potential curves from BMP tests. (A) gathers mostly BMP tests results from SD treated with consortium solutions coming from the LAMACs, while (B) gathers only BMP tests results from SD treated with blanks.

3.5. Discussion

In this chapter, we have implemented an original way to select aerobic consortia potentially displaying specific ligninolytic activities. However, ecosystem function relationship trials clearly indicate that the obtained liquid consortia were not able to specifically degrade the lignin-like fraction of SD during a short-term aerobic treatment. Several reasons have been identified that could explain such results:

- (i) Microbial lignin degradation requires a multiplicity of oxidative enzymes and heterogeneous small molecule co-factors that are produced by ligninolytic fungi and bacteria (Brown and Chang, 2014). Currently, lignin biodegradation in nature is thought to occur in two main stages that consist in its depolymerization followed by the mineralization of resultant heterogeneous aromatics (see **figure 47**) (Kamimura et al., 2019). If bacteria are reported to be dominant and the most active in the mineralization step, it is not the case for the depolymerization step (Kamimura et al., 2017). Indeed, filamentous Basidiomycetes white-rot fungi were identified as major actors in this step due to their capacity to produce high quantities of various oxidative enzymes (e.g. laccase, lignin peroxidase...) (Leonowicz et al., 2001). In comparison to fungi, identified ligninolytic bacteria (e.g. *Pseudomonas sp.*, *Rhodococcus sp.*) have significantly lower activities during the depolymerization step (Kamimura et al., 2019).

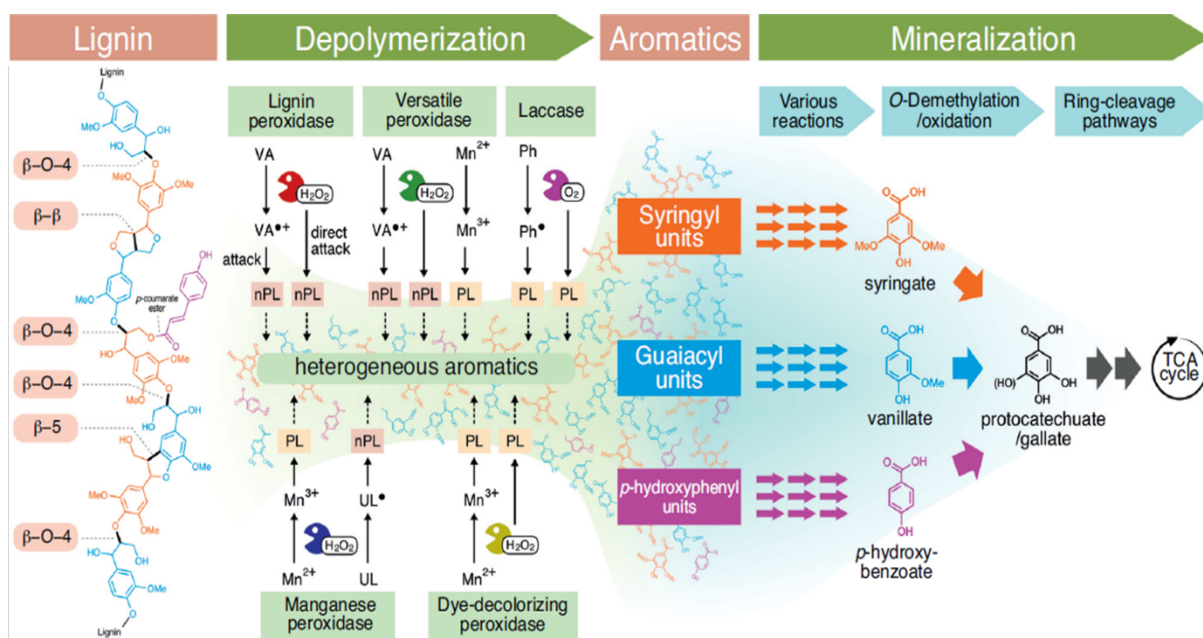


Figure 47: Schematic representation of the microbial lignin degradation system adapted from (Kamimura et al., 2019).

For this study, depolymerization of the SD lignin-like fraction is sought as aromatic units can be converted to methane during AD (Healy and Young, 1979). Assuming that most depolymerization activity is coming from dominant microbial species, it is interesting to look at those that were enriched through experiment steps. For Sordariomycetes fungi and in general for Ascomycete, it is reported that lignin depolymerization rate is slower than for white-rot fungi due to difference in enzymatic systems (e.g. lack of ligninolytic Class II enzymes) (Eichlerová et al., 2015). More generally, fungi obtained after propagation steps were mostly Ascomycetes. Basidiomycetes that were notably present in initial forest environments disappeared after the LAMACs step. One hypothesis is that liquid state during enrichment with Kraft lignin (R1) favored the grow of Ascomycetes over Basidiomycetes. Indeed, Basidiomycetes, due to their filamentous nature, are reported to grow better during solid state fermentation (Asina et al., 2017). For

bacteria, *Paenibacillus sp.* used in a consortium treating pulp and paper wastewater were reported to be able to degrade and metabolize the higher molecular weight lignin molecules (Wang et al., 2013). However, except for RU, Direct-CF and sterility-check, their abundances were relatively low. Finally, Chitinophagia, the most significantly enriched bacteria, are not ligninolytic strains. Instead their enzymatic activities are directed toward carbohydrates degradation (e.g. deconstruction of dead fungal material via chitin hydrolysis) (McKee et al., 2019; Xu et al., 2018). Thus, dominant fungi and bacteria that were enriched through this experiment are not reported as efficient actors in lignin depolymerization, notably in comparison to white-rot fungi.

- (ii) The designed approach to obtain consortia was quite unconventional in comparison to other existing studies focusing on screening of microbial ligninolytic consortia. Erlenmeyer successive transfer cultivation or sequential batch reactors containing the targeted final lignin rich substrate are often preferred (Fang et al., 2018; Lazuka et al., 2015; Liang et al., 2018). With these kinds of cultivation strategies, it is possible, notably during transfer steps, to perform precise ligninolytic activity monitoring measurement (microbial sampling, enzymatic activity, percentage of degraded lignin...). However, with the LAMACs, a system that operates in a continuous mode, reactors were closed and such activity monitoring on solids was not possible. It was thus not possible to determine during the experiment if long term LAMACs operations were sufficient to obtain reduced, stable as well as ligninolytic active microbial communities. Afterwards, it appeared that it was not the case and earlier monitoring would have been beneficial to potentially further select consortia.
- (iii) From our knowledge, studies on microbial ligninolytic consortia are always using either very classical lignocellulosic biomass, such as straw or wood, either industrial lignin derivatives (Asina et al., 2017; Fang et al., 2018). In both cases, cellulose, hemicellulose and lignin are the main molecules present. In comparison to these lignin-rich substrates, SD composition is more diverse and contains higher amount of proteins, sugars, lipids that could provide opportunity for other microbial activities than ligninolytic ones (Maynaud et al., 2017). This is rendering even more challenging the realization of specific ligninolytic activities as consortia might reorient their activity towards easier to degrade fractions. It can be hypothesized that during the short-term aerobic post-treatment, OTUs present in applied consortia and able to efficiently metabolize proteins, sugars or lipids may have outcompeted ligninolytic species with slower metabolisms (such as Sordariomycetes or *Paenibacillus*). It would have been interesting to test the obtained consortia on more classical substrates (straw, wood) to only evaluate their ligninolytic activities.

4. Conclusions

In this chapter, an original screening method for aerobic ligninolytic consortia was implemented. Within an automated continuous LAMACs system, various initial environments were enriched using Kraft lignin, then microorganisms were selected on their capacity to attach quickly and grow on wood or straw. Selective pressure was operated as alpha diversity was reduced. Besides, initial microbial communities appear to converge towards a common structure as dissimilarity distances decreased. A subsequent propagation step ensured growth under liquid conditions of all selected consortia. After these consecutive steps, Sordariomycetes fungi and Chitinophagia bacteria were the two dominant classes of microorganisms that were significantly enriched in most samples.

Finally, addition of these consortia did not increase the efficiency of short-term aerobic post-treatment of SD. Slow or lack of lignin depolymerization activity for dominant selected microorganisms, difficulty to monitor evolution of consortia ligninolytic activities during LAMACs steps and specificity of SD substrate (e.g. containing proteins) that may generate competition with other types of enzymatic activities were identified as potential reasons explaining lower methane yields obtained in comparison to the untreated control. To conclude, this chapter highlights the difficulties to constrain microbial consortia toward specific ligninolytic activities in the presence of a complex substrate, where lignocellulose structure is mixed with easier to degrade organic material.

Regarding these results, it was decided to shift our strategy and use pure culture of basidiomycetes fungi. Indeed, they should display higher lignin depolymerisation capacities and lower side activities (proteolytic) than obtained microbial consortia in this chapter. Therefore, the chances to specifically degrade the lignin-like fraction of SD may be greater.

Transition

PhD research workflow

PhD objective: Gain insights into the impact of solid digestate (SD) recirculation on agricultural biogas plants.

Three main research axis*: (A) Strategies and impact

(B) Post-treatment

(C) Full-scale

*(Associated questions → Chapter 1 section 5)

Chapter III

Answer to research axis Strategies and impact and partly to research axis Post-treatment

- ✓ Three strategies of SD recirculation have been defined: addition, replacement and partial replacement.
- ✓ For the six biogas plant evaluated:
 - A) SD direct recirculation following an addition strategy can increase plant methane production between 0,6 to 5,7%.
 - B) SD direct recirculation following a replacement strategy can offset between 64 to 1431 tons of feedstocks.
- ✓ Thermo-alkaline post-treatment costs are not compensated by potential additional methane gains/capacity offset.
- ✓ SD residual methane potential (RMP) decreased after short-term aerobic post-treatment due to unspecific endogenous activities towards the lignin-like fraction (activity reorientation using Kraft solution was not successful).

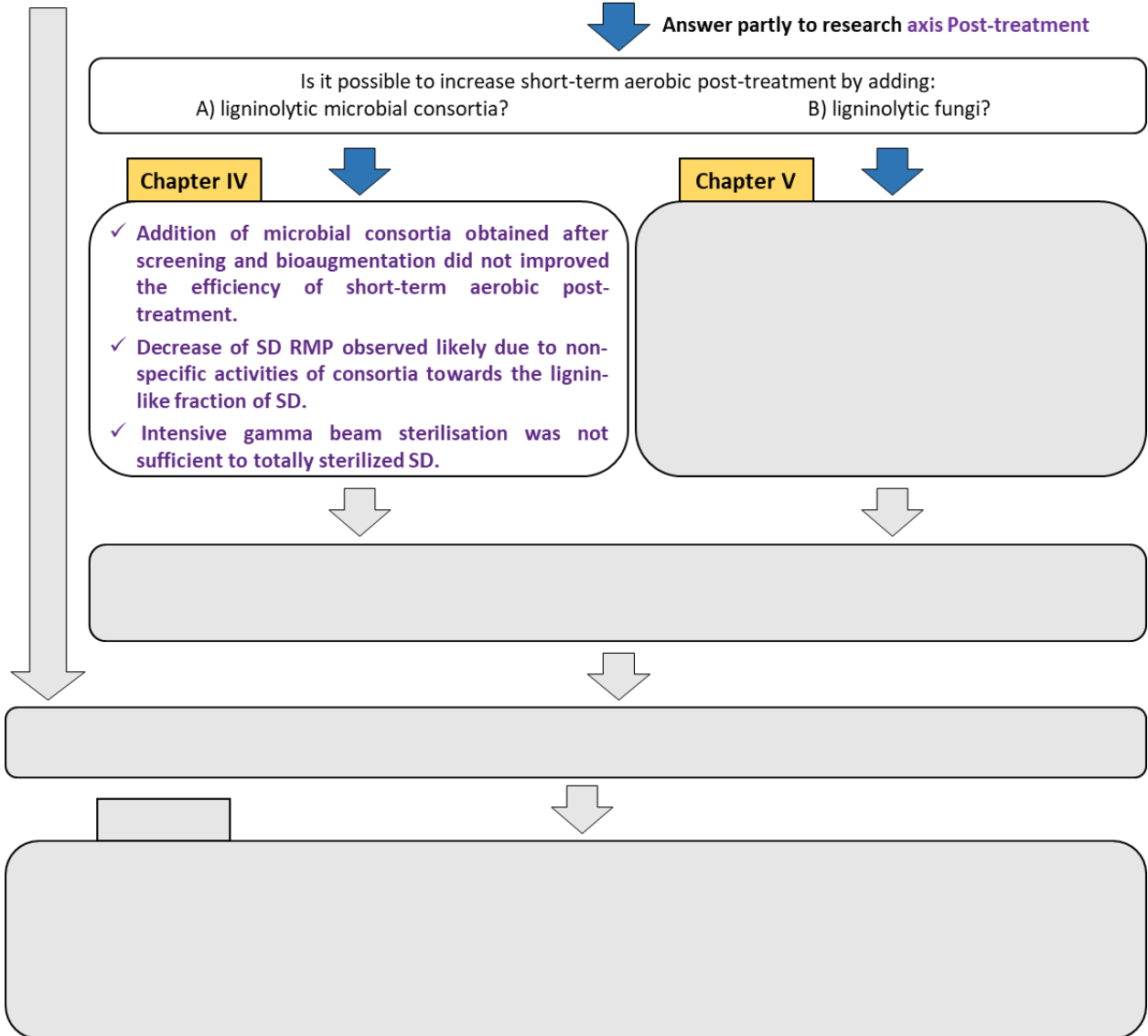
Answer partly to research axis Post-treatment

Is it possible to increase short-term aerobic post-treatment by adding:
 A) ligninolytic microbial consortia? B) ligninolytic fungi?

Chapter IV

Chapter V

- ✓ Addition of microbial consortia obtained after screening and bioaugmentation did not improved the efficiency of short-term aerobic post-treatment.
- ✓ Decrease of SD RMP observed likely due to non-specific activities of consortia towards the lignin-like fraction of SD.
- ✓ Intensive gamma beam sterilisation was not sufficient to totally sterilized SD.



Chapter V

Assessment of fungal and thermo-alkaline post-treatments of solid digestate in a recirculation scheme

Adapted from Brémond et al., Assessment of fungal and thermo-alkaline post-treatments of solid digestate in a recirculation scheme to increase flexibility in feedstocks supply management of biogas plants. Renewable Energy, 2020, 149, 641-651.

1. Chapter objectives

As described in chapter I section 4.2. fungal post-treatment of SD has been recently explored to valorize the remaining carbon into biochemical and value-added products. However, the impacts of fungal growth on SD methane yield has never been evaluated. Several fungal strains are known to be able to selectively degrade lignin and use it for their growth during SSF of straw. They can therefore potentially promote the conversion of lignin concentrated in SD into fungal biomass that will accessible OM for AD microorganisms, which can subsequently produce methane after recirculation. Thus, hypothesis at the origin of this chapter are: (i) ligninolytic fungi would be able to specifically use the complex fraction of SD for their growth and (ii) energy generation from the subsequent AD of the colonized SD will be enhanced.

Two Basidiomycetes filamentous white-rot fungi strains were identified as potentially promising in that view, *Pleurotus Ostreatus* (PO) and *Stropharia rugoso-annulata* (SRA), as they are both robust, little demanding in cultivating techniques and known to efficiently degrade lignin when growing on wheat straw (Chiavari et al., 1989; Jasmina et al., 2017). Besides, their cultivations are well known at industrial scales. Compost mushrooms such as *Agaricus bisporus* were not selected as they display lower ligninolytic activities and are generally more efficient in degrading cellulosic compounds (Wood and Leatham, 1983).

The objective of this chapter is to evaluate an original process scheme where solid digestate coming from an agricultural CSTR biogas plant undergoes a post-treatment, which corresponds to a fungal SSF, before being recirculated back into the biogas plant. Growing conditions for fungal SSF were studied. The impact of fungi colonization over time on methane yield was analyzed as well as the evolution of OM composition, especially the lignin-like fraction. Impact of the sterilization and alkalization, corresponding to a thermo-alkaline treatment alone on SD was also analyzed. Finally, feasibility at full scale of fungal SSF is discussed.

2. Materials and methods

2.1. Solid digestate selected and fungal mycelium

Solid digestate used in this chapter came from plant A. This plant was selected for the following reasons: (i) a ration that is relatively rich in lignocellulosic biomass; (ii) the use of a screw press, a covered storage tank and an intermediate HRT which are representative of the process conditions applied in the agricultural biogas sector (Guilayn et al., 2019b). Information about SD sampling procedure can be found in section 1.1. of chapter II. Besides, details about the biogas plant A can be found in **table 8**, in section 2.1. of chapter III. Finally, information about fungal mycelium ordered and its storage can be found in section 1.2. of chapter II.

2.2. Fungal cultivation method

An isolated wooden box was designed. Inside the box, a system using a vacuum pump KNF Laboport™ (Freiburg-im-Breisgau, Germany) bubbling air in vessels containing water ensured efficient air humidification. Temperature within the box was lowered and regulated using a Minichiller Huber (Offenburg, Germany) and piping system. Temperature and air relative

humidity inside the box were measured via a Hygrasgard® RPFTF – 20 – Modbus sensor from S+S Regeltechnik (Nürnberg, Germany) and values were recorded every 10 minutes via a software developed internally. During experiments, relative humidity was maintained close to 75% while box temperature varied between 20-25°C as a function of the variation of the external temperature with day-night cycle. Finally, the box could be closed ensuring a dark environment. These conditions were inside the range for good substrate colonization according to both *Mycelia bvba* strains factsheets and previous studies (Sánchez, 2009; Wang et al., 2001).

2.3. Screening of fungal solid-state fermentation conditions

Screening was performed to determine SSF conditions on SD of *Pleurotus ostreatus*. Two conditions were applied to SD before inoculation: (i) no treatment; (ii) quicklime (addition of calcium oxide, CaO) at 2% w/w and then autoclave for 30 minutes at 121 °C. Following these treatments, four spawn levels were tested: 5, 10, 15 and 20% w/w of SD. Fungal SSF was carried out by mixing 10 grams of SD, potentially treated, with the corresponding amount of *PO* spawn within Corning-Gosselin® 40mL polypropylene tubes (Borre, France). A control, where SD was replaced by moisturized and sterilized wheat straw, was added to be sure that *PO* spawn was active. Tubes were then placed inside the box in the dark. After 10 days, colonization inside the tubes, due to SSF, were visually assessed according to the following scale: (0) no colonization at all; (+) some area of SD are colonized; (++) SD is totally colonized. Selected SSF conditions for *PO* were similarly applied to *SRA*.

2.4. Evaluation of optimal fungal solid state fermentation duration

Selected conditions for efficient SSF were applied, which consisted of 2% w/w CaO and 20 minutes autoclaving at 121 °C of SD followed by a 20% w/w spawn addition. Following inoculation, different SSF times under aerobic conditions were evaluated. For *PO*, five durations were tested: 5.5, 7.5, 10, 15 and 21 days. The first four ones reach the incubation/colonization phase, while the latter is in the fructifying phase. In that case, the wooden box was slightly opened after day 15 to ensure half-light and trigger fruiting. For *SRA*, three durations were tested: 5.5, 7.5 and 15 days. Two controls were also added, consisting in: (i) untreated SD, *PO* spawn and *SRA* spawn left under aerobic conditions inside the wood box for 5.5 and 15 days (ii) SD quicklimed at 2% w/w limed and autoclaved for 20 minutes, left under aerobic conditions inside the wooden box for 5.5 and 15 days. A summary of the experimental set-up is given in **table 21**. For every 16 conditions, Corning-Gosselin® 40 mL tubes containing 2 grams of VS of corresponding substrates were prepared and placed for the given duration inside the wooden box that was subsequently closed.

Table 21: Experiments performed to evaluate optimal fungal SSF duration

Aeration duration	5.5 days	7.5 days	10 days	15 days	21 days
Tested substrates					
Untreated SD	x3			x3	
CaO + autoclaved SD	x3			x3	
PO spawn untreated	x3			x3	
SRA spawn untreated	x3			x3	
PO spawn + treated SD	x3	x3	x3	x3	x3
SRA spawn + treated SD	x3	x3		x3	

2.5. Respiration assessment

VS loss due to respiration of fungi or endogenous SD microorganisms inside the wooden box was evaluated using TS/VS measurement. Initial VS in each tube was known. At the end of each post-treatment, 3 tubes were first placed at 105 °C to determine TS. Dry samples were then entirely transferred into ceramic crucibles and placed at 550 °C to determine remaining VS contents. Difference between the initial VS and the remaining VS content corresponds to VS loss from respiration.

2.6. Calculations of methane yield and biodegradability

Biomethane Potential (BMP) tests were performed in triplicate to simulate recirculation of SD within the biogas plant after post-treatment for each condition of **table 21**.

Residual methane potential (RMP in mL CH₄.g⁻¹ VS initial) is defined as the accumulated amount of methane produced as a function of the initial VS content of the sample before post-treatment (≈2 g of VS), while BMP of final post-treated matter (BMP_{FPTM} in mL CH₄.g⁻¹ VS final) is defined as the methane produced by the remaining matter. BMP_{FPTM} can be assimilated to biodegradability. It was expressed as a function of the final VS content of the sample after the post-treatment and respiration (less than 2 g of VS). BMP values obtained were compared to two types of control: (i) direct codigestion: untreated SD and spawn are directly anaerobically digested without contact time and (ii) treated codigestion: controls where treated SD (lime and autoclaving) and spawn are separately stored under aerobic conditions for 5.5 or 15 days before being anaerobically digested without contact time. In both cases, proportions between SD and spawn corresponded to identical VS ratios obtained in SSF trials with 20% w/w spawn levels. Overall BMPs for the controls were obtained as follows:

$$BMP_{direct_codigestion} = BMP_{spawn} * [VS]_{spawn} + RMP_{SD} * [VS]_{SD} \quad (16)$$

$$BMP_{treated_codigestion_Xdays} = BMP_{aerated_spawn_Xdays} * [VS]_{spawn} + RMP_{treated_SD_Xday} * [VS]_{SD} \quad (17)$$

3. Results and discussion

3.1. Substrates' features

Several substrate factors are known to affect mushroom growth such as C/N ratio, moisture, pH, microbial load and spawn level (Bellettini et al., 2016). SD was characterized in order to determine if it was a suitable substrate for fungus growth (**table 22**). Moisture and pH were slightly higher than what is usually stated as optimal for growth. In the case of *Pleurotus* species: (i) moisture contents between 50-75% are often targeted as higher levels can favor fungus diseases and microbial competition and (ii) an initial pH of 6.5-8.7 is preferred (Bellettini et al., 2016; Hernandez et al., 2003). However, high nitrogen content (C/N ratio below 40 in the case of synthetic medium) was identified as a potential way to enhance ligninolytic activities of *Pleurotus* species, which was relevant for the tested post-treatment (Knop et al., 2015). It can be noticed that the pH of spawn, also characterized in **table 22**, were around 5.5 due to the fact that mycelium colonization led to an acidification of the bulk growth substrate. Initial trials at different spawn levels (5-20% w/w) for *PO* did not lead to any significant colonization of untreated SD. This can be seen in **figure 48**. This is not due to a problem in *PO* spawn activity, as wheat straw control was partly colonized. Treatment of the SD is thus necessary to allow its colonization by *PO*.

Table 22: Substrate features

Substrate	[TS] (%FM)	[VS] (%FM)	%C/g TS	%N/g TS	C/N	pH
Solid Digestate (SD)	20.5 ±0.3	15.5 ±0.1	33.2 ±0.2	1.7 ±0.1	19.3	9.1
SD quicklime & autoclaved	22.9 ±0.2	15.2 ±0.1	32.4 ±0.2	1.4 ±0.1	23.6	11.4
<i>PO</i> spawn	48.9 ±0.1	43.1 ±1.5	39.7 ±0.3	2 ±0.1	19.6	5.7
<i>SRA</i> spawn	26.7 ±0.5	24.3 ±0.4	31.2 ±0.3	1.4 ±0.1	22.9	5.4

3.2. Determination of fungal SSF conditions

Sterilization appears to be the most important parameter to work on as the microbial load of SD was high and *PO* is reported to be sensitive to bacteria competition during its development (Válková et al., 2017). Results of the qPCR on untreated SD displayed an absolute abundance of 7.3×10^{11} ($\pm 3.9 \times 10^{11}$) numbers of 16S gene copies per gram of fresh matter (FM). This corresponds to an average of 1.8×10^{11} bacterial cells.g⁻¹ FM, which is in the range of values found in literature for similar digestates coming from AD plants (Braun et al., 2011). However, this amount is high in comparison to other growth media for fungi such as bulk soil (10^8 cells.g⁻¹) or even unsterilized straw (10^9 cells.g⁻¹) (Raynaud and Nunan, 2014; Tian et al., 2017).

The sterilization strategy was based on the combination of an alkaline and thermal treatment to reduce microbial load and activity and results are displayed in **figure 48**. Indeed, alkaline conditions appear to reduce activity of competing microorganisms, while mycelium development of *PO* is not greatly affected (Hernandez et al., 2003; Romero-arenas et al., 2012). Therefore, a 2% w/w quicklime powder was used to alkalinize SD as it is a cheap chemical, generally used on farms for agricultural practice, it is more concentrated than slacked lime previously successfully used by Hernandez et al., (2003) and it has an additional delignification effect (Ramos-Suárez et al., 2017). Autoclaving was subsequently performed to

reduce microbial load before inoculation. Following this treatment, moisture was close to 75%, C/N ratio increased slightly, as nitrogen probably volatilized under ammonia form during autoclaving, and pH was alkaline (see **Table 22**). Only a high 20% w/w of *PO* spawn succeeded in colonizing efficiently SD after 10 days of SSF, a white coating was formed around the SD. According to these results, selected growth conditions for *PO* and *SRA* consisted in addition of quicklime (2% w/w), autoclaving and a 20% w/w spawn level. Full-scale feasibility of such a strategy will be discussed thereafter.

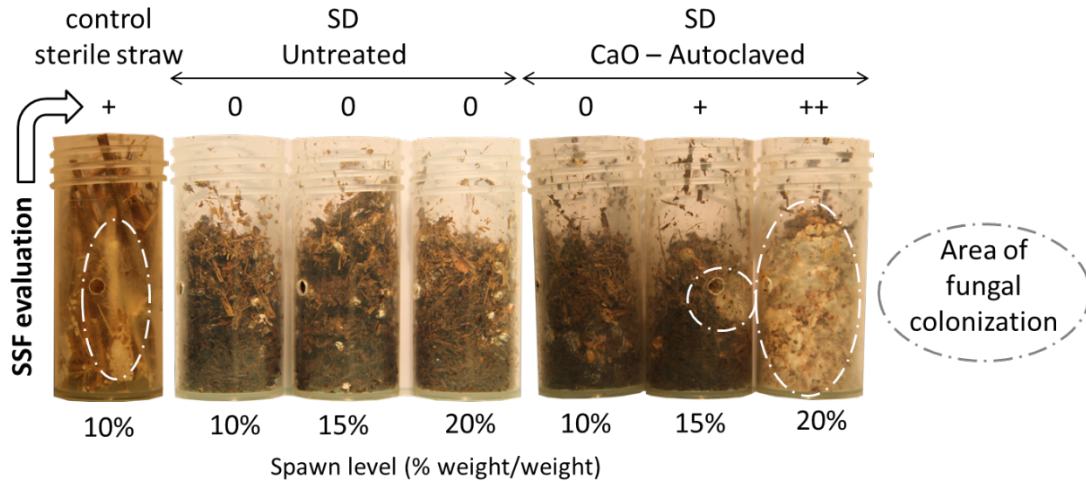


Figure 48: Screening of colonization conditions during SSF of *Pleurotus ostreatus* on solid digestate and visual assessment according to predefined evaluation scale (5% spawn level is not shown as no colonization occurred).

3.3. Impact of fungal SSF duration on volatile solids loss

Selected growth conditions led to colonization of SD by *PO* during SSF. For each SSF duration, from 5.5 to 21 days, all tubes had successful colonization with a mycelium that tightens and forms an increasingly strong coating around the SD in time. Some tubes at 21 days even showed some fructification starting with small carpophores growth at the top of SD. For SSF of *SRA*, growing and SD colonization was less obvious than for *PO*. At 5.5 and 7.5 days colonization was not significant at sight, and at 15 days some white mycelium had developed but it was not comparable with *PO* colonization. To illustrate these observations, some tubes are shown in **figure 49**.

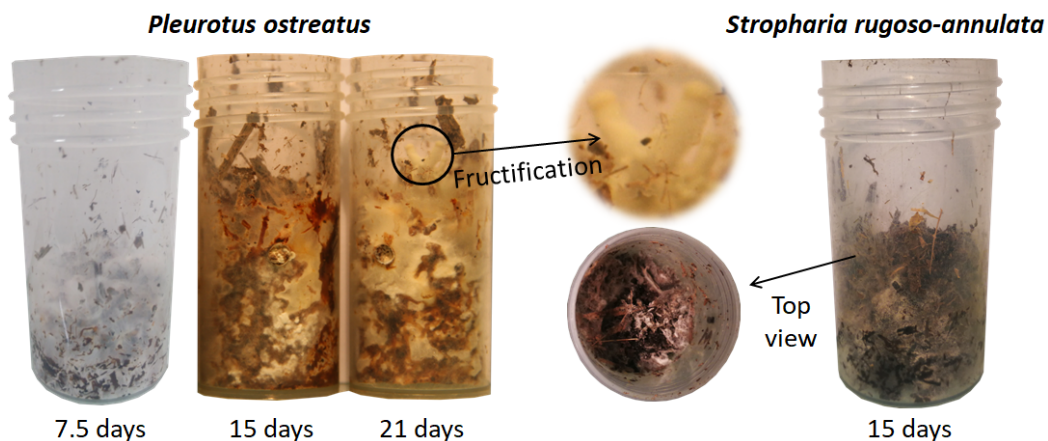


Figure 49: Photographs of solid digestate colonized by *PO* and *SRA* after SSF at different times

The trend of VS loss for each condition was plotted in time in **figure 50**. VS is lost in time for every condition except at 5.5 days in the case of quicklime and autoclaved SD (thermo-alkaline post-treatment). This is probably due to an efficient sterilization and alkaline conditions that suppressed microbial aerobic activity for a few days. However, at 15 days, thermo-alkaline post-treatment led to a VS loss higher than the SD only aerated (19% of VS loss against 12.8%). This is probably due to the fact that this treatment enhanced the amount of easily accessible VS that can be subsequently respired. Aerated spawn controls show VS losses between 13.3-23.5% after 15 days, which can be explained by further colonization and respiration by *PO* and *SRA*, respectively. For *SRA* colonizing SD, VS loss at 5.5 days was higher than the control, probably due to some colonization of SD by *SRA*. Nevertheless, after 15 days, VS loss was similar to the control showing that there were no specifically strong interactions between SD and *SRA* over time. For *PO*, VS loss was always higher than the control reflecting the colonization of the SD by *PO* and a higher activity. VS loss reaches around 20% after 15 days. Similar VS loss was observed in studies using fungi to pretreat biomass before AD (Lalak et al., 2016; Mustafa et al., 2016a; Rouches et al., 2019). VS loss directly impacts methane yield since the VS consumed for respiration is no longer available to be converted to methane.

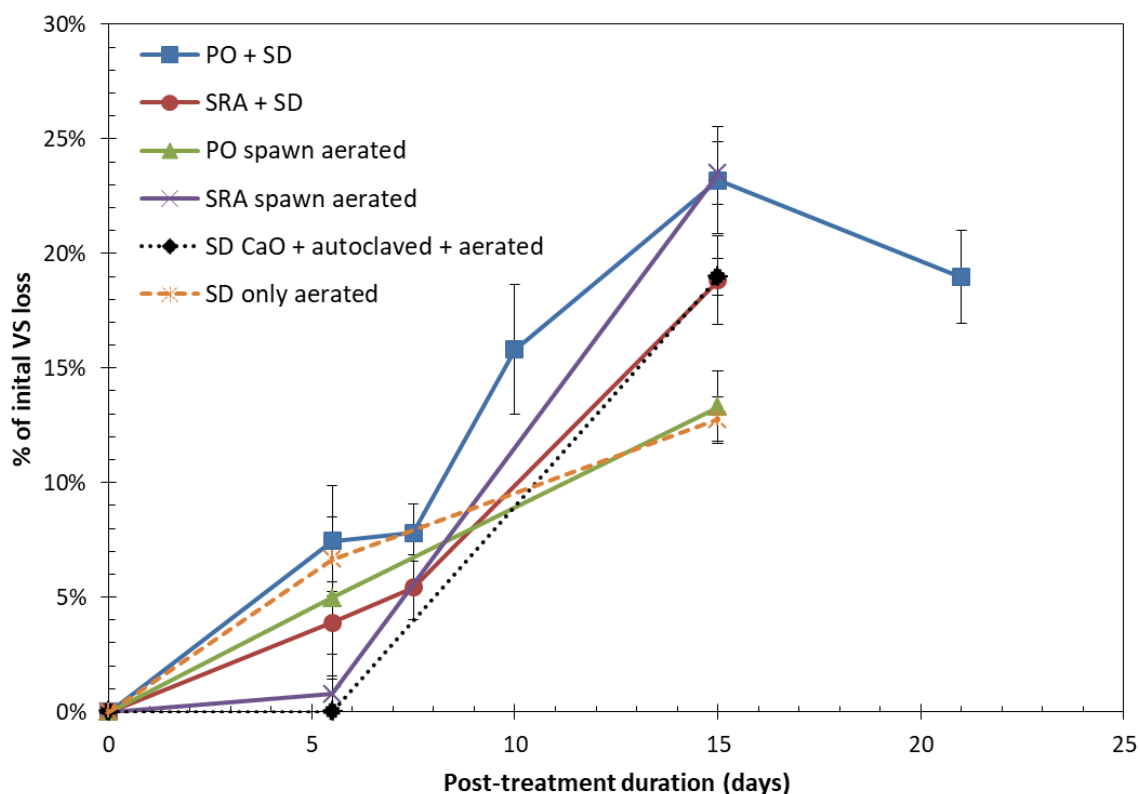


Figure 50: Volatile solids loss in time for the different conditions applied to SD

3.4. Impact of fungal SSF duration on methane yield and biodegradability

Results of *PO/SRA* SSF, thermo-alkaline post-treatment and simple aeration of spawn or SD on RMP and BMP_{FPTM} are gathered in **table 23**. Several observations can be drawn from that: (i) RMP of SD, *SRA* spawn and *PO* spawn only aerated were respectively around 10%, 20% and 30% lower than their corresponding control (no aeration). VS loss was not compensated by a

gain in biodegradability, as BMP_{FPTM} significantly decreased for *PO* (by 25%) at 5.5 and 15 days. This is also the case at 5.5 days for *SRA* (by 17%) and *SD* (by 7.6%), but BMP_{FPTM} value was not significantly different after 15 days. One hypothesis is that endogenous *SD* microorganisms or *PO* and *SRA* used *VS* which was easy to access for their growth/metabolism during this period which can explain the loss in biodegradability generally observed. Lignin-like molecules were not specifically degraded.

(ii) Thermo-alkaline post-treatment followed by a short 5.5-day aeration displayed an enhanced RMP and BMP_{FPTM} of 13% in comparison to untreated *SD*. It is likely that this kind of post-treatment acted on *VS* biodegradability and notably lignin as it was recently observed by Mustafa et al., (2018) on sugarcane bagasse. After 15 days, similarly to only aerated *SD*, RMP was significantly lower (15%) and BMP_{FPTM} was not significantly enhanced. It is likely that endogenous or/and exogenous microorganisms, after a lag time due to sterilization and increased pH, used the additional easy to degrade *VS* fraction that was released during post-treatment for their growth, explaining the biodegradability decline.

(iii) For *PO*, RMP was always lower in comparison to direct codigestion (untreated *SD* and *PO* spawn). The longer the SSF time, the lower the methane yield, with up to 50% methane yield loss after 21 days SSF. Besides, codigestion of separately treated *SD* (CaO, autoclaving and 5.5 or 15 aeration days) and *PO* spawn (5.5 or 15 aeration days) both at 5.5 and 15 days displayed higher RMP. This means that the interaction between *SD* and *PO* during colonization increased methane loss; probably due to respiration of *VS* coming from *SD* by *PO*. Colonization by *PO* of *SD* does not appear beneficial as BMP_{FPTM} is decreased over time, probably reflecting no specific ligninolytic activity of *PO* on *SD*. Similar results were observed by Rouches et al., (2015) for certain fungi strains. In their study, methane yield of pretreated wheat straw was decreased due to carbohydrate consumption by fungi.

(iv) For *SRA*, short term aeration (5.5 and 7.5 days) did not lead to any significant methane loss. However, at 15 days RMP was decreased by 16.8% as *VS* loss occurred without any significant increase of BMP_{FPTM} . Again, *SRA* interaction with *SD* is not clearly shown as methane loss is similar to the codigestion of *SRA* spawn and treated *SD* aerated 15 days separately. The lack of colonization can be due to suboptimal conditions as *SRA* is generally growing on straw, so a pH around 6.5-7.5 and C/N ratio of 50-100 is preferred compared to 11.4 and 24 in our case, respectively. Besides, growth rate is slower than *PO* and 15 days might not be long enough to see a clear colonization (Chang and Hayes, 1978).

Table 23: Volatile solids loss and BMPs results of solid digestate post-treatment

	Sample	Aeration duration	VS loss (% initial VS)	RMP (mL CH ₄ /g initial VS)	% RMP variation	BMP _{FPTM} (mL CH ₄ /g final VS)	% BMP _{FPTM} variation
Treated SD	SD control	0	0	157 (±3)	/	157 (±3)	/
	SD Cao autoclaved aerated	5.5	0 (±0.3)	177 (±2)	13	177 (±2)	+13
	SD Cao autoclaved aerated	15	19 (±0.8)	133 (±4)	-15.2	158 (±5)	Not significant
	SD only aerated	5.5	6.6 (±1)	136 (±5)	-13.3	145 (±5)	-7.6
	SD only aerated	15	12.8 (±1)	142 (±6)	-9.6	160 (±6)	Not significant
Aerated spawn	PO spawn control	0	0	365 (±30)	/	365 (±30)	/
	PO spawn aerated	5.5	5 (±3.5)	258 (±12)	-29.3	271 (±13)	-25.8
	PO spawn aerated	15	13.3 (±1.6)	238 (±3)	-34.8	269 (±4)	-26.3
	SRA spawn control	0	0	203 (±6)	/	203 (±6)	/
	SRA spawn aerated	5.5	6.5 (±0.8)	158 (±8)	-22.2	168 (±8)	-17.2
	SRA spawn aerated	15	23.5 (±1.4)	160 (±5)	-21.2	197 (±6)	Not significant
Treated SD + PO	Direct codigestion SD + PO	0	0	232 (±12)	/	232 (±12)	/
	Codigestion treated separately	5.5	2 (±1.4)	210 (±6)	-9.5	215 (±6)	Not significant
	SD + PO	5.5	7.5 (±2.4)	174 (±8)	-25	187 (±9)	-19.4
	SD + PO	7.5	7.8 (±1.3)	170 (±5)	-26.7	183 (±5)	-21.1
	SD + PO	10	15.8 (±2.8)	143 (±14)	-38.4	166 (±16)	-28.4
	Codigestion treated separately	15	17.2 (±1)	197 (±4)	-15.1	207 (±4)	-10.8
	SD + PO	15	23.2 (±2.3)	149 (±13)	-35.8	184 (±16)	-20.7
	SD + PO	21	19 (±2)	114 (±5)	-50.9	136 (±6)	-41.4
Treated SD + SRA	Direct codigestion SD + SRA	0	0	167 (±3)	/	167 (±3)	/
	Codigestion treated separately	5.5	1.5 (±0.2)	173 (±3)	Not significant	175 (±3)	+3.6
	SD + SRA	5.5	3.9 (±1.4)	158 (±9)	Not significant	164 (±10)	Not significant
	SD + SRA	7.5	5.4 (±1.4)	169 (±8)	Not significant	178 (±9)	Not significant
	Codigestion treated separately	15	19.9 (±0.9)	139 (±4)	-16.8	166 (±5)	Not significant
	SD + SRA	15	18.8 (±1.9)	139 (±7)	-16.8	165 (±9)	Not significant

3.5. Volatile solids fractions: evolution following post-treatments

To better understand the impact of these different treatments on VS, fractionation of 8 samples was carried out and results were gathered in **figure 51**. Impact of fungal treatment on VS was evaluated at 15 days as methane yield was lower both for *PO* and *SRA*. Controls A and C, which correspond to direct codigestion of untreated SD and spawn, allow to have an insight into VS evolution after thermo-alkaline treatment, aeration and fungal colonization. Controls B and D correspond to codigestion of thermo-alkaline post-treated SD and untreated spawn. They enable to distinguish the effect of the thermo-alkaline treatment from aeration and fungal colonization.

Firstly, untreated SD is in range with previously analyzed agricultural SD using the same method, notably with a NEOM close to 45% showing that agricultural SD can accumulate a large quantity of hardly degradable molecules such as lignin (Maynaud et al., 2017). Thermo-alkaline post-treatment (Treated* SD in **figure 51**) had a strong effect on NEOM reducing it by 26% while SPOM content was increased by 123% (see **table 24**). Fluorescence spectroscopy analysis was used to have insight into the SPOM composition and the complexity ratio was slightly increased by 8% (from 1.42 to 1.54) due to higher lignin-like and humic acid-like compounds. This validates our previous hypothesis and indicates that NEOM get more accessible and potentially released soluble compounds as well as embedded holocelluloses explaining the 13% increase in RMP. Mustafa et al., (2018) similarly obtained a delignification of 46% and a decrease in hemicellulose of 83% by a combination of a hydrothermal treatment at 180 °C and addition of 8.5% Ca(OH)₂ on sugarcane bagasse. On rice straw, 5% Ca(OH)₂ combined with 6 hours 80°C thermal treatment also led to 31% lignin and 15% hemicellulose content reduction, as well as a 25% biogas production increase (Du et al., 2019).

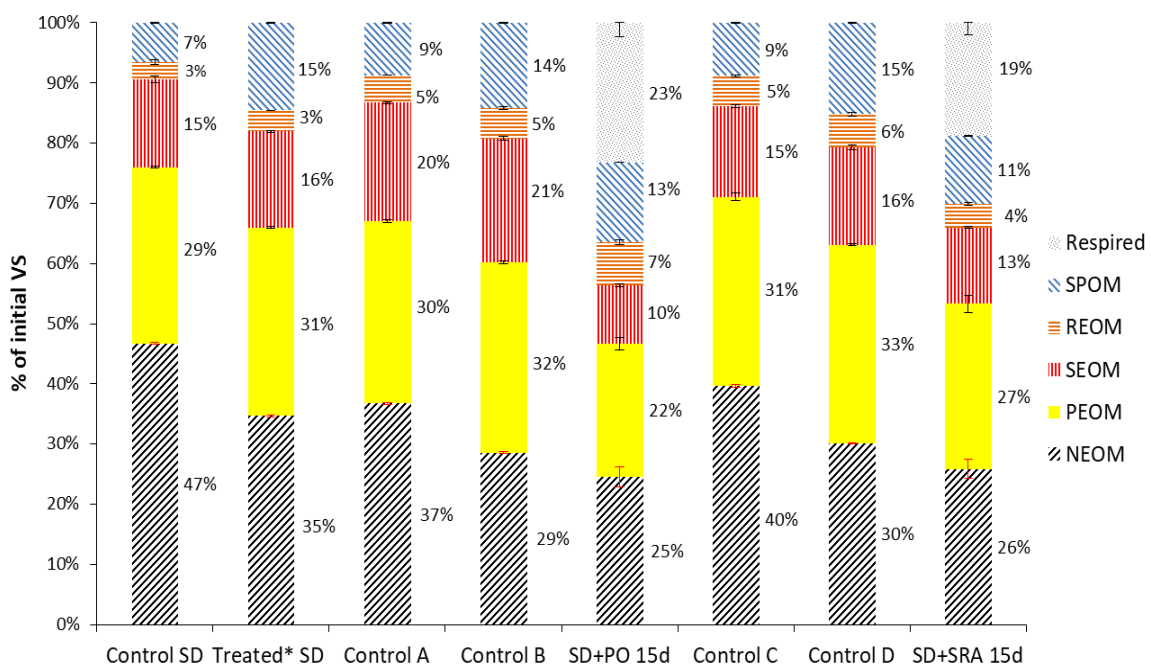


Figure 51: Distribution of the different VS fractions in percentage of initial VS for various samples. (Treated* SD) SD CaO + autoclave + 5.5 aeration days; (Control A) untreated SD and PO; (Control B) Treated* SD + untreated PO; (Control C) untreated SD and SRA; (Control D) Treated* SD + untreated SRA

Table 24: Impact of treatments on the evolution of the different fractions of the VS in comparison to the untreated SD

	SPOM	REOM	SEOM	PEOM	NEOM
Thermo-alkaline post-treatment	+123%	+22%	+9%	+7%	-25,77%
<i>PO</i> colonization	-7%	+44%	-53%	-30%	-14%
<i>SRA</i> colonization	-26%	-28%	-21%	-17%	-14%

Control A and C have lower NEOM contents than SD which can be explained by the fact that *PO* and *SRA* spawns had lower contents of lignin-like molecules. Between Control A and B as well as C and D, decrease in NEOM contents and increase in SPOM contents are due to the thermo-alkaline post-treatment on SD which degrades lignin-like molecules into soluble ones. When comparing control B (treated SD and untreated *PO*) to the colonized digestate with *PO*, it can be observed in **table 24** that *PO* activity was not really selective towards lignin (only -14% for NEOM) but rather proteolytic (-53% for SEOM) and hemicellulolytic (-30% for PEOM). This non-specific activity can explain the measured loss in BMP_{FPTM} and in RMP. Similarly, for *SRA*, activities were not specific as all VS fractions were used (between -14% and -28%). Contrary to *PO*, SPOM and REOM fractions were degraded up to 25%. This could be linked to the activity of endogenous microorganisms such as proteolytic bacteria. It might indicate that *SRA* probably did not entirely colonize SD and is still competing or coexisting with endogenous SD flora.

A hypothesis to explain low lignin selectivity of fungi strains could come from the sterilization process (thermo-alkaline post-treatment) that released soluble compounds such as sugars, which can be then preferentially used by fungi. It has been previously shown that the addition of glucose can limit delignification by fungi (Rouches et al., 2015). Besides, to our knowledge, it is the first time that effects of post-treatments, different from composting, on SD lignin-like content are clearly evaluated using the fractionation method. This method seems particularly adapted to better understand the effect of various post-treatments on SD.

3.6. Correlations between VS fractions and methane yield/biodegradability

Data on RMP (called here methane yield), BMP_{FPTM} (called here biodegradability) from **table 23** and VS fraction percentages from **figure 51**, for control SD, CaO+autoclave+5.5d aeration SD, SD+PO 15d aeration and SD+SRA 15d aeration samples, were used to perform a PCA analysis presented in **figure 52**. Looking at the PCA representation, several observations can be made.

The two major components were sufficient to describe 92.5% of the data variability. Component 1 explains 62% of the total variability. It can be shown that SD biodegradability (BMP_{FPTM}) is negatively correlated with the NEOM content. Anaerobic biodegradability of lignin-like molecules are reported to be low, therefore this correlation appears to make sense. SD control which has the highest lignin content (47% initial VS) has logically the lowest biodegradability (157 mL CH₄.g⁻¹ final VS). Besides, SPOM content appears to be positively correlated with SD biodegradability. On that point, similar observations were already found

on recent studies, pointing that a significant positive correlation exists between soluble content and BMP values (Jimenez et al., 2017; Maynaud et al., 2017). For methane yield (RMP), it can be shown that it is negatively correlated with respiration. Indeed, during respiration VS are lost, that would otherwise give methane.

Regarding these results, a conclusion that can be drawn is that SD post-treatment that aims to improve methane yield SD should avoid too important VS losses due to aeration and increase soluble molecules (SPOM) content while reducing lignin-like (NEOM) content. Fungal SSF post-treatment does not follow these recommendations.

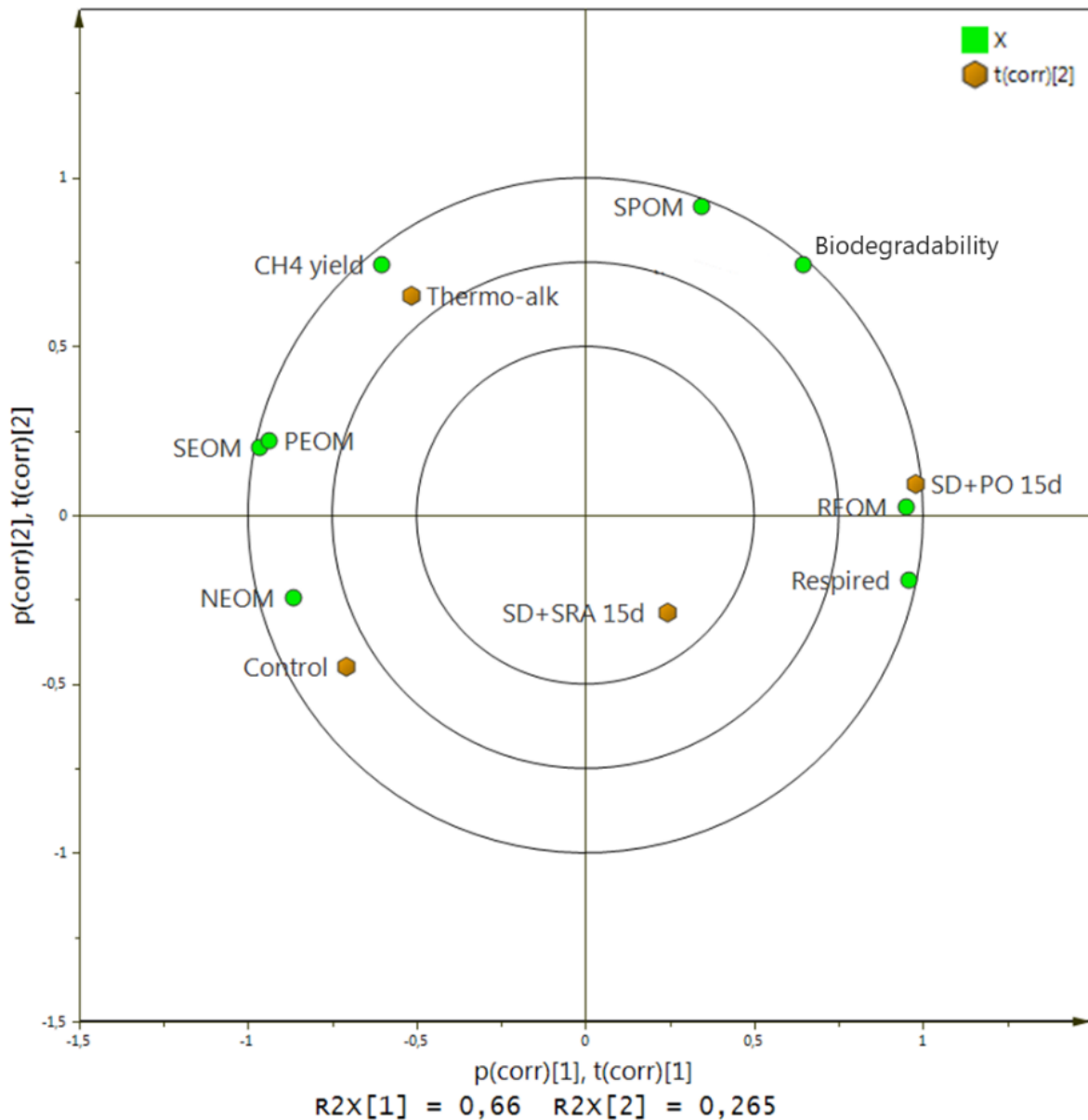


Figure 52: Correlation circle (scores) obtained from PCA analysis of control SD, CaO+autoclave+5.5d aeration SD and fungal post-treated SD (SD+PO 15d & SD+SRA 15d) samples.

3.7. Discussion over feasibility of fungal SSF post-treatment

Within the tested conditions, feasibility of fungal SSF at full scale to enhance methane yield of agricultural biogas plants does not appear as viable. Alternatives to thermo-alkaline sterilization treatment might be explored to reduce costs and potentially get a higher specific activity toward lignin-like fraction: (i) Trials performed directly on-site, using fresh SD from thermophilic plant would be of interest to determine if long term anaerobic and thermophilic conditions can create an available ecological niche in SD for subsequent aerobic mesophilic fungal SSF. (ii) Spawn could be propagated on-site by colonizing low-cost substrate such as weed plants without sterilization (Das and Mukherjee, 2007) or miscanthus pellets (Rouches et al., 2019) in order to apply higher spawn levels at affordable cost. However, it is likely that extensive labor would counterbalance the economic gains. (iii) Explore mild alkaline or acidic treatment without sterilization on SD as lower or higher pH may inhibit endogenous bacterial activity while favoring fungal growth (Zadrazil and Puniya, 1994). Nevertheless, it is foreseeable that optimization of the fungal SSF at full scale will be difficult to manage and remain too costly. Tracks that explore production of higher value products than methane (enzymes, biomolecules...) is likely to be the only way to enhance economic profitability and render viable this strategy.

4. Conclusions

Fungal solid-state fermentation of SD before recirculation into agricultural biogas plants has never been evaluated until now. It appears not to be a viable strategy to enhance energy recovery from SD within the tested conditions. For the two strains studied, sterilization and a high spawn level were needed to ensure fungal colonization. Besides, fungal activities during SSF were not specific to the most complex fraction, leading to uncontrolled VS loss and subsequent decrease of biodegradability and methane yield from SD. Overall, looking at the energetic loss (sterilization and lower methane yield) and the additional economic cost (mycelium and labor) this strategy will not be profitable at full scale. Finally, sterilization process, consisting in a thermo-alkaline post-treatment, showed an increase in energy recovery from SD of 13% due to the reduction of the SD lignin-like content. However, even with optimisation, it is unlikely that additional economic gains from this post-treatment outperforms additional post-treatment CAPEX and OPEX. **Figure 53** gives an overview of the results obtained in this chapter.

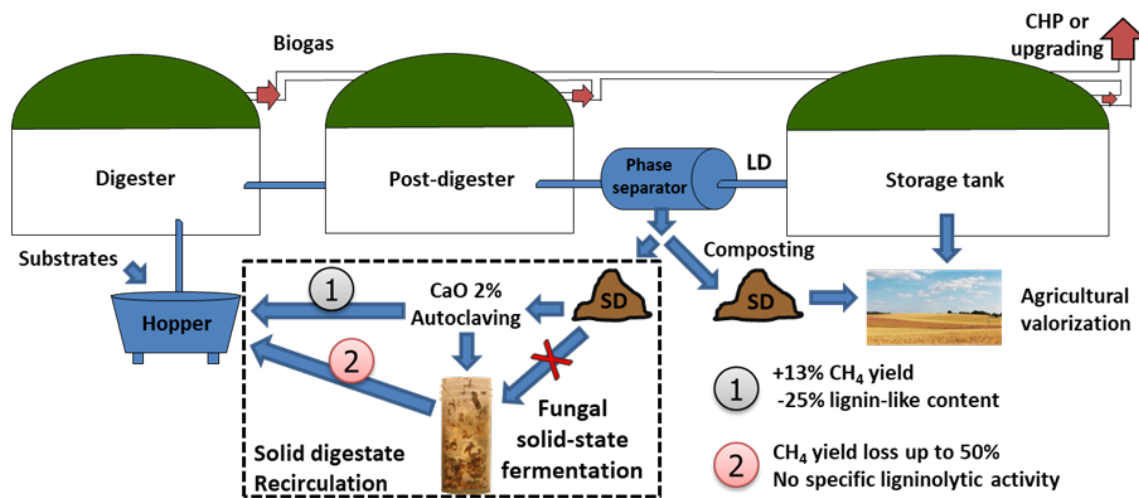


Figure 53: Overview of chapter V results

Regarding results from chapters III, IV and V it is possible to answer to several research questions of the PhD thesis.

- 1) Is it possible to increase the efficiency of aerobic post-treatment by adding ligninolytic consortia or fungal strains?

In both cases, efficiency of short-term aerobic post-treatment was not improved. Addition of obtained microbial consortia or basidiomycetes strains, renowned for their ligninolytic capacities, led to lower SD RMP. This is due to unspecific activities towards the lignin-like fraction of SD.

- 2) What are the impact of aerobic and thermo-alkaline post-treatments on organic matter?

For aerobic post-treatments, when organic matter was characterized afterwards (simple aeration or fungal treatment) it appears that all fractions of organic matter had been degraded. In addition to some reduction of the lignin-like fraction, consumption (conversion to carbon dioxide through microbial respiration) of relatively easy to access organic matter fractions that would have otherwise given methane (proteins, sugars, cellulose and hemicelluloses) were observed. Lower SD RMP are explained by the fact that this loss of easily accessible organic matter due to respiration is not compensated by a sufficient gain in biodegradability from reduction of the lignin-like fraction.

For thermo-alkaline post-treatment, chapter V organic matter characterization shows well that lignin-like fraction is specifically reduced and converted towards more accessible fractions. This explains why post-treated SD RMP is higher.

3) Is solid digestate a suitable feedstock for aerobic post-treatment?

SD in comparison to more conventional lignocellulosic biomass feedstocks has very specific features. On a physical aspect, it is a complex feedstock that notably still contains large amount of proteins. On a microbial aspect, it is a feedstock with a high load and a high diversity of endogenous microorganisms. These microorganisms display a wide range of hydrolytic activities under aerobic conditions. Specific endogenous ligninolytic activities were not observed. Orientation of the endogenous community towards only specific ligninolytic activities are unlikely to be feasible. Therefore, an expensive sterilization step is required to allow colonization by microorganisms that might have more specific ligninolytic activities (consortia, fungi). But, finally even these microorganisms are not specifically degrading the lignin-like fraction. It is likely due to SD organic matter composition that might favor other microbial activities (proteolytic...). For all these reasons SD does not appear to be a suitable feedstock for aerobic post-treatment as potential gain in biodegradability will not compensate respiration loss. If an increase in SD RMP is sought thermo-chemical post-treatments displayed better results.

4) What is the best conditions for SD recirculation implementation at full-scale?

Looking at the obtained results, both addition of aerobic post-treatments (decrease SD RMP) and thermo-alkaline post-treatments (gain in methane yield are not compensating cost) appear to be not worth it in the case of a SD recirculation. Best strategy is finally the simplest: direct SD recirculation. Besides, observations on short-term aerobic post-treatments indicate that SD RMP can decrease relatively quickly under aerobic conditions. Therefore, it is advised to perform recirculation of SD quickly after the exit of the screw press (within maximum a few days) to avoid any potential methane loss. On a daily basis, direct recirculation of a part of the SD produced during the day is likely to be the best solution.

Chapter VI

Conditions for solid digestate recirculation implementation at full-scale: Theory and practice

Adapted from Brémond et al., Recirculation of solid digestate to enhance or maintain steady biogas production at full scale: Strategies and conditions. Submitted to Applied Energy, 2020.

1. Chapter objectives

Regarding results from chapters III, IV and V, we have seen that the best strategy for solid digestate recirculation is the direct one, without any post-treatment. Indeed, tested post-treatments either decrease residual methane yield of SD (biological post-treatment), either their costs are not compensated by potential gains from improvement of SD RMP (thermo-chemical post-treatment). Thus, in this chapter, we will only focus and discuss direct recirculation of SD for agricultural CSTR biogas plants.

In several studies, recirculation of SD at lab scale has shown to be feasible and allowed a stable AD process (Biswas et al., 2012; Jagadabhi et al., 2008; Wagner et al., 2018). However, conditions, at full scale, that are technologically and economically viable need to be clearly defined. The aim of this chapter is first to provide insights into the theoretical aspects of SD direct recirculation and notably its potential impact on viscosity linked with the economic cost of stirring. Both addition and replacement recirculation strategies will be considered and discussed. The second part will describe in details, a full-scale trial where SD direct recirculation following a replacement strategy was implemented. Finally, general conclusions concerning conditions of implementation for SD recirculation for agricultural CSTR biogas plants will be provided.

2. Material and methods

2.1. Impact of SD recirculation on solid retention time and digester TS content

For agricultural CSTR biogas plants, hydraulic retention time (HRT), expressed in days, represents retention time of the liquids in the system and can be calculated according to two ways. The most common and simplest way uses digester and post-digester volumes (RV_{digester} and $RV_{\text{post-digester}}$ in m^3) as well as the feedstock intake ($\dot{m}_{\text{feedstock}}$ in tons/year divided by the days of operation during the year $D_{\text{Operation}}$ in days/year) to calculate HRT ($HRT_{\text{Classical}}$) according to the **Eq. 18**. It is generally assumed that feedstock densities are one and therefore tons can be converted to cubic meters.

$$HRT_{\text{Classical}} = \frac{(RV_{\text{digester}} + RV_{\text{post-digester}})}{\frac{\dot{m}_{\text{feedstock}}}{D_{\text{operation}}}} \quad (18)$$

HRT calculated in table 8 of chapter III are calculated in that way and are close to values provided by operators. However, with this approach, calculations do not take into account the gas production from the feedstock, which leads to inaccurate results. However, in these biogas plants, the amount of biogas produced ($V_{\text{biogas_year}}$ in Nm^3/year) as well as its average composition is known (x_{CH_4} defined as the methane percentage in the biogas). From that, the mass of biogas produced (\dot{m}_{biogas} in tons per year) can be approximated using average methane and carbon dioxide densities (d_{CH_4} - 0.67 and d_{CO_2} - 1.87 kg/Nm^3 at 15°C and atmospheric pressure), according to the following equation:

$$\dot{m}_{\text{biogas}} = \frac{V_{\text{biogas_year}} * x_{\text{CH}_4} * d_{\text{CH}_4} + V_{\text{biogas_year}} * (1 - x_{\text{CH}_4}) * d_{\text{CO}_2}}{1000} \quad (19)$$

This new value can be used to perform a more precise HRT calculation (**Eq. 20**), which takes into account the biogas production and really reflects the time spent in the digester by the

outgoing undigested matter. For this reason, $HRT_{accurate}$ will be used instead of $HRT_{classical}$ in the rest of this chapter.

$$HRT_{accurate} = \frac{(RV_{digester} + RV_{post-digester})}{\frac{(\dot{m}_{feedstock} - \dot{m}_{biogas})}{D_{operation}}} \quad (20)$$

Without any SD recirculation, $HRT_{accurate}$ corresponds to the solid retention time (SRT), also expressed in days. However, when SD recirculation is performed, HRT and SRT will increase but the extent will depend of the strategy selected (addition or replacement).

2.1.1. Direct SD recirculation - Addition strategy

In the case of an addition strategy, HRT will slightly increase due to biogas production from the recirculated SD. The new mass of biogas produced ($\dot{m}_{biogas}(R)$) can be calculated as a function of the percentage of total SD produced that is recirculated (R), according to **Eq. 21** that is based on **Eq. 19**, the amount of SD recirculated determined in chapter III ($\dot{m}_{SD_recirculated}$ that depends of R), the VS content of SD ($[VS]_{SD}$) as well as SD residual methane potential (RMP_{SD} in Nm^3/ton VS). From that $HRT_{accurate}(R)$, can be recalculated using **Eq. 22**.

$$\dot{m}_{biogas}(R) = \dot{m}_{biogas}(0) + \frac{\dot{m}_{SD_recirculated}(R) * [VS]_{SD} * RMP_{SD} * (d_{CH_4} + \frac{(1-x_{CH_4})}{x_{CH_4}} * d_{CO_2})}{1000} \quad (21)$$

$$HRT_{accurate}(R) = \frac{(RV_{digester} + RV_{post-digester})}{\frac{(\dot{m}_{feedstock} - \dot{m}_{biogas}(R))}{D_{operation}}} \quad (22)$$

According to calculations and hypotheses developed in Appendix n°1, **Eq. 23** was found; it allows to calculate the impact of SD recirculation on SRT as a function of the percentage of recirculated digestate (R dimensionless), the efficiency of the separation unit (SI dimensionless), the repartition factor of mass flow between SD and LD (α dimensionless), digester and post-digester volumes ($RV_{digester}$ and $RV_{post-digester}$ in cubic meters), the feedstock intake ($\dot{m}_{feedstock}$ in tons/year), and the mass of biogas produced ($\dot{m}_{biogas}(R)$ in tons/year). Based on a similar framework than **Eq. 23**, impact of recirculation on digester TS content ($[TS]_{digester}$ in ton TS/ton raw digestate) could be calculated according to **Eq. 24** that uses notably the initial TS content of feedstocks ($[TS]_{feedstock}$ in ton TS/ton of feedstock). More details about the obtaining of **Eq. 23** and **Eq. 24** can be found in Appendix n°1.

$$SRT = \frac{(1 + \alpha - R)}{(1 + \alpha) * (1 - R * SI)} * \frac{(RV_{digester} + RV_{post-digester})}{\frac{(\dot{m}_{feedstock} - \dot{m}_{biogas}(R))}{D_{operation}}} \Leftrightarrow SRT = \frac{(1 + \alpha - R)}{(1 + \alpha) * (1 - R * SI)} * HRT_{accurate}(R) \quad (23)$$

$$[TS]_{digester} = \frac{(1 + \alpha - R)}{(1 + \alpha) * (1 - R * SI)} * \frac{(\dot{m}_{feedstock} * [TS]_{feedstock} - \dot{m}_{biogas}(R))}{(\dot{m}_{feedstock} - \dot{m}_{biogas}(R))} \quad (24)$$

For the rest of this chapter, SI and α will be fixed but further details about their validity range for the general formula can be found in Appendix n°1. As we are working with agricultural biogas plants using screw presses that are low-efficiency phase separator, we can use the average mass distribution profiles provided from a recent study on digestate mechanical separation (Guilayn et al., 2019b). Thus, SI can be set at 38% and α at 9 (90% of flow weight goes to the LD and 10% to the SD). Previous equations can thus be simplified to:

$$SRT = \frac{(1 - \frac{R}{10})}{(1 - 0.38 * R)} * HRT_{accurate}(R) \quad (25)$$

$$[TS]_{digester} = \frac{(1 - \frac{R}{10})}{(1 - 0.38 * R)} * \frac{(\dot{m}_{feedstock} * [TS]_{feedstock} - \dot{m}_{biogas}(R))}{(\dot{m}_{feedstock} - \dot{m}_{biogas}(R))} \quad (26)$$

In these equations, R will be set at 30%, 50% and 80% of total SD produced. It can be added that to determine $[TS]_{feedstock}$, we will first use **Eq. 24** and $[TS]_{digester}$ provided by plant operators and obtained without recirculation. Finally, it is interesting to notice that SRT and $[TS]_{digester}$ will be increased to the same extent as the same coefficient is applied for both equations.

2.1.2. Direct SD recirculation - Replacement strategy

In the case of a replacement strategy, $\dot{m}_{feedstock}$ has to be replaced by the new amount of feedstock ($\dot{m}_{1_{feedstock}}$) in **Eq. 22**, **Eq. 23**, **Eq. 24**, **Eq. 25** and **Eq. 26**. This new amount of feedstock can be determined using **Eq. 27** and the feedstock offset ($\Delta_{feedstock}$ in tons per year) calculated in chapter III, that depends on the amount of digestate recirculated. Besides, as feedstock intake is decreased, TS of the initial feedstock ($[TS]_{feedstock}$) and initial biogas produced ($\dot{m}_{biogas}(0)$) is also reduced. It was assumed that TS of the feedstocks and biogas produced decreased linearly as a function of the amount of feedstock intake (homogenous repartition of the impact of feedstocks on TS and biogas production). New calculated values of $\dot{m}_{biogas}(0)$ and $[TS]_{feedstock}$ were used in **Eq. 21** and in **Eq. 26** respectively.

$$\dot{m}_{1_{feedstock}} = \dot{m}_{feedstock} - \Delta_{feedstock}(R) \quad (27)$$

2.2. Full-scale trial

A full-scale experiment of SD recirculation in a replacement strategy was performed on plant A (Digester and post-digester: 4000 m³). The test was carried out for 308 days, the equivalent of 2.5 HRT_{accurate}. During 1.2 HRT_{accurate} (147 days comprised between the 29/10/2018 and the 24/03/2019), which consisted in the control phase, no SD was recirculated within the digester. The quantity of feedstock used every day was known thanks to the hopper and liquid tank values. The ratio of solids incorporated (expressed in total mass of solids incorporated) was fixed during the control period: 28% manure, 33% catch crops, 22% beet pulp and 17% cereal dust. However, quantities incorporated could vary over time. For liquid feedstock (whey and cattle slurry), quantities incorporated could also vary over time.

Then, SD was integrated to the solids ration for 119 days (almost 1 HRT_{accurate}, comprised between the 25/03/2019 and the 21/07/2019) according to this new ratio: 8% SD, 25% manure, 31% catch crops, 20% beet pulp and 15% cereal dust. Again, solid quantities can vary in time but not the ratio that was fixed during the test period. On average, 30% of SD produced daily (1.5 tons out of the 5 tons produced) was picked up and directly poured into the hopper, while the remaining fraction was set apart for composting before land spreading. For the last 42 days, initial feedstock supply was set back (no more SD incorporation). A summary of these different steps is described in **figure 54**.

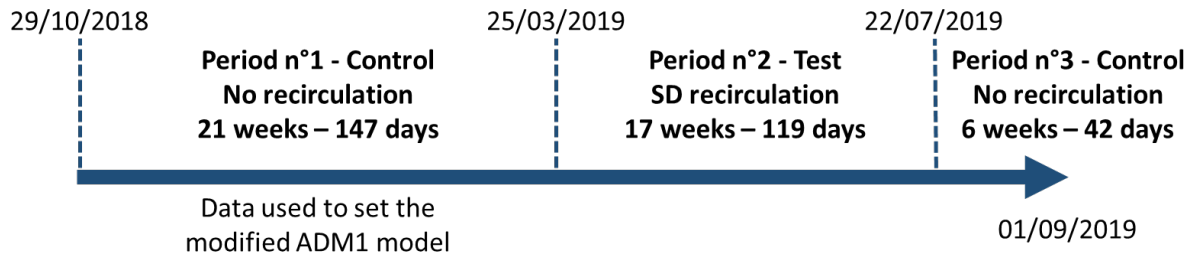


Figure 54: The different periods of the full-scale trial

In addition to the feedstock supply, several other parameters were followed during the experiment to evaluate the impact of SD recirculation. Methane production was measured over time. Extraction yield of the upgrading unit (η_{CH_4}), defined as the ratio of methane molecules valorized in the biomethane after upgrading over methane molecules initially contained in the biogas, was calculated weekly thanks to measurement of: (i) the concentration of CH_4 (in %) in biogas: $x_{CH_4_biogas}$; (ii) the concentration of CH_4 (in %) in the injected biomethane: $x_{CH_4_biomethane}$; (iii) the concentration of CH_4 (in %) in the off-gas: $x_{CH_4_offgas}$ (measured every week). Data were then used in the following equation:

$$\eta_{CH_4} = \frac{((x_{CH_4_biogas} - x_{CH_4_offgas}) * x_{CH_4_biomethane})}{((x_{CH_4_biomethane} - x_{CH_4_offgas}) * x_{CH_4_biogas})} \quad (28)$$

To determine as accurately as possible the total volume of methane produced by plant A in $Nm^3/week$ (M), the following parameters were measured each week: (i) the volume of biomethane injected in the gas grid in $Nm^3/week$ (Q_{bioCH_4}); (ii) the extraction rate of the upgrading unit (η_{CH_4}); (iii) flare operation ratio in hours per week (T_{flare}); (iv) the nominal flow rate of biogas applied to the flare (Q_{flare}) was $370 Nm^3$ biogas/hour; (v) the concentration of CH_4 (in %) in the dry biogas flow rate: $x_{CH_4_biogas}$; (vi) boiler operation ratio in hours per week: (T_{boiler}); (vii) the operating power of the boiler (P_{boiler}) was 150 kWh; (viii) as previously defined, higher heating value (HHV) of biomethane was set at $10.8 kWh/Nm^3$. The total volume of methane produced weekly by plant A corresponds thus to the following equation:

$$M = \frac{Q_{bioCH_4}}{\eta_{CH_4}} + T_{flare} * Q_{flare} * x_{CH_4_biogas} + \frac{T_{boiler} * P_{boiler}}{HHV} \quad (29)$$

A simplified visualization of the different elements used in these calculations is given in **figure 55**. It can be added that all these calculations are required as the biogas plant is not equipped with biogas flow meters.

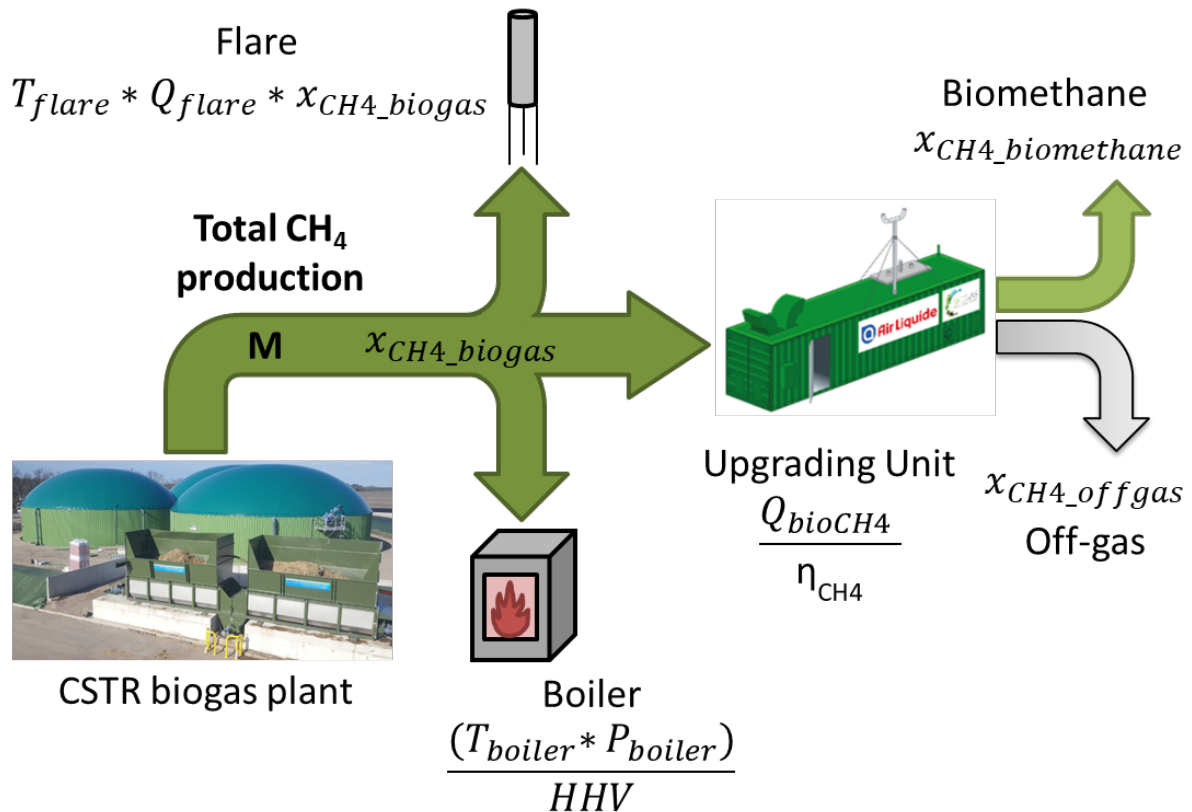


Figure 55: Elements to calculate total methane production from a biogas plant

Monitoring of AD biological parameters in the digester and post-digester was important to evaluate the stability of the process, notably during the recirculation: (i) the pH and FOS/TAC were measured intermittently as a function of the availability of the plant operator. Alkalosis and acidification risks were thus evaluated; (ii) similarly, TS in the digester and post-digester were measured during the control phase, at the beginning of the test phase (recirculation) and at the end of the trial.

2.3. Modelling of biogas plant A methane production based on the ADM1 model

The digestion model used in this chapter is the IWA Anaerobic digestion model No. 1 (ADM1). All equations, matrices and parameters have been previously described in detail (Batstone et al., 2002). The conversion processes modelled are: (i) biochemical processes, considered as irreversible, that corresponds to the AD stages: disintegration, hydrolysis, acidogenesis, acetogenesis and methanogenesis; (ii) Physicochemical processes, considered as reversible, that corresponds to ion association/dissociation (pH) and gas-transfer. The ADM1 model is based on COD balance and takes into account that during AD part of the COD is not degradable under anaerobic conditions. Once parameters are set, it models feedstocks conversion into methane taking into account feedstock anaerobic biodegradability and potential biological inhibitions. It can be implemented to model simple fixed volume CSTR and it will be therefore further used to model AD of biogas plant A.

2.3.1. Modelling of the digester and post-digester

Data from the feedstock supply and methane production from period n°1 (without recirculation) were used to set the initial parameters of the biogas plant A ADM1 model, which

corresponds to the feedstock features. On this period, on average every day, 42.6 tons of feedstocks were sent in the digester, 6.9 tons of biogas were produced from the digester and the post-digester, and 35.7 tons of digestate went out of the post-digester. Methane content of the biogas was in average 60.3% during this period. Using methane density and COD of methane (4 kg COD/kg CH₄), it was possible to determine an average daily content of COD content of biogas. Besides, we know from chapter III and measurements performed from received LD (see section 1.2. of chapter II), COD content of solid digestate (266 kg O₂/m³) as well as liquid digestate (75 kg O₂/m³) produced. Efficiency of the screw press was determined based on plant data. It was set that 16% of the raw digestate flow goes into SD while 84% goes into LD. Knowing daily COD of the biogas and the digestate outlet, it was possible to determine an average daily COD content of the inlet feedstock as well as the digester and post-digester efficiency. Inlet feedstock had a COD content of 335 kg O₂/m³ and plant efficiency was 68% (32% of the feedstock COD content remains into digestate, the rest goes into biogas). **Figure 56** provides an overview of the system. Besides, these estimations are based on several strong hypotheses:

- (i) Densities of solids equal the densities of liquids, it is therefore possible to go from tons to cubic meters. The latter unit will be used for the rest of the modelling step.
- (ii) It was assumed that the storage tank had a fix biogas production of 6% of total biogas produced over time, that was retrieved from the total biogas production of the plant measured.
- (iii) It was assumed that estimated COD contents of SD and LD are constant over time as well as the efficacy of the screw press.
- (iv) There is no change in the quality of the initial feedstocks and their determined features are constant over time (COD content, biodegradability).

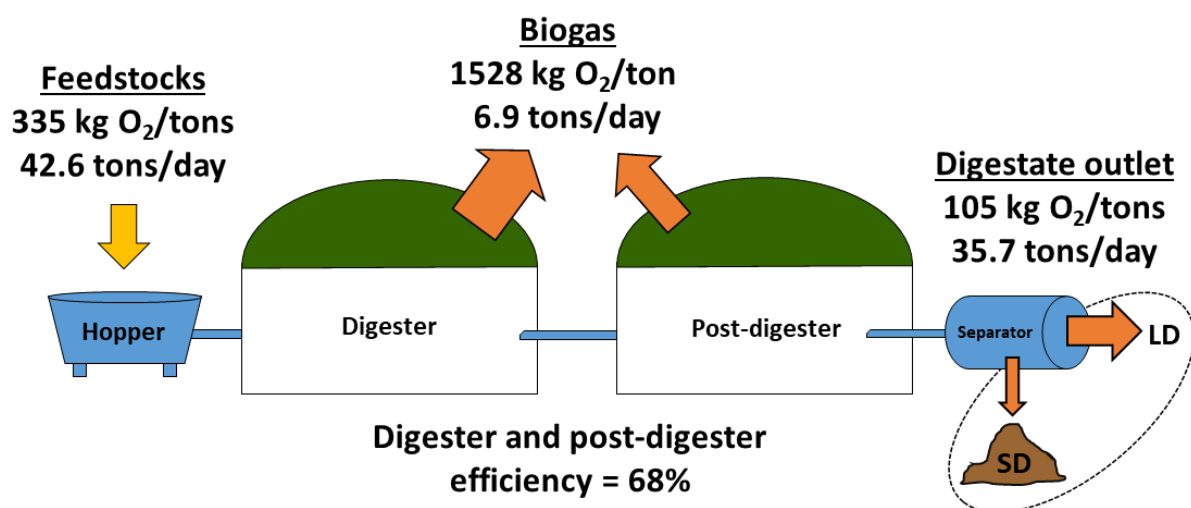


Figure 56: Biogas plant system – mass and COD balance as a function of the different flows

Then, for the 6 feedstocks based on their COD values (COD_{theoretical,x}) found in the literature and internal database as well as their average content in the daily feedstock supply ([x]_{supply} express in % of the total ration), it was possible to determine an average theoretical ratio of

their COD content (COD_{ratio_x}) in the ration using **Eq. 30**, where x corresponds to a given feedstock:

$$COD_{ratio_x} = \frac{COD_{theoretical_x} * [x]_{supply}}{\sum_{x_i=1}^6 COD_{theoretical_x_i} * [x_i]_{supply}} \quad (30)$$

These ratios were subsequently used to recalculate new COD values for each feedstock (COD_{model_x}), based on the previously determined total feedstock COD content (340 kg O₂/m³). Moreover, a percentage of COD biodegradability (%BD_{ANA}) was also attributed to each feedstock based on three different biodegradability categories. Whey was defined as a feedstock with a high biodegradability and it was assumed that 90% of its COD can be converted to methane. Beet pulp, cereal dust and catch crops were defined as feedstocks with an intermediate biodegradability and it was assumed that 62% of their COD can be converted to methane. Finally, cattle slurry and manure were defined as feedstocks with a low biodegradability and it was assumed that only 32% of their COD can be converted to methane. These values were also selected in order to obtain a biodegradability of the feedstock supply of 68%, similar to the value previously determined for the digester and post-digester. Again, selection of these values are strong hypothesis for the model, but they were required as no information were available about feedstocks used in plant A. All these values are provided in **table 25** and were further used in the ADM1 model.

Table 25: Feedstocks features, and associated model parameters

Feedstocks	Whey	Cattle slurry	Beet pulp	Cereal dust	Catch crop	Manure
Type of feedstock	liquid	liquid	solid	solid	solid	solid
[x] _{supply} (% total feedstock supply)	47.22	11.88	8.97	7.73	12.97	11.23
COD _{theoretical_x} (g O ₂ /kg m ³)	75	107	336	1,057	315	280
COD _{ratio_x} (% of feedstock supply COD)	15.25	5.46	12.98	35.18	17.59	13.54
COD _{model_x} (g O ₂ /kg m ³)	108	154	484	1,522	454	403
%BD _{ANA} (% of COD _{model_x})	90	32	62	62	62	32

In our case, ADM1 model was slightly modified, compared to the initial one, based on previous work (Jimenez et al., 2014). First, the disintegration step was removed. Then, it was assumed that each feedstock has a fraction of its COD content that is non-biodegradable (it corresponds to 1-%BD_{ANA}), while the rest of its COD content can be converted into dissolved organic matter. Then, based on previous works and database, a specific content in soluble sugars (S_{sugar}), proteins (S_{amino_acids}) and lipids (S_{fatty_acids}) was attributed to the dissolved organic matter of each feedstock. Values are provided in **table 26**.

Table 26: Feedstocks and associated modified ADM1 model parameters; DOM stands for dissolved organic matter.

Feedstocks	Whey	Cattle slurry	Beet pulp	Cereal dust	Catch crop	Manure
S_{sugar} (% total DOM)	80	67	76	80	80	32
S_{amino_acids} (% total DOM)	14	28	22	20	20	53
S_{fatty_acids} (% total DOM)	6	5	2	0	0	15

From that point, which corresponds to end of the hydrolysis, each soluble fraction is further converted into biogas through modelled acidogenesis, acetogenesis and methanogenesis stages. Biomass growth, conversion of death biomass into methane (that corresponds to the endogenous production) as well as potential inhibitions are taken into account and modelled. Based on plant A features, initial pH of the system was set at 8.1, HRT was set at 100 days and the temperature at 40°C. All other parameters (e.g. kinetic) were set according to IWA recommendations (IWA, 2002). Modelling under dynamic conditions was performed (feedstock supply varies every day) using the MATLAB software (Mathworks, 2019). Finally, **figure 57**, gives a simplified overview of the modified ADM1 model used to model digester and post-digester AD.

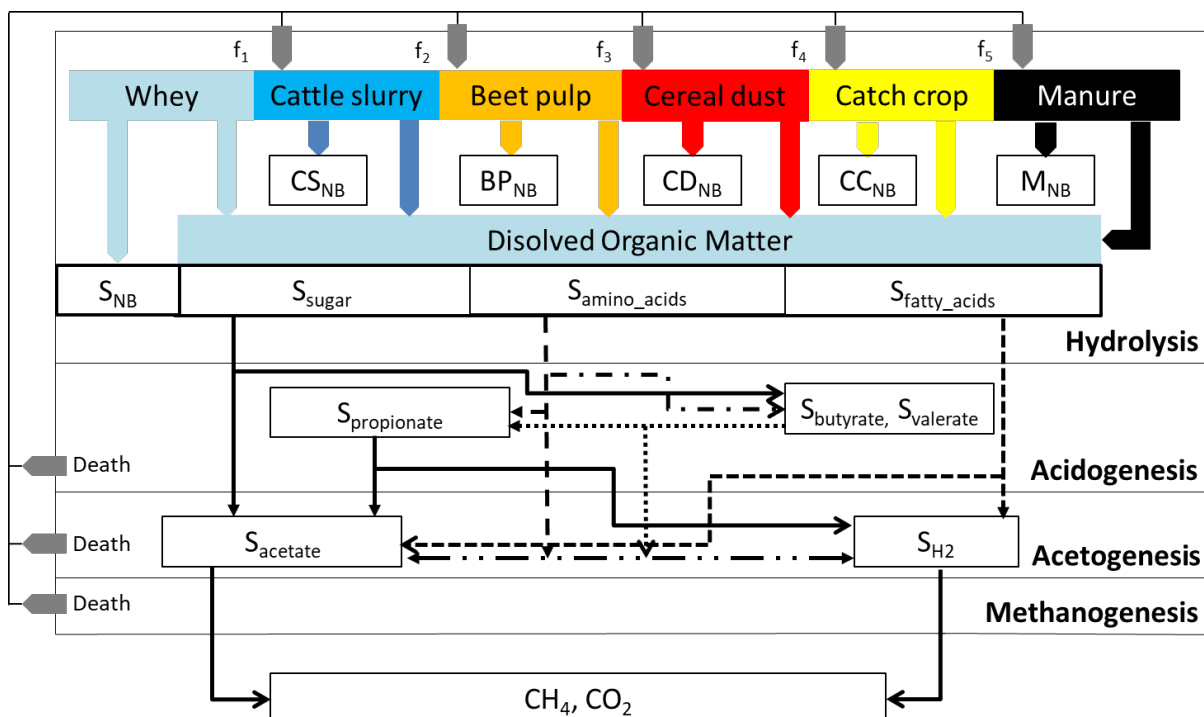


Figure 57: Modified ADM1 simplified representation scheme adapted to model digester and post-digester AD; NB stands for non-biodegradable fraction; S stands for solubles

2.3.2. Modelling of the storage tank

Storage tank is covered and biogas production from the LD can be recovered. However, contrary to the digester and post-digester this tank is not heated and thus biogas production can vary as a function of the outdoor temperature. Thanks to sensors placed within the storage tank, it was possible to record LD flow based on liquid height variation and internal temperature. Here, storage tank was modelled as a fed-batch. Based on experimental data on LD from biogas plant A (TS: 8.5% of FM, VS: 4.5% FM, BMP: 154 Nm³/ton VS), as well as internal database containing organic matter fractionation details for agricultural biogas plant LD, it was possible to provide to the ADM1 model a total percentage of biodegradability (BD_{ANA} corresponds to 24%) and the features of each LD organic matter fractions (% COD total, content in non-biodegradable COD and various ratios of S_{sugar} , S_{amino_acids} , S_{fatty_acids}). **Figure 58**, provides a simplified overview of the modified ADM1 model adapted to the storage tank AD.

As previously, pH was set at 8.1 but a temperature threshold was implemented and below 20°C, methanogens were inhibited. Besides, all other parameters (e.g. kinetic) were set according to IWA recommendations (IWA, 2002). Modelling of the storage tank under dynamic conditions was performed (LD amount in the tank varies every day) using the MATLAB software (Mathworks, 2019).

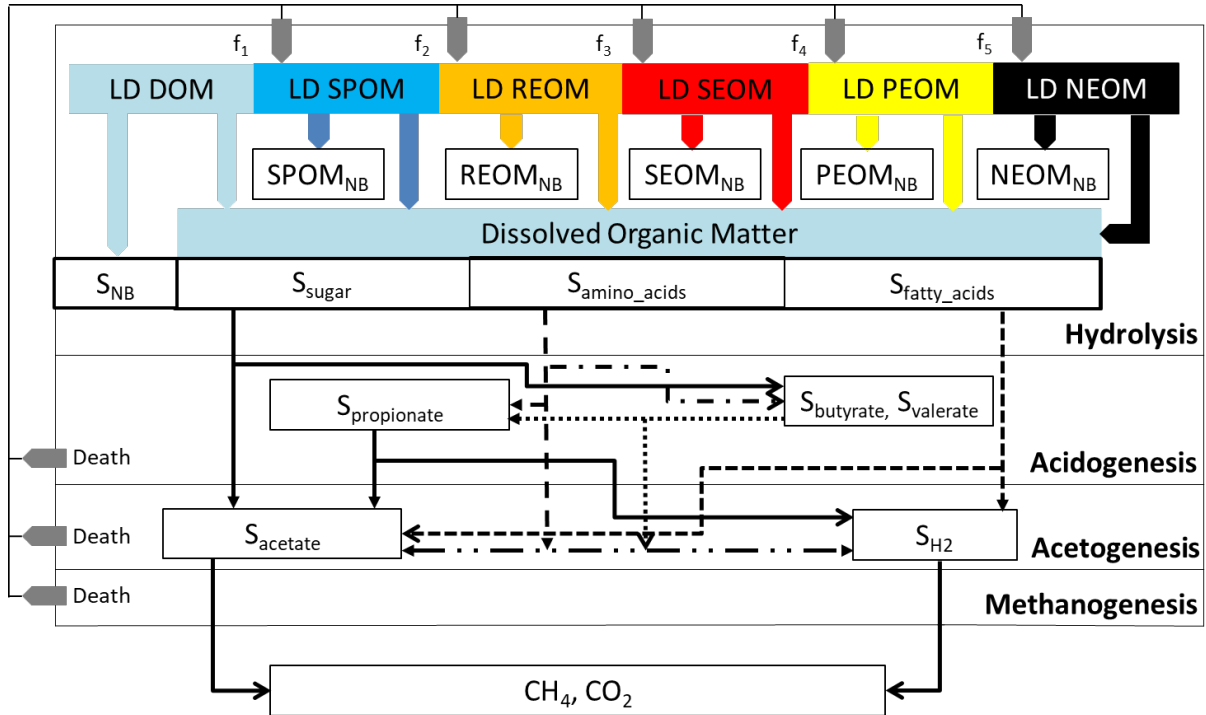


Figure 58: Modified ADM1 simplified representation scheme adapt to model storage tank AD; LD, stands for liquid digestate, NB stands for non-biodegradable fraction; S stands for solubles.

3. Results

3.1. Considerations over SD recirculation impact on HRT, SRT, digester TS and mixing cost

Evolution of CSTR plant HRT_{accurate} and SRT as a function of the recirculation applied strategy and for three different percentages of SD recirculated (30-50 and 80% of \dot{m}_{SD}) are presented in **table 27**. For the five CSTR biogas plants studied and in the case of an addition strategy, HRT_{accurate} only slightly increase (less than one percent on average) as additional methane production from SD is relatively low in comparison to total methane production. However, for SRT, increase is more significant. SRT are on average 10%, 18% and 33% higher, comparatively to SRT without recirculation, when respectively 30%, 50% and 80% SD are recirculated. For a replacement strategy, HRT_{accurate} and SRT improvements are slightly better in comparison to an addition strategy as initial feedstock supply was reduced. Thus, comparatively to HRT_{accurate} and SRT without recirculation, HRT_{accurate} are on average 2%, 3% and 4% higher and SRT are on average 11%, 21% and 38% higher, when respectively 30%, 50% and 80% SD are recirculated.

Table 27: Evolution of plant HRT and average SD HRT as a function of recirculation strategy applied

Plant Name	Plant A	Plant B	Plant C	Plant D	Plant E
RV_{digester} (m ³)	2,000	2,200	2,000	3,700	1,200
$RV_{\text{post-digester}}$ (m ³)	2,000	2,200	2,000	2,700	1,200
$\dot{m}_{\text{feedstock}}$ (tons/year)	13,600	18,000	10,500	25,000	20,000
$HRT_{\text{classical}}$ (days)	100	84	130	88	40
$\dot{m}_{\text{biogas}(0)}$ (tons/year)	2,494	3,184	2,547	2,749	1,641
$HRT_{\text{accurate}} = \text{SRT}$ (days) for R=0%	123	102	172	98	45
Strategy	Addition				
HRT_{accurate} for R=30% (days)	123	102	172	99	45
HRT_{accurate} for R=50% (days)	124	102	173	99	45
HRT_{accurate} for R=80% (days)	124	103	173	99	45
SRT for R=30% (days)	135	111	189	108	49
SRT for R=50% (days)	145	119	202	116	53
SRT for R=80% (days)	164	135	229	131	59
Strategy	Replacement				
HRT_{accurate} for R=30% (days)	125	103	173	101	46
HRT_{accurate} for R=50% (days)	126	104	174	102	47
HRT_{accurate} for R=80% (days)	127	105	176	105	48
SRT for R=30% (days)	137	113	190	110	50
SRT for R=50% (days)	148	122	205	120	55
SRT for R=80% (days)	169	139	232	139	63

For both strategies, recirculation of a fraction of total SD produced has a negligible impact on the HRT_{accurate} but allows an increase of the SRT as SD remains longer within the biogas plant. These higher SRT lead to higher methane recovery that can explain the increase in plant methane production previously determined as well as the higher biogas plant efficiency. Finally, it has been reported that 200 days of AD are required to reach 95% methane recovery from most complex feedstock to degrade, such as manure (Muha et al., 2015). Getting plant SRT close to such value via SD recirculation would be interesting for biogas plant treating complex feedstocks (e.g. plant A, B or D) in order to allow a high biogas plant efficiency. However, impact of recirculation on the digester TS content should also be taken into account.

Indeed, digester TS will be modified when SD recirculation is performed. This is due to the fact that in CSTR biogas plants, the volume of the digester (RV_{digester}) and the post digester ($RV_{\text{post-digester}}$) cannot be increased as it is definitively set once constructed. In general, this feature is due to: i) an overflow system that corresponds to a pipeline between the digester and post-digester in which digestate passes from the former to the latter; ii) a sensor system set in the post-digester that switches on the phase separator when a certain height of digestate is reached (generally corresponding to the height of the overflow system) and switches off when it goes down to a lower pre-defined digestate height (e.g. switch on at 5.30m and switch off at 5.20m). This system allows digestate to go from the digester to the post-digester. The localization of the overflow pipeline corresponds to the maximum height that the digestate can reach and thus sets the volume of the CSTR biogas plant. Thus, if SRT is increased and biogas plant volume is fixed TS content of digester will increase.

Table 28 displays the evolution of digester TS as a function of applied recirculation strategy and for different percentages of SD recirculated. It can be seen that for the five CSTR biogas plants studied when the amount of recirculated SD increases, digester TS is also increased for both recirculation strategies. Thus, for an addition strategy, initial digester TS content is increased on average by 7%, 14% and 25% (representing on average +0.8, +1.5, +2.8% increase in initial digester TS value), when respectively 30%, 50% and 80% of total produced SD is recirculated. In the case of a replacement strategy digester, initial digester TS content is increased on average by 6%, 11% and 20% (representing on average +0.6, +1.2, +2.2% increase in initial digester TS value), when respectively 30%, 50% and 80% of total produced SD is recirculated. Slightly lower increases in TS values for the replacement strategy than for the addition strategy can be explained by the removal of some feedstocks from the initial feedstock supply. Such digester TS content increases are not negligible and will modify digestate viscosity and potentially affect digester and post-digester mixing. Digestate rheology and mixing costs need to be further discussed, to better define conditions of application for SD recirculation.

Table 28: Evolution of digester TS as a function of applied recirculation strategy and for different percentages of SD recirculated

Plant Name	Plant A	Plant B	Plant C	Plant D	Plant E
Initial $[TS]_{\text{digester}}$ (%TS)	12.0	10.5	10.5	14.0	7.6
Calculated Initial $[TS]_{\text{feedstock}}$ (%TS)	28.1	26.3	32.2	23.5	15.2
Strategy	Addition				
$[TS]_{\text{digester}}$ for R=30% (%TS)	12.9	11.3	11.3	15.1	8.1
$[TS]_{\text{digester}}$ for R=50% (%TS)	13.7	11.9	12.0	16.0	8.5
$[TS]_{\text{digester}}$ for R=80% (%TS)	15.1	13.1	13.2	17.7	9.3
Strategy	Replacement				
$[TS]_{\text{digester}}$ for R=30% (%TS)	12.8	11.1	11.2	14.7	7.9
$[TS]_{\text{digester}}$ for R=50% (%TS)	13.4	11.7	11.9	15.4	8.2
$[TS]_{\text{digester}}$ for R=80% (%TS)	14.7	12.7	13.0	16.6	8.7

Rheological studies have been historically focused on activated sludge as well as raw manure and it is only recently (since 2010) that several studies have been performed on digestate from agricultural biogas plants (Chen, 1986; Ratkovich et al., 2013; Schneider, 2018). In most of

these studies, agricultural digestate comes from a co-digestion process, and consists in a mix of livestock manure and various biomass that displays a non-Newtonian shear-thinning flow behaviour, frequently modelled via the power-law model (Garuti et al., 2018; Hreiz et al., 2017; Kress et al., 2018; Mbaye et al., 2014; Montgomery et al., 2016; Schneider, 2018). A study performed in a full-scale plant using an in-line viscometer showed that when the TS content of the digester increases, apparent viscosity of digestate also increases. Associated power-law flow-behaviour model for this biogas plant digestate shows that at a low shear-rate (10 s^{-1}), a shift from 11.2% TS to 11.7% TS in the digester (+0.5% TS) increases apparent viscosity of digestate by 35% (Mönch-Tegeger et al., 2015). Similarly, digestates with higher TS content were also reported in other studies to have a higher viscosity than digestates with lower TS content (Kamarád et al., 2013; Mbaye et al., 2014). However, contrarily to manure or activated sludge, it has been shown that the TS content only was not sufficient to provide a reliable estimation of agricultural digestate rheological properties (Björn et al., 2018). Indeed, agricultural digestate has a complex structure with notably a large quantity of particulate matter. Additional parameters have to be taken into account to fully determine rheological properties of agricultural digestate such as: particle size, particle size distribution and gel forming structure (e.g. mucilage) (Schneider, 2018). To illustrate the impact of these additional parameters on viscosity, it has been reported that size reduction of solids via mechanical treatments could decrease digestate viscosity (Liu et al., 2019; Tian et al., 2014). Thus for SD recirculation, it is not possible, knowing only the increase in TS content, to precisely determine to which extent the digestate viscosity will be increased, but a significant increase is expected.

According to a long-term study performed on a full-scale research biogas plant, on average 4% of the total electricity produced (CHP unit) is used for mixing (Naegele et al., 2012). This value corresponds to 1.6% of the total methane produced with an electrical efficiency of 40% and is in the same range as potential gains or capacity offset in methane production (0.61 to 6.25% of total methane produced). If this value is used as an example and specific to one digester, it shows that mixing energy consumption is not negligible and should be taken into account when evaluating SD recirculation strategy. If TS content and digestate viscosity increase following SD recirculation, it is likely that the mixing energy consumption increases as well. But again, it is difficult to determine precisely and even more to generalize to which extent energy consumption will increase. As an example, it was shown that for 13 full-scale biogas plants, the average TS content during operation was not correlated with mixing energy consumption and biogas plant since the highest TS content did not display the highest mixing energy consumption (Singh et al., 2019). This can be explained by the fact that mixing energy consumption is not only depending on TS content as well as digestate viscosity but also on the type of mixers, their numbers, the type of impellers, the agitation frequency and the agitation speed (Singh et al., 2019). All these parameters can greatly vary from one biogas plant to another and therefore sensitiveness of mixing energy consumption to an increase TS is also likely to be very variable as a function of the biogas plant.

Regarding these conclusions, the impact of SD recirculation implementation on digestate viscosity and mixing cost can be very variable from one biogas plant to another. Therefore,

interest of SD recirculation has to be determined on a case-by-case basis. Nevertheless, regarding potential increase in digester TS content displayed in **table 28**, recirculation above 50% of total SD produced is likely to be unreasonable and not economically beneficial. Interesting potential trade-off, between additional methane or offset capacity and higher mixing cost may lie in low to medium recirculation percentage values ($R \leq 0.5$).

3.2. A potential approach to determine the interest of SD recirculation for a given biogas plant

To evaluate the interest of SD recirculation for a given biogas plant, the following approach can be followed. First, the operator has to determine the VS content and RMP of its SD as well as the annual quantity of SD produced from the phase separation system ($\dot{m}_{SD\text{year}}$). From that, potential gains/offset capacity can be calculated. Then, potential TS increase as a function of the % of SD recirculated (R) can be calculated based on average $[TS]_{\text{digester}}$, annual quantity of feedstock incorporated ($\dot{m}_{\text{feedstock}}$), annual quantity of biogas produced (\dot{m}_{biogas}) and using **Eq. 24** or directly **Eq. 26** if a screw press is used. New calculated digester TS values should be compared with the working range of installed mixing equipment. **Table 29** gives an overview of the estimated upper TS content range that a certain type of mixer can handle. It can be seen that, except submersible motor mixers with propeller, most of the existing mixing technologies can handle TS contents above 10%. If new calculated digester TS are in the working range, the operator may determine (from the historical monitoring of the plant) what could be potentially the impact of such TS increase on mixing cost. When applicable, observations from past periods with similar TS content might be used to assess to which extent electrical consumption or maintenance rate were increased. Besides, the presence of a solid size-reduction device on-site has to be taken into account as it will reduce impact of SD recirculation on digestate viscosity. If the plant operator considers that potential increase on mixing costs will be low enough in comparison to potential gains/offset capacity, then he can start the implementation of SD recirculation.

Table 29: Features of principal mixer types for CSTR adapted from (FNR, 2012; Singh et al., 2019)

Type of mixer	Upper TS content range	Approx. operation speed (rpm)	Installed max. power (kW)
Submersible motor mixer with propeller	8%	500 – 1,500	35
Submersible motor mixer with large-blade rotor	12%	50 – 120	20
Central mixer	12%	12 – 18	25
Paddle mixer	14%	10	30
Shaft mixer	18%	40 – 50	11

It should be mentioned that during the implementation of SD recirculation, as digester TS increases, it is likely that SD production from the screw press will increase as well. Thus, if SD recirculation is performed on a long term basis, it could be expected that the total produced SD in the initial year ($\dot{m}_{SD\text{year}}$) will be lower than the total produced SD in the following years ($\dot{m}_{SD\text{year}+n}$). Therefore, on a long-term, the biogas plant operator should maintain the initial amount of SD recirculated (determined in the first year) instead of recalculating it every year

as a percentage of the new total SD produced ($\dot{m}_{SD\text{year}+n}$). This way, it avoids a progressive increase over time in the amount of SD recirculated and allows a stable recirculation process. Finally, **figure 59** summarizes our findings under the form of a decision diagram for biogas plant operators that would like to implement SD recirculation strategies.

Two examples of the procedure to be adopted regarding potential TS increase and installed mixing system can be provided after discussion with plant A and B operators. For plant A, digester is equipped with 3 Amaprop submersible motor mixer with large-blade rotors (KSB, Frankenthal, Germany). The average TS content in the digester without recirculation (12%) is in the high range of mixing equipment working capacity. At this TS, maintenance operations are already twice more frequent than for the post-digester that has the same mixing equipment but with a lower TS due to biogas production (in average close to 10.5% TS). Therefore, recirculation does not appear for the plant operator as an interesting strategy or it should remain at low level ($R \leq 0.1$). For plant B, digester is equipped with one Flyght 4430 submersible motor mixer with large-blade rotors (Xylem, New York, United-States) and two Biogator HPR 1 paddle mixers (REMA GmbH, Hausen, Germany). For this plant, average TS content in the digester without recirculation (10.5%) is in the low range of mixing equipment working capacity (13 to 14% TS). Besides, presence of a PreMix incorporator, equipped with a shredder RCX Rotacut (Vogelsang, Essen, Germany) may potentially reduce the impact of SD recirculation on digestate viscosity. Here, recirculation can be envisioned as a potential practice as TS in the digester even at a high percentage of recirculation ($R=0.8$) will remain in the working range. The interest in this strategy remains to be determined by the plant operator regarding potential gain/offset capacity and estimated increase on mixing costs.

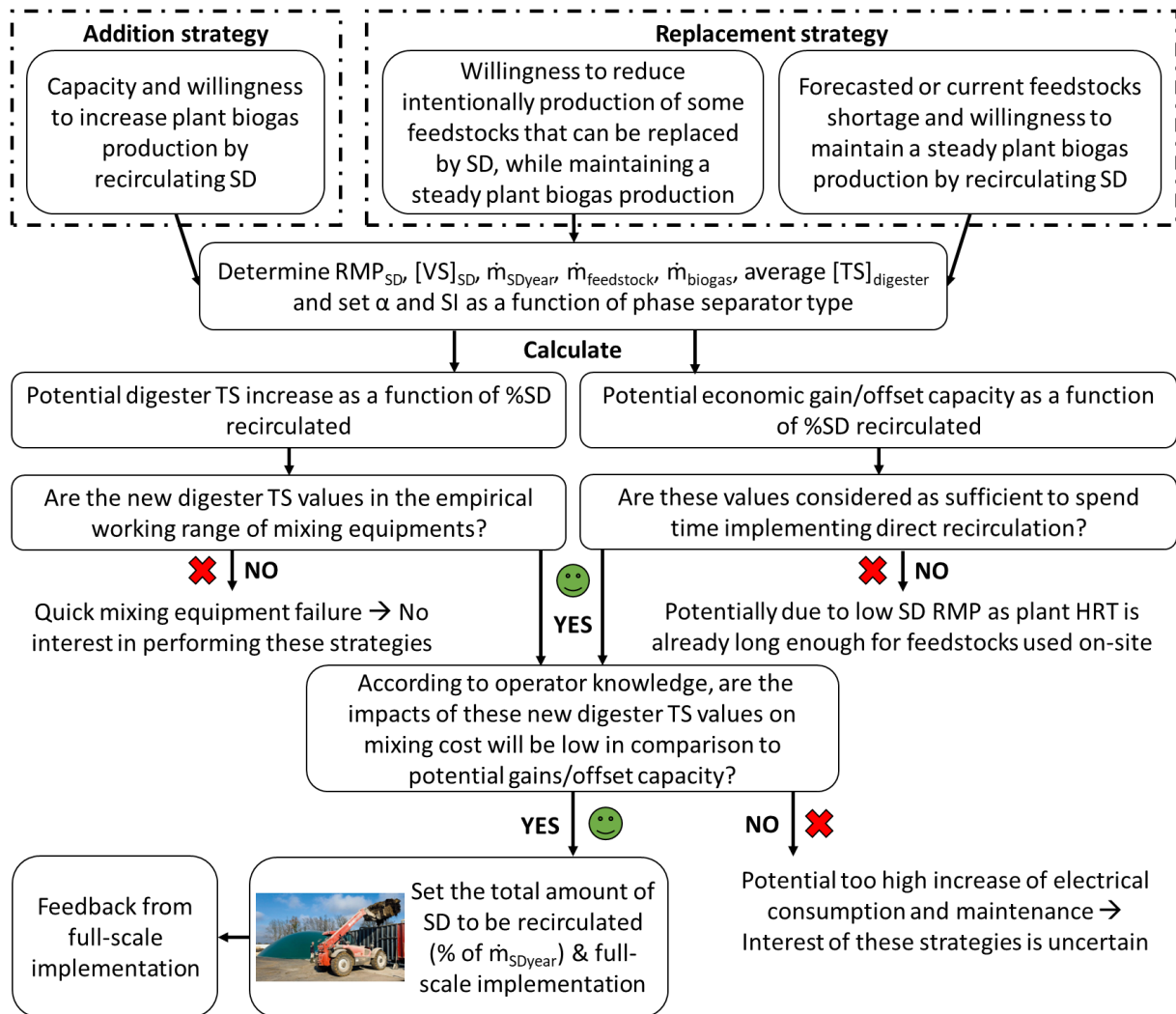


Figure 59: Decision diagram for plant operators to determine if SD recirculation can be an interesting and viable strategy.

3.3. Full-scale trial results

3.3.1. Expected results and recovered data

Plant A operator was interested in testing SD recirculation on his plant as he has never performed it before. However, as previously indicated, potential increase in TS following a recirculation or a replacement strategy was not an option for him. Besides, produce more biogas is not very interesting for him, as his biogas plant is at the maximum of the upgrading unit capacity and during summer he already needs to flare up a part of its biogas production due to gas network saturation. A trade-off was found in the form of the “partial replacement strategy”, and 8% of the weight of plant solid feedstock supply was replaced with the same weight of SD. Here, contrary to a replacement strategy amount of recirculated SD was not higher than the amount of solid feedstock replaced (normally allowing to maintain a constant methane production). Therefore, it is expected that the methane production of the biogas plant will be slightly reduced as SD produces less methane in average than plant A solid feedstocks (e.g. catch crops). Such strategy allows only to limit partially biogas production losses.

Figure 60 displays weekly feedstock supply obtained from whey and cattle slurry storage tanks and hopper as well as calculated weekly plant methane production. On the first period (control n°1) average weekly methane production was almost at 27,000 Nm³, during the second period (recirculation) average weekly methane production was closed to 25,600 Nm³ (5% decrease) and finally during the last period (control n°2) average weekly methane production was at 22,300 Nm³ (13% decrease in comparison to control n°1). This trend is correlated with a decrease in the solid feedstock supply that started from week 19 and an average increase in the liquid feedstock supply. Thus, weekly incorporated solid feedstock supply was reduced on average by 25% during period n°2 and n°3 in comparison to period n°1 (93 m³/week versus 122 m³/week), while, for liquids in the contrary, content incorporated weekly was increased on average by 31% during period n°2 and n°3 in comparison to period n°1 (231 m³/week versus 176 m³/week). In total, 135 m³ of SD were recirculated during period 2, which represents 2.5% of the total feedstock supply and 8% of the solids feedstock supply of this period. Due to this small amount recirculated as well as the variation of the feedstock supply over time, it is difficult to distinguish any potential effects of SD recirculation on the plant methane yield.

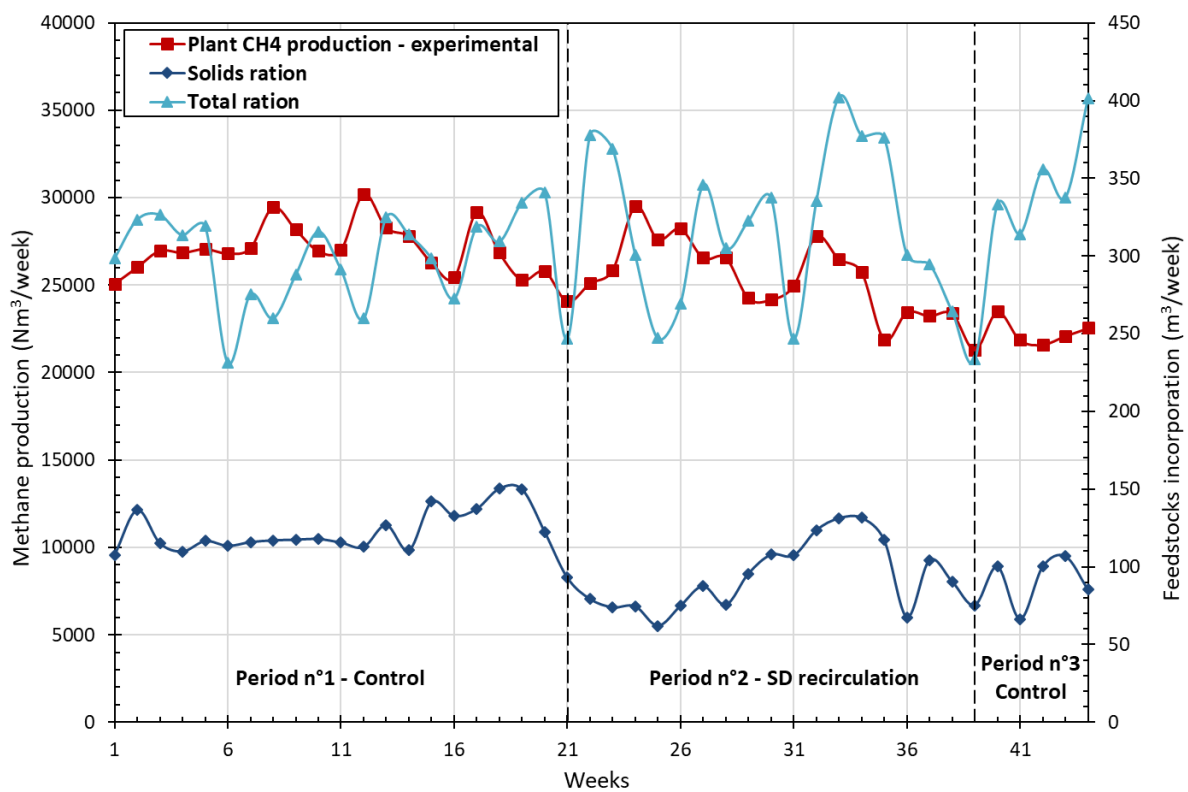


Figure 60: Weekly feedstocks supply and methane production data from full-scale trial on biogas plant A

3.3.2. Modelling of methane production from biogas plant A

In order to have a better insight on the impact of SD recirculation on the methane yield, it was decided to model biogas production of plant A, based on data from period n°1 and several hypotheses that have been described previously (see section 2.4.). The main goal of this modelling is to compare experimental methane production obtained during period 2 with modelled biogas production of the same plant with the same feedstock supply and without recirculation.

In **figure 61**, the experimental and modelled methane production from biogas plant A as well as the feedstock COD load are represented over time. Modelled methane production aggregates production from the digester and post-digester system as well as from the storage tank. Modelled methane production is following the same trend as the feedstock COD load. This is expected as COD conversion of whey, directly located in the DOM, is instantaneous. Besides, COD conversion kinetics for the other feedstocks were set to be fast (days) as it was assumed that with 100 days of HRT, all biodegradable COD will be converted to methane. For the period n°1, it can be seen that the modelled methane production is lower than the experimental methane production (in average 15% percent lower). Besides, variations of the modelled production do not correlate with the variation of the experimental production. Thus, it can be observed that model setting on the first period was not optimal and could only approximately reproduce methane production from plant A. For the period n°3, modelled values and their variations were closer to the experimental values. Finally, on the period n°2, two phases can be distinguished: (i) first, the modelled methane production decreased due to a decrease in COD loading while experimental methane production increased; (ii) COD load is then progressively increased from day 175 leading to higher modelled methane production that meet decreasing experimental methane production. Despite the limits of the model, already highlighted, it appears that at the beginning of the recirculation, experimental methane production does follow an opposite trend to model without recirculation. Methane production values higher than expected are found in reality.

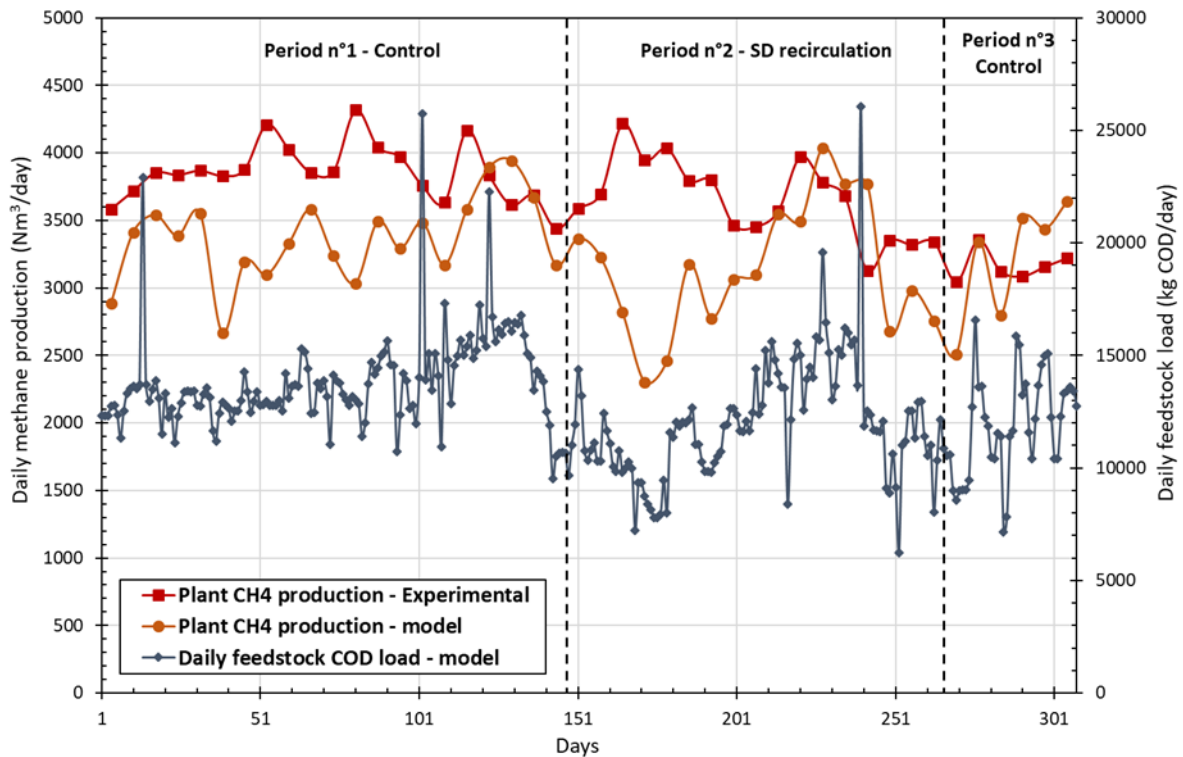


Figure 61: Comparison between experimental and modelled methane production without recirculation for biogas plant A

Another way to illustrate this phenomenon is to calculate the weekly conversion efficiency of the biogas plant, which corresponds to the experimental methane production during the week divided by the estimated theoretical COD content of the feedstock supply incorporated during the week. This ratio was represented over time in **figure 62**. First, it is interesting to see that despite SD recirculation, no decrease on conversion efficiency of the biogas plant can be observed. Indeed, average conversion efficiency value on period n°2 and n°3 (not represented on **figure 62**) is 6% higher than during control period n°1. This indicates that SD recirculation does not seem to have a negative effect on AD as previously reported at lab scale (Biswas et al., 2012; Jagadabhi et al., 2008; Wagner et al., 2018). Besides, at the starting of the recirculation, a peak can be seen that correspond to a strong increase in biogas plant conversion efficiency and on average, the conversion efficiency values during SD recirculation were increased by 10% in comparison to control period n°1. This peak shows that, in reality, a higher anaerobic degradation of the organic matter occurs during this period. The model does not take into account this phenomenon.

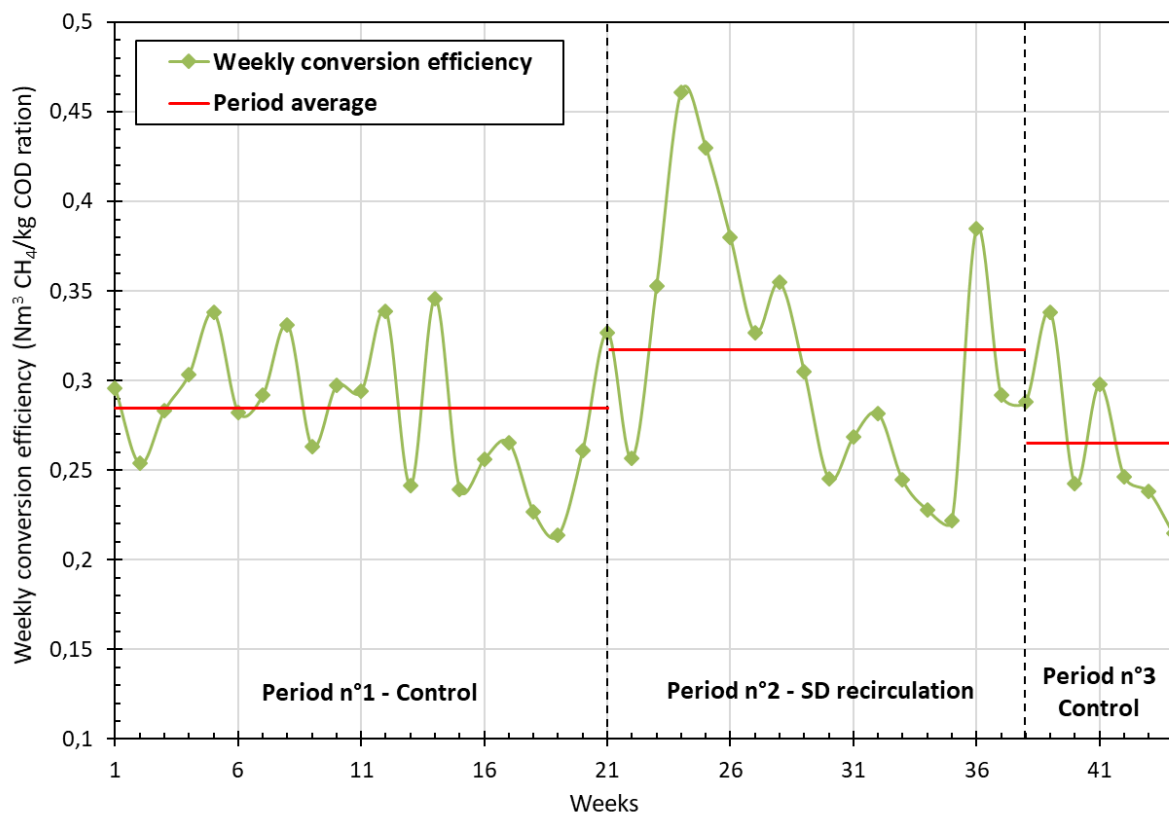


Figure 62: Weekly conversion efficiency of the biogas plant based on a fixed COD content for each feedstock determined from period n°1

Several hypotheses were identified that could explain together or individually these average higher conversion efficiency values:

- (i) The initial hypothesis on a constant feedstock quality is very strong. It is known that feedstock quality on a biogas plant could vary over time (e.g. manure is different as a function of the season and the livestock ration). Such increase could

- be explained by the use of feedstocks that have a better methane potential or biodegradability than initial set values;
- (ii) The storage tank is not heated. Thus, during winter, the biology slows down as temperature is low. However, during spring, increase in temperature can lead to high production of methane from accumulated undigested liquid digestate. This phenomenon was observed several times by the operator in the previous year. Therefore, it was decided to model potential methane production of the storage tank using 20°C as an activity threshold for the biology. **Figure 63** displays modelled methane production, temperature and filling level of the storage tank over time. On average, the modelled methane production from the storage tank represents 5% of the modelled methane production from the digester and the post-digester. It can be seen that in February, and early March storage tank temperature went below 20°C and therefore, no methane was produced. However, slightly before the beginning of the recirculation, temperature went up and a production peak was effectively modelled. This modelled peak (representing 20% of the modelled methane production of the digester and post-digester) is in advance in comparison to the previously described phenomenon that occurred three to four weeks after and is therefore, unlikely to be the cause of it. Thus, hypothesis that the increase in conversion efficiency of the biogas plant is due to the sudden AD of accumulated LD during winter cannot be totally eliminated, but modelling seems to indicate that it is not the cause.
 - (iii) SD recirculation, despite the small amount recirculated, could potentially have a role on conversion efficiency by increasing the microbial load in the digester, as the SRT of the biogas plant is increased. A better AD of sludge have been previously reported when SRT of the reactor was increased that is likely to be due to additional microbial activities towards recalcitrant organic matter (Jimenez et al., 2010). A similar phenomenon could occur in agricultural biogas plant as SD is recirculated and SRT increased. However, we could expect that such phenomenon would be constant over time, but it was not the case as just a peak is observed and then data goes back to the average conversion efficiency values of the control period.
 - (iv) It could also be another phenomenon that affected the biogas plant and that was not reported nor identified.

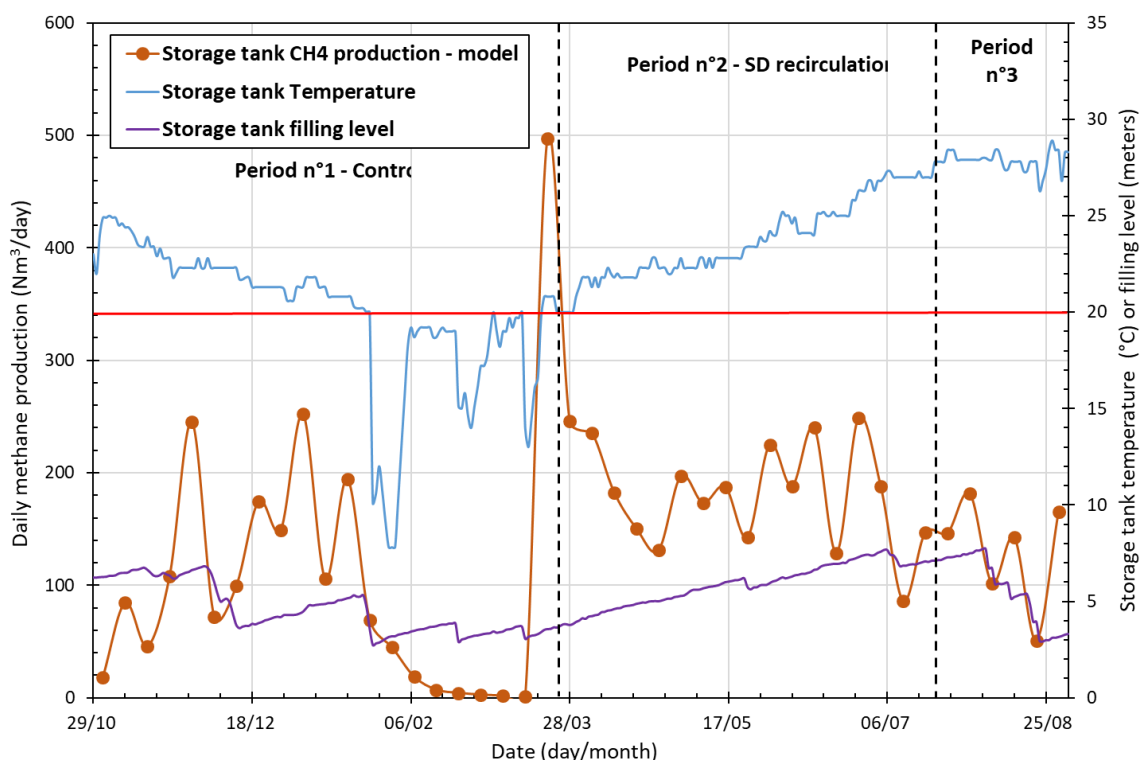


Figure 63: Storage tank modelled methane production, temperature and filling level over time

3.3.3. Additional analysis performed on biology and TS content

Finally, biological analysis presented in **table 30** shows that, during the recirculation period, no significant change was observed in the digester and post-digester in comparison to control periods. Besides, no crash due to acidogenesis or alkalosis was observed during the recirculation period. Besides, TS content was relatively stable close to 12% in the digester and 11% in the post-digester until the end of March. However, diminution in the solid feedstock supply as well as increase in liquid incorporation during period n°2 and n°3 led to a strong decrease in TS content, that was by the end of August at 9.9% for the digester and 8.6% for the post-digester.

Table 30: Average of the biological analysis performed on the digester and post-digester as a function of the period type

	Parameter analysed	Control periods	Recirculation period
Digester	pH	8.2 ± 0.20	8.4 ± 0.14
	FOS (eq. CH ₃ COOH mg/L)	6,800 ± 690	7,060 ± 990
	TAC (eq. CaCO ₃ mg/L)	16,780 ± 2,220	14,800 ± 320
	FOS/TAC	0.41 ± 0.05	0.48 ± 0.07
Post-digester	pH	8.4 ± 0.16	8.6 ± 0.15
	FOS (eq. CH ₃ COOH mg/L)	4,600 ± 570	5,830 ± 1,230
	TAC (eq. CaCO ₃ mg/L)	17,730 ± 300	16,672 ± 800
	FOS/TAC	0.26 ± 0.03	0.35 ± 0.09

3.3.4. Discussion on the full-scale trial

From this full-scale trial, we can learn several lessons about SD recirculation:

- (i) Under the tested conditions, SD recirculation does not have a negative impact on the biological parameters of the AD. This was expected regarding the low amount of SD recirculated (2.5% of the volume of the total feedstock supply).
- (ii) Regarding evolution of the feedstock supply over time (decrease in solid content) and based on our hypothesis on the initial feedstock supply, the plant conversion efficiency was not negatively affected by SD recirculation. Plant conversion efficiency even slightly increased, but this can be due to several phenomena previously described that could occur simultaneously during this period.
- (iii) Among these phenomena, a potential one would be that SD recirculation and SRT increase lead to a higher microbial load in the digester and that activities toward recalcitrant organic matter occur. Data and model presented in this chapter allow us to imagine this phenomenon but they are far from confirming it scientifically.

Thus, this trial did not allow to detect any potential negative effect of SD recirculation on biogas plant AD. However, regarding the small amount recirculated, it was not possible to clearly distinguish potential effects of SD recirculation on plant methane yield as other phenomena occur at full scale as a lot of parameters vary and cannot be controlled. Potential co-benefits of SD recirculation on the AD biochemical processes (increase in hydrolysis due to higher microbial load?) and the AD physicochemical processes (buffering and stabilization capacities that have been reported at lab scale for composted SD (Wagner et al., 2018) and by biogas plant operator performing SD recirculation) require more attention as they might improve the AD process. Thus, if it is the case, for a recirculation strategy, these co-benefits could counterbalance SD lower methane yield than feedstocks (reduce the amount of SD that needs to be recirculated to offset missing feedstock) or, for an addition strategy, they could further enhance plant methane efficiency and additional methane gains.

Additional full-scale trials should be performed to have better insights in the effect of SD recirculation. Best conditions would be to perform SD recirculation following an addition strategy (if TS content allows it), during a period where the feedstock supply is stable, with higher amounts of SD recirculated (at least $R \geq 0.1$) and with a better knowledge of feedstock features.

4. Conclusions

In this chapter, conditions for the full-scale implementation of SD recirculation have been explored. New equations have been introduced that allows to calculate potential impact of SD recirculation on HRT, SRT and TS content of the biogas plant. It has been shown that direct SD recirculation will lead to an increase in TS content of the biogas plant as volume is set. This will increase digestate viscosity and mixing cost. However, the extent of this increase will greatly vary as a function of the amount of SD recirculated, the biogas plant mixing system and the feedstock features. Decision concerning the interest of recirculating SD for a biogas plant has to be done on a case-by-case basis. A decision diagram, based on equations developed in this chapter, was provided to allow operators to estimate potential interest of this practice for their biogas plant and two concrete examples of this approach were provided.

Finally, the main conclusion that can be drawn from the full-scale trial reported in this chapter, where SD replaced 8% of the solid feedstocks, is that SD recirculation did not have any negative effect on biogas plant AD. Additional potential co-benefits on AD process such as enhanced hydrolysis or higher buffered capacity might require more attention and be studied in the future. Recommendations to better distinguish SD recirculation effect during potential new full-scale trials were provided.

Transition

PhD research workflow

PhD objective: Gain insights into the impact of solid digestate (SD) recirculation on agricultural biogas plants.

Three main research axis*: (A) Strategies and impact

(B) Post-treatment

(C) Full-scale

*(Associated questions → Chapter 1 section 5)

Chapter III

Answer to research axis Strategies and impact and partly to research axis Post-treatment

- ✓ Three strategies of SD recirculation have been defined: addition, replacement and partial replacement.
- ✓ For the six biogas plant evaluated:
 - A) SD direct recirculation following an addition strategy can increase plant methane production between 0,6 to 5,7%
 - B) SD direct recirculation following a replacement strategy can offset between 64 to 1431 tons of feedstocks
- ✓ Thermo-alkaline post-treatment costs are not compensated by potential additional methane gains/capacity offset.
- ✓ SD residual methane potential (RMP) decreased after short-term aerobic post-treatment due to unspecific endogenous activities towards the lignin-like fraction (activity reorientation using Kraft solution was not successful).

Answer partly to research axis Post-treatment

Is it possible to increase short-term aerobic post-treatment by adding:
A) ligninolytic microbial consortia? B) ligninolytic fungi?

Chapter IV

- ✓ Addition of microbial consortia obtained after screening and bioaugmentation did not improved the efficiency of short-term aerobic post-treatment.
- ✓ Decrease of SD RMP observed likely due to non-specific activities of consortia towards the lignin-like fraction of SD.
- ✓ Intensive gamma beam sterilisation was not sufficient to totally sterilized SD.

Chapter V

- ✓ Addition of ligninolytic fungi did not improved the efficiency of short-term aerobic post-treatment. Decrease of SD RMP observed.
- ✓ Fungal activities were again not specific towards the lignin-like fraction of SD.
- ✓ Fungal colonization of SD required sterilization
- ✓ Thermo-alkaline post-treatment (used as sterilization) degraded SD lignin-like fraction.

- ✓ Specific features of SD (rich in endogenous microorganisms, difficult to sterilize, rich in proteins) makes it a not suitable feedstock for aerobic post-treatments (no possibility to reach specific activities towards the lignin-like fraction).

Answer to research axis Post-treatment and partly to research axis Full-scale

- ✓ Quick and direct recirculation appears to be the best condition for SD recirculation at full-scale.
- ✓ Post-treatments appear to be not necessary.

Chapter VI

Answer to research axis Full-scale

- ✓ Direct SD recirculation will increase digester TS content, digestate viscosity and likely mixing cost. Extent will greatly vary as a function of the amount of SD recirculated and the biogas plant mixing system.
- ✓ Equation to calculate potential increase in TS content as well as Decision diagram for plant operator were provided. The main no-go is based on the capacity of the biogas plant mixing system to handle an increase in TS content.
- ✓ Full-scale SD recirculation had no negative effect on biogas plant AD process. Further trials need to be performed to fully evaluate the interest of SD recirculation and notably potential co-benefits that may exist (increase hydrolysis efficiency?, AD stabilization).

Chapter VII

Conclusions and Perspectives

1. Outcomes of the PhD thesis: Insights into solid digestate management.

The European biogas sector, after several years of significant growth mainly driven by strong subsidy policies and energy crops cultivation, is currently entering in a new era of its development. Higher sustainability and economic competitiveness of the sector, notably in comparison to solar or wind energies, are required by the European politics. Biogas production cost reduction will mainly be based on: (i) the use of cheaper and more sustainable feedstocks; (ii) the improvement of the AD process; (iii) the development of additional sources of revenues (commercialization of digestate/nutrients, CO₂ valorization, monetization of co-benefits...). The two former points are strongly intertwined in particular for agricultural AD. Indeed, future sustainable agricultural feedstock deposit is mainly based on various crop related feedstocks (sequential crops, residues) and manure. These feedstocks are rich in lignocellulose that is hardly degraded during AD. Therefore, optimization of the preparation step (e.g. pretreatments), the co-digestion process and the final digestate management is essential to recover as much as possible biogas from these various feedstocks.

This PhD thesis, based on a public-private partnership, was set in line with this context as it aims to explore innovative digestate management solutions to improve the anaerobic digestion process efficiency and reduce biogas production costs. In the literature review (chapter I), it was reported that coverage and heating of storage tanks that contains LD or raw digestate have been identified as good management practices for the agricultural biogas sector (Bacenetti et al., 2016; Lijó et al., 2017). Indeed, such practices, now often implemented at full-scale, allow additional biogas recovery, which leads to a higher efficiency and a lower environmental footprint of agricultural biogas plants. However, for SD management, no good practices have clearly been reported. Among existing valorization strategies, SD recirculation has been identified as a relatively low-cost and easy to implement way to further anaerobically digest feedstocks and improve biogas plant efficiency (Monlau et al., 2015). However, economic viability of post-treatments applied to SD as well as recirculation conditions within the biogas plant have been relatively unexplored in the scientific literature.

Thus, this PhD thesis aimed to provide further insights into the impact of SD recirculation on agricultural biogas plants. Results obtained can be grouped in three main research axes: (i) strategies and impact of SD recirculation; (ii) interest of additional post-treatments; (iii) condition for SD recirculation at full-scale.

1.1. Strategies and impact of SD recirculation

Three different strategies for SD recirculation were first defined (chapter III): (i) the addition case (SD is added to the ration) that allows additional biogas production and an increase in biogas plant efficiency; (ii) the replacement case (SD replaces missing or unproduced feedstocks) that allows to offset partially (“partial replacement strategy”) or totally (“replacement strategy”) potential methane loss.

For six agricultural and territorial biogas plants, we quantified the potential impact of direct SD recirculation on plant methane production, based on SD characterization and biogas plant features (chapter III). Methane produced from the direct recirculation of SD was estimated to range between 0.6% and 5.7% of the total plant methane production, as a function of the plant studied (plant size, SD residual methane potential) and the percentage of total SD

recirculated. In the case of an addition strategy, it corresponds to additional economic gains between 8 k€ and 83 k€ per year, which are not negligible for biogas plant operators regarding the ease and low-cost of SD direct recirculation. In a replacement strategy, it could entirely offset methane loss from the shortage of 64 to 1,431 tons of feedstocks that can be an interesting alternative for farmers to buying feedstocks as SD is produced for a very low cost (phase separator OPEX and damping).

1.2. Interest of additional post-treatments

The second step was to evaluate the interest of additional post-treatments to improve the initial results obtained with the direct recirculation. Thermo-chemical and biological aerobic post-treatments were studied on two SD that were coming from two plants representative of agricultural biogas plant treating complex feedstocks (e.g. manure).

Thermo-alkaline post-treatments tested (NaOH and CaO) efficiently improved the biodegradability of SD due to the degradation of the most complex fraction of the organic matter (lignin-like fraction). Recirculation of this post-treated SD subsequently increased between 13% and 46% the additional economic gains or offset capacity in comparison to direct SD recirculation (chapters III and V). However, such increases do not compensate for the additional expenditure generated by these post-treatments (e.g. cost for chemicals, post-treatment tank, additional labour). Therefore, addition of a thermo-alkaline post-treatment before SD recirculation does not appear to be economically viable under the tested conditions.

Biological aerobic pretreatments are reported to be less expensive and more environmentally friendly than thermo-chemical pretreatments, while displaying interesting results notably on lignocellulosic feedstocks (chapter I). It was therefore, decided to study aerobic post-treatment as a potential cheaper alternative to thermo-alkaline post-treatment. Short-term aeration of SD has been tested under several conditions: simple aeration (chapter III), addition of Kraft lignin solution (chapter III), addition of ligninolytic consortia (chapter IV), addition of ligninolytic basidiomycetes fungi (chapter V). However, in all conditions, SD biodegradability was not improved as no specific activities towards the most complex fraction of the organic matter (lignin-like fraction) could be obtained. Most accessible fractions of SD organic matter (e.g. proteins), that would have given methane under anaerobic conditions, were always respired (carbon loss under the form of CO₂) during aerobic post-treatment leading to a lower residual methane potential. These results are due to the specificity of SD organic matter composition (rich in proteins) that might favor other microbial activities (proteolytic...) over ligninolytic ones. Thus, SD does not appear to be a suitable feedstock for aerobic post-treatment as potential gain in biodegradability will not compensate respiration loss.

Regarding these results, fast and direct recirculation of solid digestate without any post-treatment seems to be the best condition to apply at full-scale.

1.3. Conditions for SD recirculation at full-scale: Theory and practice

Several equations have been introduced that allow to calculate potential impact of SD direct recirculation on HRT, SRT and TS content of the biogas plant (chapter VI). It has been shown that this practice leads to an increase in TS content of the biogas plant as volume is set. Higher TS content will increase digestate viscosity and mixing cost. However, extent of this increase

will greatly vary as a function of the amount of SD recirculated, the biogas plant mixing system and the feedstock features. The interest in solid digestate recirculation has to be determined on a case-by-case basis and a decision support scheme was provided for biogas plant operators (chapter VI). The main no-go is based on the incapacity of the biogas plant mixing system to handle an increase in TS content.

Finally, a full-scale trial on an agricultural biogas plant was performed over ten months. SD was directly recirculated following a partial replacement strategy and replaced 8% of the solid feedstocks ration. The main outcome is that SD recirculation does not have any negative effect on biogas plant AD process. Besides, potential existence of co-benefits of this practice on AD process, such as enhanced hydrolysis or higher buffered capacity, was raised and should require more attention in the future.

1.4. Take-home message

This PhD thesis demonstrates that direct solid digestate recirculation can be a simple and low-cost way to increase agricultural CSTR biogas plant efficiency. It notably provides the keys to determine on a case-by-case basis the interest of this practice for a given biogas plant. Under certain conditions, it can be considered as a good management practice that might allow to increase biogas production in the order of a few percent without any additional CAPEX and a low OPEX (short daily labour time, associated tractor operations and additional low mixing costs). Such potential increase is not negligible for biogas plant operators and can be seen as a relevant way to reduce biogas production cost. **Figure 64** gives an overview of the main conclusions drawn from this PhD thesis.

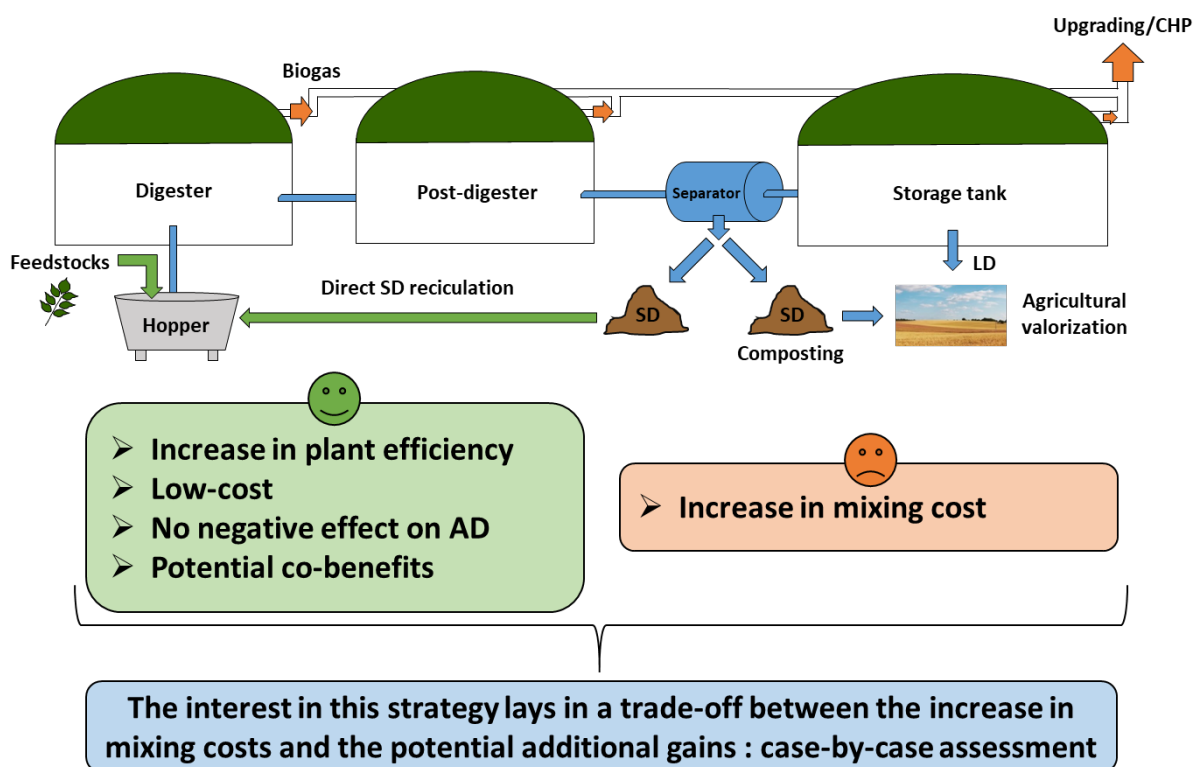


Figure 64: Main PhD thesis conclusions

2. Perspectives of the PhD thesis

Following this work, several perspectives emerge, either to gain further knowledge into SD direct recirculation practice, either on the different steps of the biogas production, or concerning the biogas sector in general:

- ❖ It would be interesting to study more precisely potential co-benefits of SD recirculation. Lab or pilot scale studies should be performed to clearly demonstrate potential higher hydrolysis activities due to a higher microbial load or AD process stabilization due to pH buffering. Such phenomena would be difficult to evaluate at full-scale as lots of parameters are varying and could interfere.
- ❖ Further full-scale studies should be performed to fully confirm that direct SD recirculation following an addition strategy can effectively enhance plant methane production. Optimally, it would be appropriate to perform this strategy during a period where the ration is stable, with higher amount of SD recirculated (at least $R \geq 0.1$, if digester TS content allows it) and with a better knowledge of feedstock features.
- ❖ Besides, in this PhD thesis, we mainly focused on the impact of SD direct recirculation practice on the biogas plant efficiency. However, it would also be interesting to study the impact of this practice on plant sustainability and notably on potential greenhouses gas emissions, as it was done for LD (Baldé et al., 2016). It is likely that this strategy will equally be beneficial, as additional carbon converted to methane during recirculation would have been otherwise released under the form of carbon dioxide during the composting of SD.
- ❖ A similar study on the evolution of digestate agronomic properties following this practice would be interesting to perform notably at full-scale. It might answer the following question: “Will the recovered SD and LD be richer in complex as well as humified compounds and display better agronomic properties?”
- ❖ Concerning pretreatment of lignocellulosic agricultural feedstocks several strategies appear to be promising: (i) NaOH is the most efficient alkaline chemical to delignify biomass. However, the presence of salt is a true issue for the subsequent agricultural valorization. Dilute pretreatment, strategies to recover sodium (e.g. washing step before entering the digester), or combination with other alkaline chemicals that have positive properties on soil (KOH, CaO) should be further explored. (ii) For biological pretreatments the use of enzymes, despite their costs, remains an interesting track. The best results may not be obtained with easy to degrade feedstocks (energy crops) but with agricultural residues rich in lignin (straw) and with cocktails containing not only cellulase and hemicellulase but also laccase or peroxidase (Herrero Garcia et al., 2019). Besides, enzyme addition may have additional positive effects such as a reduction of mixing costs by decreasing digestate viscosity. (iii) The combination of alkaline or enzymatic pretreatments with conventional existing biological storage strategy (ensiling) might be an efficient way to improve pretreatment efficiency as well as biodegradability of agricultural residues or sequential crops. Thus, in the case

of enzymes, contrary to their direct addition in the digester, their use under acidic conditions in an environment without the abundant presence of protease (contrary to digestate) might allow a higher activity as well as a longer lifespan.

- ❖ Within the digestate management area, recirculation of liquid digestate or raw digestate were also identified as potential interesting practices. They are already performed at full-scale to homogenize biology between tanks or to control TS content. Post-acidification during recirculation of raw digestate, via notably CO₂ injection, to enhance its methane yield was explored in Appendix n°2. The recirculation and the use of liquid digestate as a biological vector to pre-hydrolyse lignocellulosic biomass also appear as a promising pretreatment (Guan et al., 2018; Liu et al., 2019).
- ❖ Likewise to digestate management, mixing has been identified in this PhD thesis as an important parameter in agricultural biogas plants, as its optimization could lead to a strong reduction in plant OPEX (Kress et al., 2018; Nsair et al., 2019). However, relationships between TS content, digestate viscosity and mixing cost remain poorly understood as a great number of parameters could vary from one biogas plant to another (e.g. feedstocks, pretreatments, particle size, mucilage, mixing equipment, temperature). Fortunately, this subject is gaining more and more interest (Singh et al., 2019). Studies that would allow to adapt and to apply optimal mixing conditions as a function of the variation in TS content as well as digestate viscosity would be of high interest. Indeed, their results would be important to optimize potential benefits of direct SD recirculation and critical for the sector as a way to reduce biogas production costs.
- ❖ Increase in biogas plant efficiency implies mainly to produce more biogas. However, for biogas plant operators, it is not a priority. Indeed, today for most biogas plants performing upgrading, a small part of the production is already flared up as they cannot inject into the gas network when it is saturated. This is the main bottleneck envisioned for the development of incremental innovation and continuous improvement in biogas plants. To answer the question: “Why should I optimize my process and produce more biogas than I already do, if I cannot valorize it?” solutions are currently emerging that are either storage or backfeeding installations that makes gas networks bidirectional. Thus, the LiLiBox system developed by Azola (Bois-Colombes, France), allows to liquefy and to store up to 60,000 Nm³ biomethane that can be reinjected when the network is no longer saturated. Besides, within the West Grid Synergy project conducted by GRTgaz (Bois-Colombes, France), the first backfeeding installation in France in Noyal-Pontivy (France) should be operational by the end of 2019. These new solutions should enable biogas plant operators, in the future, to bypass network constraints and to perceive more interest towards the optimization of the whole chain of the biogas production process (pretreatment, AD co-digestion, digestate management) in order to increase plant efficiency as well as biogas production.

- ❖ Finally, this PhD highlights the interest for the agricultural anaerobic digestion sector of efficient collaborative research projects between farmers, public research and industries in order to find ways to optimize existing AD process and reduce biogas production costs. This is one of the keys to ensure long-term development of the AD sector.

Appendix n°1

Calculations to determine impact
of solid digestate recirculation
on solid retention time and
digester total solids content

In the case of a CSTR biogas plant equipped with a phase separator and producing SD and LD, it is possible to describe the system according to **figure 65**:

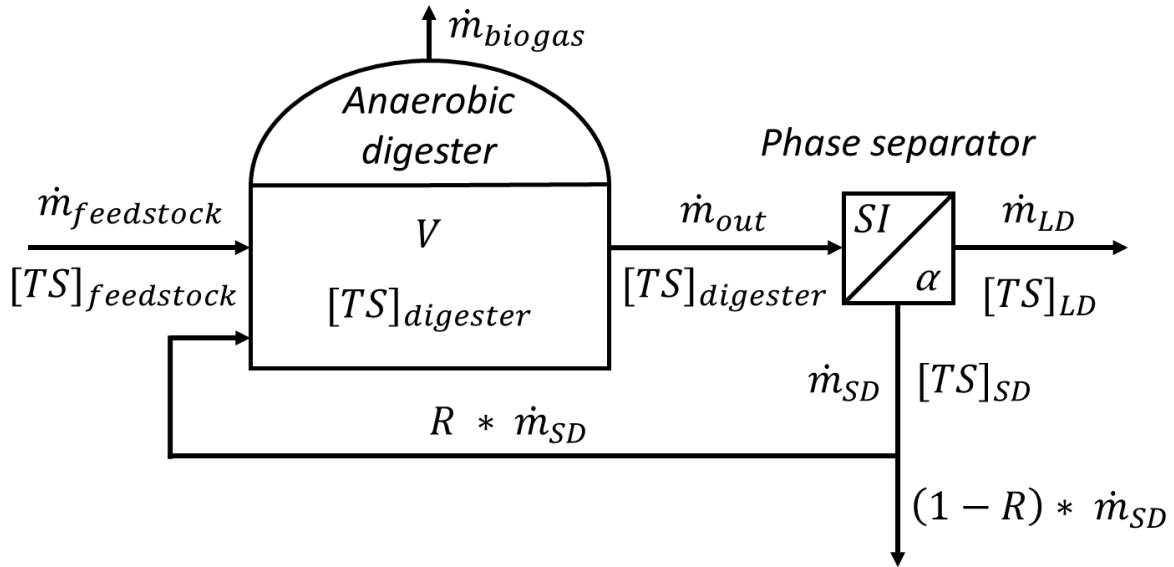


Figure 65: CSTR biogas plant equipped with a phase separator and performing solid digestate recirculation

With the following known variables:

- $\dot{m}_{feedstock}$: mass of feedstocks incorporated per day (tons/day)
- \dot{m}_{biogas} : mass of biogas produced per day (tons /day)
- V : volume of the anaerobic digester (m^3)
- $[TS]_{feedstock}$: concentration in total solids of feedstock (ton TS/ton feedstock)
- SI : efficiency of the separation unit - Defined
- α : the repartition factor of mass flow between solid digestate and liquid digestate -
- R : the percentage of recirculated solid digestate - Defined

As well as, the following unknown variables:

- \dot{m}_{out} : mass of raw digestate leaving the digester per day (tons/day)
- \dot{m}_{LD} : mass of liquid digestate produced per day (tons/day)
- \dot{m}_{SD} : mass of solid digestate produced per day (tons/day)
- $[TS]_{digester}$: concentration in total solids of raw digestate (ton TS/ton raw digestate)
- $[TS]_{LD}$: concentration in total solids of liquid digestate (ton TS/ton liquid digestate)
- $[TS]_{SD}$: concentration in total solids of solid digestate (ton TS/ton solid digestate)

It is important to precise that for the rest of the calculations the following strong hypothesis were made:

- Densities of all feedstocks and digestate were equal to one. This allows to switch freely from mass to volume.
- $[TS]_{digester}$ was considered to be the total solid content of the raw digestate entering in the phase separator (\dot{m}_{out}). Ideally, $[TS]_{post-digester}$ should be considered.

The seven following equations can be defined based on system balance and existing definitions:

$$\dot{m}_{feedstock} - \dot{m}_{biogas} = \dot{m}_{LD} + (1 - R) * \dot{m}_{SD} \quad (1)$$

$$\dot{m}_{feedstock} * [TS]_{feedstock} - \dot{m}_{biogas} = \dot{m}_{LD} * [TS]_{LD} + (1 - R) * \dot{m}_{SD} * [TS]_{SD} \quad (2)$$

$$\dot{m}_{out} = \dot{m}_{LD} + \dot{m}_{SD} \quad (3)$$

$$\dot{m}_{out} * [TS]_{digester} = \dot{m}_{LD} * [TS]_{LD} + \dot{m}_{SD} * [TS]_{SD} \quad (4)$$

$$\alpha * \dot{m}_{SD} = \dot{m}_{LD} \quad (5)$$

$$SI = \frac{\dot{m}_{SD} * [TS]_{SD}}{\dot{m}_{out} * [TS]_{digester}} \quad (6)$$

$$1 - SI = \frac{\dot{m}_{LD} * [TS]_{LD}}{\dot{m}_{out} * [TS]_{digester}} \quad (7)$$

It is known from previous works on sludge recirculation (Degremont, 2007) that solid retention time corresponds to the following equation:

$$SRT = \frac{V * [TS]_{digester}}{\dot{m}_{LD} * [TS]_{LD} + (1 - R) * \dot{m}_{SD} * [TS]_{SD}}$$

Based on **Eq. (6)** and **Eq. (7)** we can write that:

$$\dot{m}_{SD} * [TS]_{SD} = SI * \dot{m}_{out} * [TS]_{digester} \quad \& \quad \dot{m}_{LD} * [TS]_{LD} = (1 - SI) * \dot{m}_{out} * [TS]_{digester}$$

It is therefore possible to express the denominator of the SRT equation as a function of \dot{m}_{out} and get rid of $[TS]_{digester}$.

$$SRT = \frac{V}{(1 - R * SI) * \dot{m}_{out}}$$

Now by combining **Eq. (1)** and **Eq. (5)** we can write that:

$$\dot{m}_{feedstock} - \dot{m}_{biogas} = (1 + \alpha - R) * \dot{m}_{SD}$$

And by combining **Eq. (3)** and **Eq. (5)** we can write that:

$$\dot{m}_{out} = (1 + \alpha) * \dot{m}_{SD}$$

Thus, \dot{m}_{out} can be expressed as a function of $\dot{m}_{feedstock}$, \dot{m}_{biogas} , α and R:

$$\dot{m}_{out} = \frac{(1 + \alpha) * (\dot{m}_{feedstock} - \dot{m}_{biogas})}{(1 + \alpha - R)}$$

This new expression of \dot{m}_{out} can be reinjected in the SRT equation and we obtain as final equation:

$$SRT = \frac{(1 + \alpha - R)}{(1 + \alpha) * (1 - R * SI)} \frac{V}{(\dot{m}_{feedstock} - \dot{m}_{biogas})}$$

Concerning α , it is a positive real number. The higher the value, the lower the quantity of SD produced. SI index is comprised between $1/(1 + \alpha)$ and 1. When SI equals $1/(1 + \alpha)$ it means that SD is not enriched in TS in comparison to LD and that raw digestate is only separated in two flows (according to α). When SI equals 1 it means that the efficiency of the phase separation is maximal and all total solids go in the SD. We can select α and SI values based on the average mass and total solids distribution profiles provided from a recent study on digestate mechanical separation (Guilayn et al., 2019b). In the case of a low efficiency separator (e.g. screw press) typical values for α and SI are 9 and 0.38 respectively. In the case of a high efficiency separator (e.g. centrifuges) typical values for α and SI are 2.45 and 0.81 respectively. R is comprised between 0 and 1. When R equals 0 it means that no SD is recirculated and SRT equals HRT. When R equals 1 it means that all SD produced is recirculated.

It is also possible to determine the impact of SD recirculation on digester TS content based on **Eq. (2)**. Using **Eq. (6)** and **Eq. (7)** it is possible to transform **Eq. (2)** into:

$$\dot{m}_{feedstock} * [TS]_{feedstock} - \dot{m}_{biogas} = \dot{m}_{out} * [TS]_{digester} * (1 - R * SI)$$

Based on the previous expression of \dot{m}_{out} expressed as a function of $\dot{m}_{feedstock}$, \dot{m}_{biogas} , α and R, we can express $[TS]_{digester}$ according to this final equation:

$$[TS]_{digester} = \frac{(1 + \alpha - R)}{(1 + \alpha) * (1 - R * SI)} * \frac{(\dot{m}_{feedstock} * [TS]_{feedstock} - \dot{m}_{biogas})}{(\dot{m}_{feedstock} - \dot{m}_{biogas})}$$

As generally, $[TS]_{digester}$ without recirculation (R=0) is known, the following equation obtained from the one above can be used to determine the $[TS]_{feedstock}$:

$$[TS]_{feedstock} = \frac{[TS]_{digester} * (\dot{m}_{feedstock} - \dot{m}_{biogas}) + \dot{m}_{biogas}}{\dot{m}_{feedstock}}$$

Finally, for more precision in the SRT and $[TS]_{digester}$ calculations, \dot{m}_{biogas} can be modified as a function of R. Indeed, when SD is recirculated additional biogas is produced that should be added to the initial biogas from feedstocks (\dot{m}_{biogas}). In this equation $\dot{m}_{biogas}(R)$ is expressed as a function of $\dot{m}_{SD_recirculated}(R)$, that corresponds to a fraction (R) of the estimated amount of SD produced per year (from biogas plant operator knowledge). $\dot{m}_{biogas}(R)$ is also expressed as a function of SD residual methane yield, volatile solids contents, methane density as well as carbon dioxide density. Thus, it gives the following equation:

$$\dot{m}_{biogas}(R) = \dot{m}_{biogas}(0) + \frac{\dot{m}_{SD_recirculated}(R) * [VS]_{SD} * RMP_{SD} * (d_{CH_4} + \frac{(1 - x_{CH_4})}{x_{CH_4}} * d_{CO_2})}{1000}$$

This can be reinjected in the SRT and [TS] equations and leads to these two final equations used in chapter VI:

$$SRT = \frac{(1 + \alpha - R)}{(1 + \alpha) * (1 - R * SI)} \frac{V}{(\dot{m}_{feedstock} - \dot{m}_{biogas}(R))}$$

$$[TS]_{digester} = \frac{(1 + \alpha - R)}{(1 + \alpha) * (1 - R * SI)} * \frac{(\dot{m}_{feedstock} * [TS]_{feedstock} - \dot{m}_{biogas}(R))}{(\dot{m}_{feedstock} - \dot{m}_{biogas}(R))}$$

Appendix n°2

Post-acidification and
hydrolysis/acidogenesis of
digestate before its recirculation
to increase biogas production
from agricultural biogas plants

1. Introduction

On agricultural biogas plants performing biomethane upgrading, purified carbon dioxide is produced at the same cost as biomethane. Valorizing this carbon dioxide is an actual issue for plant operators. To get an order of magnitude, for a site such as biogas plant A, which upgrades 145 Nm³/h of biomethane and with a percentage of carbon dioxide in the biogas of 47%, there is a production of 1,054,000 Nm³ per year (8,200 hours of operation) of purified carbon dioxide, the equivalent of 105,400 large compressed gas cylinders (10 Nm³ per cylinder). However, the relatively small size and dispersion of these biogas plants currently limit the application of existing industrial recovery solutions (e.g. methanol or ethanol production, applications for the food sector). Thus, the idea would be to provide to biogas plants performing upgrading a way to valorize its purified carbon dioxide directly on site to improve methane production.

Carbon dioxide bubbling into anaerobic digester has been reported to double AD sludge methane production rate and increase yield of AD food waste by 13% (Al-mashhadani et al., 2016; Bajón Fernández et al., 2014). Precise biological mechanisms uptake of carbon dioxide and its conversion to methane remains unclear and several hypotheses have been emitted (e.g. it enhances acetoclastic pathway, it boosts VFAs formation). Carbon dioxide during bubbling is also reported to dissolve in digestate and subsequently decrease pH of the anaerobic digestion. Thus in a case of sludge at a pH of 7.5, carbon dioxide bubbling allowed its reduction to 6.9; getting closer to carbon dioxide pKa value in water defined at 6.4 (Bajón Fernández et al., 2014). From these articles two interesting points were identified: (i) In these experiments not all carbon dioxide injected is converted to methane or dissolved inside the AD. If this strategy is applied in a digester or post-digester its economic interest is reduced as it will require to upgrade several times the reinjected carbon dioxide. Therefore, the use of a satellite reactor where carbon dioxide could be reinjected is of interest; (ii) Concerning the acidification capacity of carbon dioxide, it is potentially interesting to use it to trigger hydrolysis/acidogenesis in raw digestate, in order to subsequently enhance hydrolysis of refractory/complex compounds such as lignocellulosic materials (Amin et al., 2017). Indeed, hydrolysis/acidogenesis optimal pH for this step is slightly acidic, comprised between 5.5 and 6.5 (Mao et al., 2015). Combination of hydrolysis/acidogenesis with additional carbon dioxide biological uptake may enhance raw digestate methane yield. Based on these two points an original post-treatment scheme was imagined.

The purpose of this Annex chapter is to evaluate an original post-treatment scheme represented in **figure 66**. Purified carbon dioxide is used as an acidifying agent of the raw digestate coming from the post-digester that will be recirculated (only a fraction of all digestate) in the digester. It aims to promote hydrolysis and acidogenesis of remaining recalcitrant material in recirculated raw digestate during an intermediate AD batch performed in a satellite digester. Besides, recirculation of post-digester raw digestate towards digester is a practice that is already performed in several biogas plant to homogenize and maintain AD biology. Finally, aside from CO₂, two additional acidification vectors were identified as

promising candidate: (i) silage juice was selected as potential low-cost way to acidify raw digestate as it can be recovered directly on biogas plant site; (ii) Phosphoric acid was selected due to its strong acidifying power and the fact that it is bringing phosphate to the system that could be further valorized as an agricultural nutrient.

Three main points will be evaluated and discussed in this annex: (i) The proof of concept that carbon dioxide can effectively be used as a cheap way to preliminary acidify post-digester raw digestate; (ii) The evaluation of using silage juice or H_3PO_4 as additional raw digestate acidification vectors; (iii) The impact of acidification, pH ranges as well as duration of the hydrolysis/acidogenesis step on post-digester raw digestate residual methane potential.

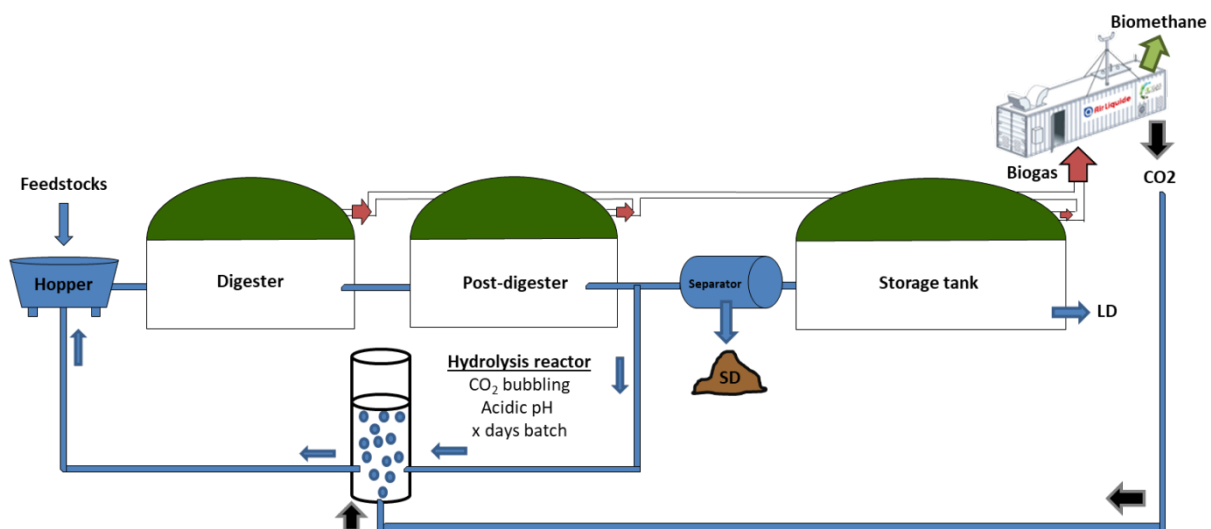


Figure 66 : Scheme of the potential full-scale implementation of the digestate post-treatment tested in this Annex chapter

2. Material and Methods

2.1. Post-digester digestate and silage juice sampling

Raw digestate coming from biogas plant A was sampled from the post-digester thanks to a sampling point in a pipeline. This is a substrate that has several advantages: it is liquid, homogeneous and the most easily biodegradable fractions have already been transformed into methane. Silage juice was obtained from the Pot-au-Pin biogas plant. It was sampled from the silage juice recovery pit located below silage silos. Samples were shipped quickly to the lab. They were used the same day as reception for characterization and post-treatment. No freezing nor storage at 4°C were performed to avoid modification of the endogenous digestate microbial community.

2.2. Raw digestate and silage juice characterization methods

Raw digestate and silage juice were fully characterized after reception. For raw digestate, TS/VS, pH and alkalinity (TAC) were measured on the raw fraction. Besides, centrifugation (18,650 g for 25 minutes) was carried out to separate solid and liquid fractions of raw digestate. COD and ammonium content were measured on the liquid fraction as described in chapter II. VFAs and soluble metabolites were measured according to this two protocols:

VFAs concentration was determined by gas chromatography measurement. Measurement was performed by using a GC (Perkin Clarus 580) composed of an injector at 250°C, a capillary column (Elite-FFAP crossbond carbowas) at 200°C and a flame ionization detector at 280°C. The carrier gas was nitrogen at a flow rate of 6 mL.min⁻¹. Before analysis, all samples were mixed at a proportion 1:1 volume with a solution of ethyl-2-butyric acid (1 g.L⁻¹) acting as an internal standard.

Soluble metabolites (lactate, ethanol...) were measured using an HPLC. The HPLC was made of a refractive index detector (Waters R410), an autosampler (Water 717 plus), a pre-column (Micro-Guard cation H refill cartridges, Bio-rad) for filtering potential remaining residues and a column Aminex HPX-87H, 300 x 7.8 mm (Bio-Rad). The temperature of the column was 35°C and H₂SO₄ at 4 mM was used as a mobile phase at a flow rate of 0.4 ml.min⁻¹.

Finally, for silage juice, COD measurement, VFAs as well as soluble metabolites analysis were performed.

2.3. Post-treatment conditions

Several conditions of post-treatment were tested. Three main parameters varied: the acidification vector (either gaseous carbon dioxide, silage juice or H₃PO₄), the pH applied (2, 5.5 or 6.4) and the duration of the post-treatment (24 hours, 2 days or 5 days). A large range of acidic pH was tested to fully evaluate interest of acidification on raw digestate methane yield. Finally, tested durations are closed to duration applied in a full-scale hydrolysis reactor (a few days) that are generally sufficient for an efficient hydrolysis/acidogenesis of the organic matter.

Acidification step using carbon dioxide sparging were performed in a closed and mixed Sartorius reactor. Carbon dioxide was sparged until that pH stabilized. For acidification using silage juice or H₃PO₄, they were directly mixed with digestate in beakers. Amounts of acids added until to reach wanted pH were measured. Finally, acidified raw digestate was poured in BMP flasks that were flushed with carbon dioxide to ensure anaerobic conditions, closed and subsequently placed in a rotary shaker under agitation (120 rpm) at 38°C for the given duration. **Table 31** gives an overview of the twelve conditions tested.

Table 31: Overview of the different post-treatment conditions tested

Hydrolysis/acidogenesis duration ⇔ ☞ Selected pH & acidification vector	24 hours	48 hours	5 days
pH 6.4 with CO ₂ bubbling	x4	x4	x4
pH 5.5 with addition of H ₃ PO ₄	x4	x4	x4
pH 5.5 with addition of silage juice	x4	x4	x4
pH 2 with addition of H ₃ PO ₄	x4	x4	x4

2.4.Characterization of digestate following post-treatment

For each condition, four flasks were prepared and they were stopped at the end of each defined hydrolysis/acidogenesis period. Gas analysis of the headspace to determine the amount of methane produced during the post-treatment were performed for all flasks. Then, one flask was sacrificed to carry out VFAs analysis on liquid fraction of post-treated digestate. A similar protocol as described in section 2.2 was applied to recover the liquid fraction (centrifugation, separation, analysis). The three other flasks were used to launch BMPs according to standard protocol. It should be highlighted that before addition of the inoculum, when necessary, pH was corrected to 7.8 using KOH in order to avoid any AD inhibitions. Additional control BMPs using untreated raw digestate as well as silage juice were launched upon their reception with the same inoculum.

3. Results and discussion

3.1. Raw digestate and silage juice features

Table 32 presents features of raw digestate and silage juice. Raw digestate from plant A post-digester has a TS content (10% TS) slightly lower than in the digester (12% TS). This lower value is not surprising as some TS have been converted to methane during AD process. Raw digestate pH value is in range with values found in literature (Monlau et al., 2015). For the liquid fraction, COD and NH_4^+ content were also in line with results from a previous study characterizing liquid digestate (Akhlar et al., 2017). Only a small quantity of acetate (300 mg/L) could be found in the liquid fraction of raw digestate and no other metabolites were analysed. Finally, silage juice has an acidic pH mainly due to a high content in VFAs and lactate.

Table 32: Raw digestate and silage juice features

Substrates	Raw digestate	Liquid fraction of raw digestate	Silage juice
TS (%Fresh matter)	9.9 ± 0.03	/	/
VS (%Fresh matter)	5.6 ± 0.05	/	/
pH	8.1 ± 0.02	/	4.7 ± 0.02
COD (gO ₂ /L)	/	24.4 ± 1.00	16.9 ± 0.20
Alkalinity (equivalent g CaCO ₃ /kg)	22.9 ± 0.10	/	/
NH ₄ ⁺ (g/L)	/	2.2 ± 0.10	
VFAs (g/L)	/	0.3 ± 0.05	4.9 ± 0.08
Lactate (g/L)	/	0	1.0 ± 0.05

3.2. Acidification tests

As shown in **figure 67**, acidification tests were performed either in a beaker for H₃PO₄ addition, either in fermentation reactor for CO₂. Results of acidification tests are displayed in **table 33**. It was possible to reach pH 2 and 5.5 with the addition of 61.3 and 22.3 mL H₃PO₄/L of raw digestate, respectively. Interestingly, when acid was added important foaming and bubbling was observed. This can be seen in **figure 67 (A)**. These gaseous emissions consisted in CO₂ produced from the conversion of buffering carbonate species under increasing acidic conditions. Silage juice despite its low pH (4.7) has a really low acidifying power (weak acid) and addition of 50%/50% volume only decreased pH of the mixture to 7.2. Therefore, additional 13 mL of H₃PO₄ were added to reach a pH of 6.25. This pH was estimated to be low

enough to trigger acidogenesis in the mixture. Finally, CO₂ was bubbled into agitated raw digestate. No precise recording of the amount injected or the duration required to decrease pH was performed as it was only a proof of concept. After a few minutes pH decreased to 7.1 and was then stable regardless of the additional amount of CO₂ added. Buffer capacity of raw digestate is high and acidifying power of CO₂ does not appear to be strong enough to get below 6.5 and trigger acidogenesis. Nevertheless, it was decided to keep this treated raw digestate for the rest of the experiment.

An additional acidification test was performed on raw digestate at 7.1 following CO₂ bubbling. H₃PO₄ was added in order to reach a pH of 5.5. Instead of 22,3 mL of H₃PO₄ to reach this pH value, 22,6 mL was required. It was therefore decided to measure the alkalinity of CO₂ treated digestate and a value of 23.4 g equivalent CaCO₃/kg raw digestate that corresponds to a slight increase of 2% of the alkalinity. This result is quite surprising and unexpected as CO₂ was initially aiming to decrease the pH, notably by increasing the FOS (acidification) but instead the TAC (alkalinity) was increased and buffered capacity of the raw digestate improved.

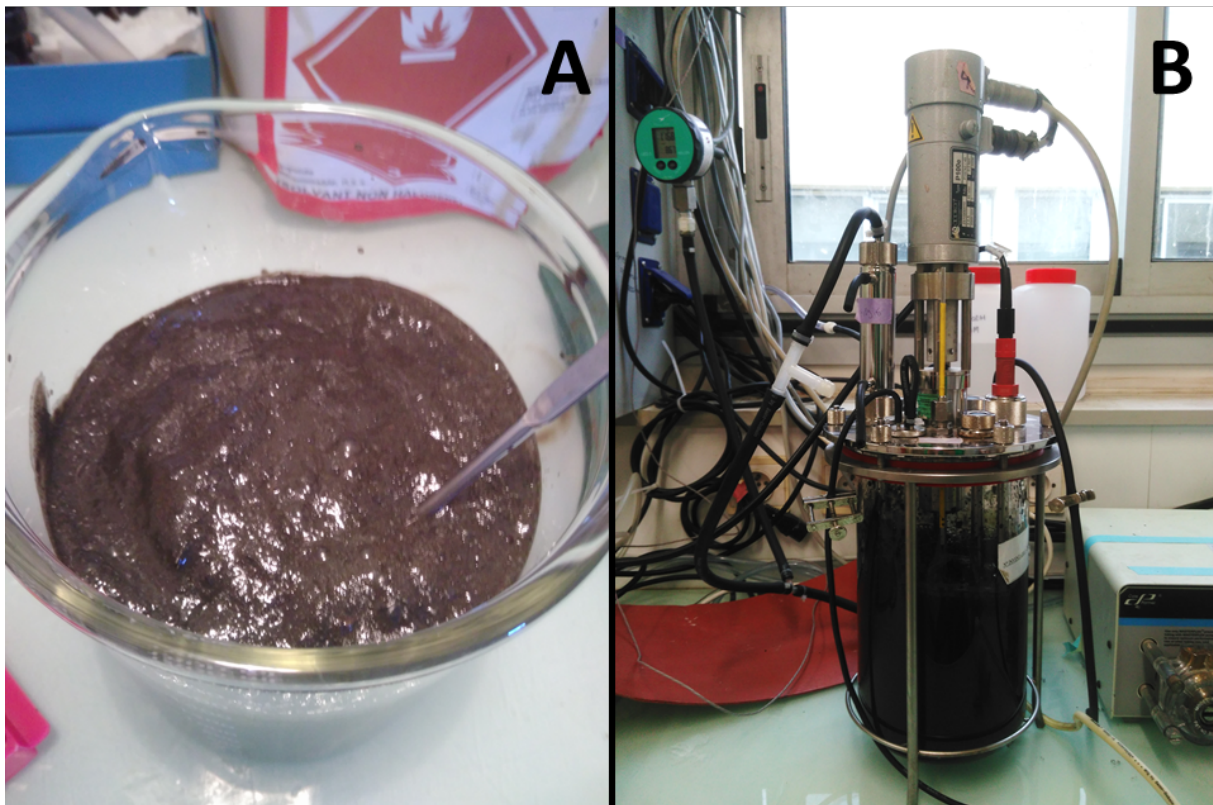


Figure 67: Acidification test vessels: (A) Addition of H₃PO₄ into raw digestate was performed into a glass beaker – Here digestate foaming can be observed; (B) Addition of CO₂ into raw digestate was performed in a fermentation reactor.

Table 33: Results of acidification tests

Targeted pH & acidic vector used	Amount of acidic vector added	Final pH value reached
pH 2 H ₃ PO ₄	Addition of 61.3 mL H ₃ PO ₄ /L of raw digestate	2
pH 5.5 H ₃ PO ₄	Addition of 22.3 mL H ₃ PO ₄ /L of raw digestate	5.5
pH 5.5 silage	Addition of 1L silage juice and 13 mL H ₃ PO ₄ /L of raw digestate	6.25
pH 6.4 CO ₂	No measurement of the amount of CO ₂ injected	Quick decrease and then get stable at 7.1

3.3. Post-treatment effect on digestate VFAs profile

VFAs profiles of the acidified raw digestate after the hydrolysis/acidogenesis step are presented in **figure 68**. For samples acidified at pH 2 with H₃PO₄ and at pH 7.1 with CO₂ no clear trend could be observed in comparison to the blank and VFAs amount remained low and close to the initial 300 mg/L. For the former group of sample (pH 2), it is likely that such low pH will inhibit most of the microbial activities, that could explain why no VFAs were produced. For the latter group of samples (CO₂), relatively high pH (7.1) did not allow acidogenesis and therefore no clear VFAs production trend was observed. Only samples acidified at pH 5.5 with H₃PO₄ displayed a beginning of acidogenesis with an increase in VFAs content (+67% in comparison to the blank after 115 h) and production of propionate, butyrate and iso-valerate. Longer duration of the hydrolysis/acidogenesis step may have been interested in that case to produce more VFAs. Finally, mixed silage juice/raw digestate blank had a higher content and diversity in VFAs than other samples (5.22 g/L dominated by acetate and butyrate) mostly due to silage. After the hydrolysis/acidogenesis step VFAs content was reduced by 20% regardless of the duration. Acetate, butyrate and valerate contents were mainly reduced. It indicates that despite the low pH, acidogenesis did not occur. Two hypotheses that could explain such decrease is that these molecules were converted to other metabolites (not measured here) or to methane as pH was not low enough to inhibit methanogens.

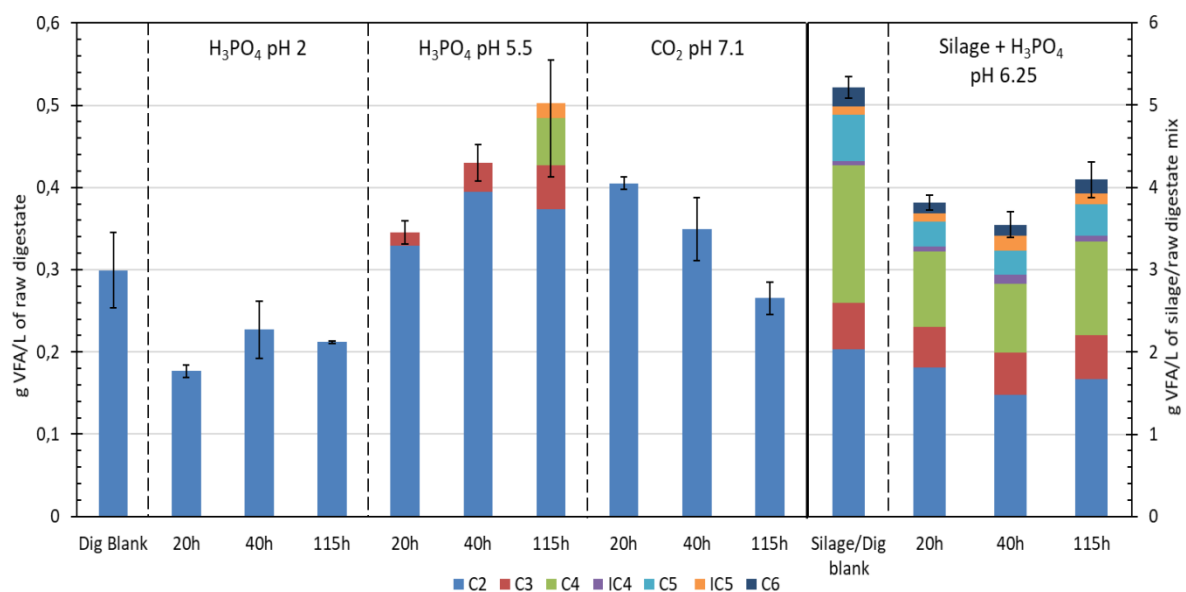


Figure 68: VFAs profile of acidified raw digestate after the hydrolysis/acidogenesis step (duration of 20h-40h or 115h)

3.4. Post-treatment effect on digestate methane yield

Table 34 gives results on the effect of acidification post-treatment on raw digestate methane yield. First, digestate blank has a methane production (132 mL CH₄/g VS) that is in the upper range of values that can be found in literature (3 mL CH₄/g VS to 126 mL CH₄/g VS) (Menardo et al., 2011b; Ruile et al., 2015). It indicates that, despite the long HRT (100 days) AD was not complete (as evidenced by the remaining content in acetate) and that some residual methane can still be recovered.

For raw digestate acidified at pH 2 and 5.5 with H₃PO₄, regardless of the hydrolysis/acidogenesis step duration, no significant increase could be observed in methane yield. A 34% decrease in methane yield was even observed in the case of 20 h and pH 5.5. Besides it can be observed that standard deviations of these samples are in average higher than the control which may indicate that AD process stability is negatively impacted when treating acidified raw digestate.

Mix of silage and raw digestate blank had a higher methane production (205 mL CH₄/g VS) than raw digestate due to silage juice high methane potential (280 mL CH₄/g COD). During the hydrolysis/acidogenesis step of silage/raw digestate mix samples a small quantity of methane was produced that indicates that methanogens was not totally inhibited at this pH. Finally, even by taking into account methane produced during the post-treatment step, methane yield of the mixture was not increased in comparison to the control.

For CO₂ acidification, small amounts of methane were produced (up to 14 mL CH₄/g VS after 115h) during the hydrolysis/acidogenesis step as pH was high enough to allow methanogens activity. If this amount of methane is taken into account, in addition to the BMP, then total methane yield was significantly increased (+16,7%) in comparison to the raw digestate blank only for samples that were subject to 115-hour post-treatment (no significant difference for

20 and 40 hours). However, if amount of methane produced during the 115-hour post-treatment was not taken into account, then methane yield was not anymore significantly higher than the blank. This highlights, the fact that this increase is very relative to the capacity to recover methane produced during the post-treatment. Besides, standard deviation of post-treated raw digestate is relatively high (almost 10% of the final methane yield), which encourages considering carefully this increase. Further trials would be required to fully demonstrate such a positive trend.

Table 34: Methane production from acidified digestate – All results are expressed in mL CH₄/g VS. Type : H/A step corresponds to the quantity of methane produce during the hydrolysis/acidogenesis step if applicable; BMP corresponds to the quantity of methane produced during BMP tests; Total corresponds to the sum of the methane production during H/A step and BMP if applicable.

Sample	Type	Duration hydrolysis/acidogenesis step (hours)			
		0	20	40	115
Raw digestate blank	BMP	132 ± 4.7	/	/	/
Silage/raw digestate blank	BMP	205.3 ± 24	/	/	/
H ₃ PO ₄ - pH 2	BMP	/	110 ± 23	123 ± 15	104 ± 26
H ₃ PO ₄ - pH 5.5	BMP	/	88 ± 12	108 ± 40	110 ± 23
Silage/raw digestate - pH 6.25	H/A step	/	2 ± 0.2	5 ± 0.7	11 ± 1.4
Silage/raw digestate - pH 6.25	BMP	/	193 ± 7.0	211 ± 6	194 ± 26
Silage/raw digestate - pH 6.25	Total	/	195 ± 7.2	216 ± 6.7	205 ± 27.4
CO ₂ - pH 7.1	H/A step	/	2 ± 0.2	3 ± 0.5	14 ± 1.9
CO ₂ - pH 7.1	BMP	/	121 ± 5.4	150 ± 20.7	140 ± 13
CO ₂ - pH 7.1	Total	/	123 ± 5.6	153 ± 21.2	154 ± 14.9

4. Conclusions and outlooks

Three main conclusions can be drawn from this experiment:

- (i) The use of CO₂ to acidify the raw digestate does not appear to be a viable strategy. Indeed, injection of CO₂ slightly increases the alkalinity, while pH is decreased as CO₂ is likely trapped by water and ammonia (forms H₂CO₃ and NH₂COOH) contained in the raw digestate. Besides, as FOS/TAC ratio is reduced, CO₂ injection is unlikely to trigger an acidogenesis of the raw digestate. Instead, buffered capacity of the raw digestate is increased.
- (ii) Raw digestate acidification (pH below 6.5) does not improve its methane yield. Acidic conditions and associated microbial activities (e.g. enhanced hydrolysis and acidogenesis) does not appear to have a significant effect on the residual matter. Besides, tested silage juice has a low acidifying power.
- (iii) Raw digestate samples treated with CO₂ and subjected to 115 h of hydrolysis/acidogenesis step displayed a higher methane yield in comparison to the blank (+16,7%). Further experiments should be performed to confirm this trend.

Regarding these conclusions several perspectives can be seen:

- (i) An alternative approach would be to use CO₂ injection as a low-cost buffering vector to limit the risk of acidogenesis/avoid crashes/stabilization in the digester. CO₂ could be injected on the exit of the hydrolysis tank (if two-stage process), or otherwise in a satellite reactor if raw or liquid digestate is recirculated on site (increase of their buffering capacity, that subsequently buffers the digester).
- (ii) The use of CO₂ could also be used to improve anaerobic digestion in the storage tank by lowering the pH and lowering the ammonia content of the liquid digestate. Potential higher content in ammonium might improve nitrogen mineralization, agronomic value of the liquid digestate, as well as reduction of NH₃ emissions. Potential implementation of this last perspective can be seen in **figure 69**.

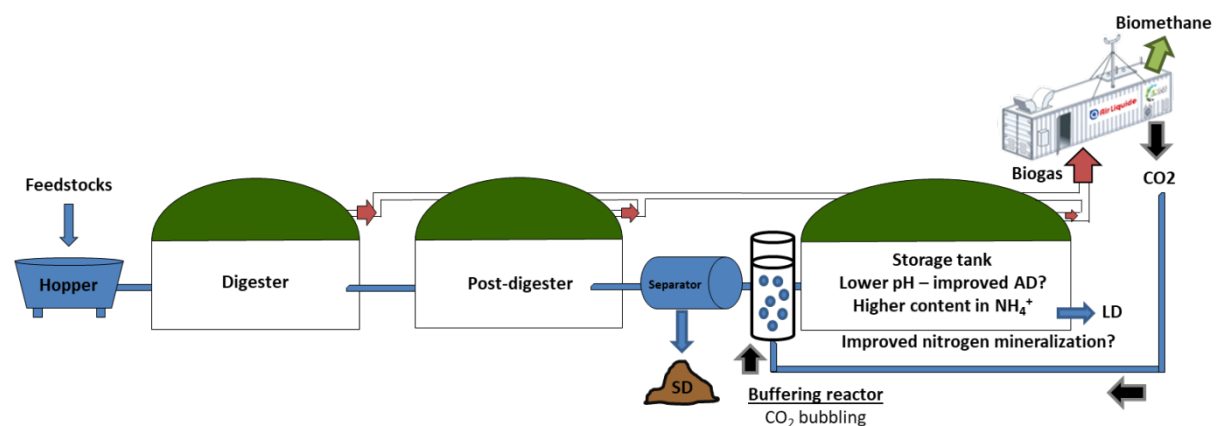


Figure 69: Scheme of the potential full-scale implementation of liquid digestate buffering system that might improve the subsequent AD and agricultural valorization.

References

- ADEME, 2018. Un mix de gaz 100 % renouvelable en 2050?
- Akhiar, A., Battimelli, A., Torrijos, M., Carrere, H., 2017. Comprehensive characterization of the liquid fraction of digestates from full-scale anaerobic co-digestion. *Waste Manag.* 59, 118–128. <https://doi.org/10.1016/j.wasman.2016.11.005>
- Al-mashhadani, M.K.H., Wilkinson, S.J., Zimmerman, W.B., 2016. Carbon dioxide rich microbubble acceleration of biogas production in anaerobic digestion. *Chem. Eng. Sci.* 156, 24–35. <https://doi.org/10.1016/j.ces.2016.09.011>
- Alberdi, A., Gilbert, M.T.P., 2019a. hilldiv: an R package for the integral analysis of diversity based on Hill numbers. *bioRxiv* 545665. <https://doi.org/10.1101/545665>
- Alberdi, A., Gilbert, M.T.P., 2019b. A guide to the application of Hill numbers to DNA-based diversity analyses. *Mol. Ecol. Resour.* 19, 804–817. <https://doi.org/10.1111/1755-0998.13014>
- Alexandropoulou, M., Antonopoulou, G., Fragkou, E., Ntaikou, I., Lyberatos, G., 2015. Fungal pretreatment of willow sawdust and its combination with alkaline treatment for enhancing biogas production. *J. Environ. Manage.* <https://doi.org/10.1016/j.jenvman.2016.04.006>
- Ali, S.S., Abomohra, A.E., Sun, J., 2017. Effective bio-pretreatment of sawdust waste with a novel microbial consortium for enhanced biomethanation. *Bioresour. Technol.* 238, 425–432. <https://doi.org/10.1016/j.biortech.2017.03.187>
- Amin, F.R., Khalid, H., Zhang, H., Rahman, S., Zhang, R., Liu, G., Chen, C., 2017. Pretreatment methods of lignocellulosic biomass for anaerobic digestion. *AMB Express* 7. <https://doi.org/10.1186/s13568-017-0375-4>
- André, L., Pauss, A., Ribeiro, T., 2018. Solid anaerobic digestion : State-of-art, scientific and technological hurdles. *Bioresour. Technol.* 247, 1027–1037. <https://doi.org/10.1016/j.biortech.2017.09.003>
- Angelidaki, I., Boe, K., Ellegaard, L., 2005. Effect of operating conditions and reactor configuration on efficiency of full-scale biogas plants. *Water Sci. Technol.* 52, 189–194. <https://doi.org/10.2166/wst.2005.0516>
- Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. *Biotechnol. Adv.* 36, 452–466. <https://doi.org/10.1016/j.biotechadv.2018.01.011>
- Angenent, L.T., Usack, J.G., Xu, J., Hafenbradl, D., Posmanik, R., Tester, W., 2018. Integrating electrochemical, biological, physical, and thermochemical process units to expand the applicability of anaerobic digestion 247, 1085–1094. <https://doi.org/10.1016/j.biortech.2017.09.104>
- Antoniou, N., Monlau, F., Sambusiti, C., Ficara, E., Barakat, A., Zabaniotou, A., 2019. Contribution to Circular Economy options of mixed agricultural wastes management : Coupling anaerobic

- digestion with gasification for enhanced energy and material recovery. *J. Clean. Prod.* 209, 505–514. <https://doi.org/10.1016/j.jclepro.2018.10.055>
- APHA, 2005. Standard methods for the examination of water and wastewater, 22.
- Appel, F., Ostermeyer-Wiethaup, A., Balmann, A., 2015. Effects of the German Renewable Energy Act on structural change in Agriculture - The case of biogas. *Util. Policy* 41, 172–182. <https://doi.org/10.1016/j.jup.2016.02.013>
- Appunn, K., 2016. EEG reform 2016 – switching to auctions for renewables [WWW Document]. *Clean Energy Wire*. URL <https://www.cleanenergywire.org/factsheets/eeg-reform-2016-switching-auctions-renewables>
- Ariunbaatar, J., Panico, A., Esposito, G., Pirozzi, F., Lens, P.N.L., 2014. Pretreatment methods to enhance anaerobic digestion of organic solid waste. *Appl. Energy* 123, 143–156. <https://doi.org/10.1016/j.apenergy.2014.02.035>
- Arreola-Vargas, J., Flores-Larios, A., González-Álvarez, V., Corona-González, R.I., Méndez-Acosta, H.O., 2016. Single and two-stage anaerobic digestion for hydrogen and methane production from acid and enzymatic hydrolysates of Agave tequilana bagasse. *Int. J. Hydrogen Energy* 41, 897–904. <https://doi.org/10.1016/j.ijhydene.2015.11.016>
- Asina, F., Brzonova, I., Kozliak, E., Kubátová, A., Ji, Y., 2017. Microbial treatment of industrial lignin: Successes, problems and challenges. *Renew. Sustain. Energy Rev.* 77, 1179–1205. <https://doi.org/10.1016/j.rser.2017.03.098>
- Association négaWatt, 2017. Scénario négaWatt 2017-2050 - Dossier de synthèse.
- Atee Club Biogaz, 2019. Calculateur - Injection du Biogaz dans le réseau [WWW Document]. URL <http://atee.fr/biogaz/injection-du-biogaz-dans-le-reseau>
- Atlas, R.M., 2010. Handbook of microbiological media. <https://doi.org/10.1201/EBK1439804063>
- Baccioli, A., Antonelli, M., Frigo, S., Desideri, U., Pasini, G., 2018. Small scale bio-LNG plant : Comparison of different biogas upgrading techniques. *Appl. Energy* 217, 328–335. <https://doi.org/10.1016/j.apenergy.2018.02.149>
- Bacenetti, J., Sala, C., Fusi, A., Fiala, M., 2016. Agricultural anaerobic digestion plants : What LCA studies pointed out and what can be done to make them more environmentally sustainable. *Appl. Energy* 179, 669–686. <https://doi.org/10.1016/j.apenergy.2016.07.029>
- Baêta, B.E.L., Lima, D., Filho, J., Adarme, O., Gurgel, L., Aquino, S.F. De, 2016. Evaluation of hydrogen and methane production from sugarcane bagasse hemicellulose hydrolysates by two-stage anaerobic digestion process. *Bioresour. Technol.* 218, 436–446. <https://doi.org/10.1016/j.biortech.2016.06.113>
- Bajón Fernández, Y., Soares, A., Villa, R., Vale, P., Cartmell, E., 2014. Carbon capture and biogas enhancement by carbon dioxide enrichment of anaerobic digesters treating sewage sludge or

- food waste. *Bioresour. Technol.* 159, 1–7. <https://doi.org/10.1016/j.biortech.2014.02.010>
- Baldé, H., VanderZaag, A.C., Burt, S.D., Wagner-Riddle, C., Crolla, A., Desjardins, R.L., MacDonald, D.J., 2016. Methane emissions from digestate at an agricultural biogas plant. *Bioresour. Technol.* 216, 914–922. <https://doi.org/10.1016/j.biortech.2016.06.031>
- Bandounas, L., Wierckx, N.J.P., de Winde, J.H., Ruijsenaars, H.J., 2011. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. *BMC Biotechnol.* 11. <https://doi.org/10.1186/1472-6750-11-94>
- Barbera, E., Menegon, S., Banzato, D., Alpaos, C.D., Bertucco, A., 2019. From biogas to biomethane : A process simulation-based techno-economic comparison of different upgrading technologies in the Italian context. *Renew. Energy* 135, 663–673. <https://doi.org/10.1016/j.renene.2018.12.052>
- Bartoli, A., Hamelin, L., Rozakis, S., Borzęcka, M., Brandão, M., 2019. Coupling economic and GHG emission accounting models to evaluate the sustainability of biogas policies. *Renew. Sustain. Energy Rev.* 106, 133–148. <https://doi.org/10.1016/j.rser.2019.02.031>
- Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S.V., Pavlostathis, S.G., Rozzi, A., Sanders, W.T.M., Siegrist, H., Vavilin, V.A., 2002. Anaerobic digestion model No 1 (ADM1). *Water Res.* 45, 65–73.
- Bayard, R., Gonzalez-Ramirez, L., Guendouz, J., Benbelkacem, H., Buffière, P., Gourdon, R., 2015. Statistical Analysis to Correlate Bio-physical and Chemical Characteristics of Organic Wastes and Digestates to Their Anaerobic Biodegradability. *Waste and Biomass Valorization* 6, 759–769. <https://doi.org/10.1007/s12649-015-9411-2>
- Belletini, M.B., Fiorda, F.A., Maievas, H.A., Teixeira, G.L., Suelen, A., Hornung, P.S., Junior, A.M., Ribani, R.H., 2016. Factors affecting mushroom *Pleurotus* spp . *Saudi J. Biol. Sci.* <https://doi.org/10.1016/j.sjbs.2016.12.005>
- Bernet, N., Buffière, P., 2016. Caractérisation de la mise en oeuvre de la méthanisation, in: *La Méthanisation*. pp. 41–68.
- Bertide, A., 2018. Recherche de relations entre le microbiote du rumen et la production laitière des brebis Lacaune. Université Paul Sabatier (Toulouse 3), Ecole Nationale Vétérinaire de Toulouse.
- Biofuels, D.A., 2016. Enzyme Products for Biogas Production - Solutions for anaerobic digesters [WWW Document]. URL <http://www.dupont.com/content/dam/dupont/products-and-services/industrial-biotechnology/documents/DuPont-BiogasEnzymes-brochure-web-EN.pdf> (accessed 4.1.17).
- Bionova-Biogas GmBh, n.d. Plant references [WWW Document]. URL <https://bionova-biogas.de/fr/references.html>
- Biswas, R., Ahring, B.K., Uellendahl, H., 2012. Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure. *Water Sci. Technol.* 66, 1751–1758. <https://doi.org/10.2166/wst.2012.298>

- Björn, A., Šafarič, L., Karlsson, A., Danielsson, A., Ejlertsson, J., Svensson, B.H., Yekta, S.S., 2018. Substrate and operational conditions as regulators of fluid properties in full-scale continuous stirred-tank biogas reactors - implications for rheology-driven power requirements. *Water Sci. Technol.* 78, 814–826. <https://doi.org/10.2166/wst.2018.352>
- Bolzonella, D., Fatone, F., Gottardo, M., Frison, N., 2018. Nutrients recovery from anaerobic digestate of agro-waste : Techno-economic assessment of full scale applications. *J. Environ. Manage.* 216, 111–119. <https://doi.org/10.1016/j.jenvman.2017.08.026>
- Bowden, J., 2019. EU sees dual gas-power energy system as best bet to reach zero carbon by 2050 [WWW Document]. 11 Febr. 2019. URL <https://knect365.com/energy/article/ac8356d3-573e-431f-8f35-1803f1bb49c7/eu-sees-dual-gas-power-energy-system-as-best-bet-to-reach-zero-carbon-by-2050>
- Braun, F., Hamelin, J., Gévaudan, G., Patureau, D., 2011. Development and application of an enzymatic and cell flotation treatment for the recovery of viable microbial cells from environmental matrices such as anaerobic sludge. *Appl. Environ. Microbiol.* 77, 8487–8493. <https://doi.org/10.1128/AEM.05549-11>
- Brijwani, K., Rigdon, A., Vadlani, P. V., 2010. Fungal Laccases: Production, Function, and Applications in Food Processing. *Enzyme Res.* 2010, 1–10. <https://doi.org/10.4061/2010/149748>
- Brown, M.E., Chang, M.C.Y., 2014. Exploring bacterial lignin degradation. *Curr. Opin. Chem. Biol.* 19, 1–7. <https://doi.org/10.1016/j.cbpa.2013.11.015>
- Brun, C., 2018. European regulation process - GRT gaz [WWW Document]. Shipp. Meet. 2018. URL <http://www.grtgaz.com/fileadmin/clients/agenda/documents/fr/Presentation-Shippers-meeting-2018.pdf>
- Budzianowski, W.M., 2016. A review of potential innovations for production, conditioning and utilization of biogas with multiple-criteria assessment. *Renew. Sustain. Energy Rev.* 54, 1148–1171. <https://doi.org/10.1016/j.rser.2015.10.054>
- Carrere, H., Antonopoulou, G., Affes, R., Passos, F., Battimelli, A., Lyberatos, G., Ferrer, I., 2016. Review of feedstock pretreatment strategies for improved anaerobic digestion: From lab-scale research to full-scale application. *Bioresour. Technol.* 199, 386–397. <https://doi.org/10.1016/j.biortech.2015.09.007>
- Cerda, A., Mejias, L., Rodríguez, P., Rodríguez, A., Artola, A., Font, X., Gea, T., Sánchez, A., 2019. Valorisation of digestate from biowaste through solid-state fermentation to obtain value added bioproducts: A first approach. *Bioresour. Technol.* 271, 409–416. <https://doi.org/10.1016/j.biortech.2018.09.131>
- Chambre d’agriculture des Landes, 2018. *Essais couverts végétaux.*
- Chandra, R., Raj, A., Purohit, H.J., Kapley, A., 2007. Characterisation and optimisation of three

- potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste. *Chemosphere* 67, 839–846. <https://doi.org/10.1016/j.chemosphere.2006.10.011>
- Chang, S.T., Hayes, W.A., 1978. The biology and cultivation of edible mushrooms.
- Chao, A., Chiu, C., Jost, L., 2014. Unifying Species Diversity , Phylogenetic Diversity , Functional Diversity , and Related Similarity and Differentiation Measures Through Hill Numbers. *Annu. Rev. Ecol. Evol. Syst.* 45, 297–324. <https://doi.org/10.1146/annurev-ecolsys-120213-091540>
- Chao, A., Chiu, C.H., 2016. Bridging the variance and diversity decomposition approaches to beta diversity via similarity and differentiation measures. *Methods Ecol. Evol.* 7, 919–928. <https://doi.org/10.1111/2041-210X.12551>
- Chen, Y.R., 1986. Rheological properties of sieved beef-cattle manure slurry: Rheological model and effects of temperature and solids concentration. *Agric. Wastes* 15, 17–33. [https://doi.org/10.1016/0141-4607\(86\)90123-X](https://doi.org/10.1016/0141-4607(86)90123-X)
- Chiavari, G., Francioso, O., Galletti, G.C., Piccaglia, R., Zadrazil, F., 1989. Characterization by pyrolysis-gas chromatography of wheat straw fermented with white rot fungus *Stropharia Rugosoannulata*. *J. Anal. Appl. Pyrolysis* 15, 129–136.
- Chiumenti, A., Borso, F., Limina, S., 2018. Dry anaerobic digestion of cow manure and agricultural products in a full-scale plant : Efficiency and comparison with wet fermentation. *Waste Manag.* 71, 704–710. <https://doi.org/10.1016/j.wasman.2017.03.046>
- Clarke, K.R., Ainsworth, M., 1993. A method of linking multivariate community structure to environmental variables. *Mar. Ecol. Prog. Ser.* 92, 205–219. <https://doi.org/10.3354/meps092205>
- COM(2018)-773, 2018. A Clean Planet for all A European strategic long-term vision for a prosperous, modern, competitive and climate neutral economy.
- Couturier, C., 2009. Techniques de production d'électricité à partir de biogaz et de gaz de synthèse.
- D'Adamo, I., Falcone, P.M., Ferella, F., 2019. A socio-economic analysis of biomethane in the transport sector: The case of Italy. *Waste Manag.* 95, 102–115. <https://doi.org/10.1016/j.wasman.2019.06.005>
- da Silva, R.R., Peduzzi, R., Souto, T.B., 2017. Exploring the bioprospecting and biotechnological potential of white-rot and anaerobic Neocallimastigomycota fungi: peptidases, esterases, and lignocellulolytic enzymes. *Appl. Microbiol. Biotechnol.* 101, 3089–3101. <https://doi.org/10.1007/s00253-017-8225-5>
- Dale, B.E., Sibilla, F., Fabbri, C., Pezzaglia, M., Pecorino, B., Baronchelli, A., Veggia, E., Gattoni, P., Bozzetto, S., 2016. Biogasdoneright™: An innovative new system is commercialized in Italy. *Biofuels, Bioprod. Biorefining* 10, 341–345. <https://doi.org/10.1002/bbb>
- Das, N., Mukherjee, M., 2007. Cultivation of *Pleurotus ostreatus* on weed plants. *Bioresour. Technol.*

- 98, 2723–2726. <https://doi.org/10.1016/j.biortech.2006.09.061>
- de Guardia, A., Mallard, P., Teglia, C., Marin, A., Le Pape, C., Launay, M., Benoist, J.C., Petiot, C., 2010. Comparison of five organic wastes regarding their behaviour during composting: Part 2, nitrogen dynamic. *Waste Manag.* 30, 415–425. <https://doi.org/10.1016/j.wasman.2009.10.018>
- Debeer, L., Velghe, F., Liebetrau, J., Müller, L., 2019. Assessing the effect of enzyme application in anaerobic digestion: experiences from the DEMETER project, in: 16th World Conference on Anaerobic Digestion.
- Degremont, 2007. *Water Treatment Handbook*, 7th ed.
- Deng, Y., Huang, Z., Ruan, W., Zhao, M., Miao, H., Ren, H., 2017. Co-inoculation of cellulolytic rumen bacteria with methanogenic sludge to enhance methanogenesis of rice straw. *Int. Biodeterior. Biodegradation* 117, 224–235. <https://doi.org/10.1016/j.ibiod.2017.01.017>
- DIRECTIVE-(EU)-2018/2001, 2018. DIRECTIVE (EU) 2018/2001 on the promotion of the use of energy from renewable sources (recast).
- Divya, D., Gopinath, L.R., Merlin Christy, P., 2015. A review on current aspects and diverse prospects for enhancing biogas production in sustainable means. *Renew. Sustain. Energy Rev.* 42, 690–699. <https://doi.org/10.1016/j.rser.2014.10.055>
- Dodd, D., Mackie, R.I., Cann, I.K.O., 2011. Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes. *Mol. Microbiol.* 79, 292–304. <https://doi.org/10.1111/j.1365-2958.2010.07473.x>
- Donoso-Bravo, A., García, G., Pérez-Elvira, S., Fdz-Polanco, F., 2011. Initial rates technique as a procedure to predict the anaerobic digester operation. *Biochem. Eng. J.* 53, 275–280. <https://doi.org/10.1016/j.bej.2010.11.007>
- Du, J., Qian, Y., Xi, Y., Lü, X., 2019. Hydrothermal and alkaline thermal pretreatment at mild temperature in solid state for physicochemical properties and biogas production from anaerobic digestion of rice straw. *Renew. Energy* 139, 261–267. <https://doi.org/10.1016/j.renene.2019.01.097>
- EBA, 2018. *Statistical Report 2018*.
- Eichlerová, I., Homolka, L., Žifčáková, L., Lisá, L., Dobiášová, P., Baldrian, P., 2015. Enzymatic systems involved in decomposition reflects the ecology and taxonomy of saprotrophic fungi. *Fungal Ecol.* 13, 10–22. <https://doi.org/10.1016/j.funeco.2014.08.002>
- Enea Consulting, 2019. *Revue des externalités positives de la filière biométhane* 1–21.
- Esposito, E., Dellamuzia, L., Moretti, U., Fuoco, A., Giorno, L., Jansen, J.C., 2019. Simultaneous production of biomethane and food grade CO₂ from biogas: An industrial case study. *Energy Environ. Sci.* 12, 281–289. <https://doi.org/10.1039/c8ee02897d>
- Eurelectric, 2018. *Decarbonisation Pathways*.

- EurObserv'ER, 2018. The state of renewable energies in europe edition 2018.
- European Commission, 2014. JEC WELL-TO-WHEELS ANALYSIS.
- Eyl-Mazzega, M.-A., Mathieu, C., 2019. Biogas and Biomethane in Europe Lessons from Denmark, Germany and Italy.
- Fabbri, D., Torri, C., 2016. Linking pyrolysis and anaerobic digestion (Py-AD) for the conversion of lignocellulosic biomass. *Curr. Opin. Biotechnol.* 38, 167–173.
<https://doi.org/10.1016/j.copbio.2016.02.004>
- Fang, W., Zhang, P., Zhang, X., Zhu, X., Lier, J.B. Van, Spanjers, H., 2018. White rot fungi pretreatment to advance volatile fatty acid production from solid-state fermentation of solid digestate : Efficiency and mechanisms. *Energy* 162, 534–541.
<https://doi.org/10.1016/j.energy.2018.08.082>
- Fang, X., Li, Q., Lin, Y., Lin, X., Dai, Y., Guo, Z., Pan, D., 2018. Screening of a microbial consortium for selective degradation of lignin from tree trimmings. *Bioresour. Technol.* 254, 247–255.
<https://doi.org/10.1016/j.biortech.2018.01.058>
- Fink, J., 2013. How Pretreatment Reduces Contamination and Aids Digestion, in: UK AD & Biogas, the Anaerobic Digestion & Biogas Association's Annual Expo and Conference.
- Florence School of Regulation, 2019. Exclusive Interview with Borchardt (EC)! From Madrid Forum to the Gas Package [WWW Document]. URL <https://fsr.eui.eu/exclusive-interview-with-borchardt-ec-from-madrid-forum-to-the-gas-package/>
- Flotats, X., 2019. Biogas: Perspectives of an Old Technology, in: *Biorefinery Integrated Sustainable Processes for Biomass Conversion to Biomaterials, Biofuels, and Fertilizers*. pp. 313–349.
- FNR, 2012. Guide to Biogas: From production to use, Fachagentur Nachwachsende Rohstoffe e. V. (FNR) FNR,.
- Frigon, J.C., Mehta, P., Guiot, S.R., 2012. Impact of mechanical, chemical and enzymatic pre-treatments on the methane yield from the anaerobic digestion of switchgrass. *Biomass and Bioenergy* 36, 1–11. <https://doi.org/10.1016/j.biombioe.2011.02.013>
- Fu, S., Chen, K., Zhu, R., Sun, W., Zou, H., Guo, R., 2018. Improved anaerobic digestion performance of *Miscanthus floridulus* by different pretreatment methods and preliminary economic analysis. *Energy Convers. Manag.* 159, 121–128. <https://doi.org/10.1016/j.enconman.2018.01.014>
- Fu, S., Shi, X., Dai, M., Guo, R., 2016. Effect of Different Mixed Microflora on the Performance of Thermophilic Microaerobic Pretreatment. *Energy Fuels* 30, 6413–6418.
<https://doi.org/10.1021/acs.energyfuels.6b00440>
- Fu, S., Wang, F., Yuan, X., Yang, Z., Luo, S., 2015. The thermophilic (55°C) microaerobic pretreatment of corn straw for anaerobic digestion. *Bioresour. Technol.* 175, 203–208.
<https://doi.org/10.1016/j.biortech.2014.10.072>

- Funke, A., Reeb, F., Kruse, A., 2013. Experimental comparison of hydrothermal and vapothermal carbonization. *Fuel Process. Technol.* 115, 261–269.
<https://doi.org/10.1016/j.fuproc.2013.04.020>
- Future biogas Systems, n.d. Economizer leaflet [WWW Document]. URL
<http://www.futurebiogas.com/wp-content/uploads/2017/02/The-Economizer.pdf> (accessed 10.1.19).
- Gallegos, D., Wedwitschka, H., Moeller, L., Zehnsdorf, A., Stinner, W., 2017. Effect of particle size reduction and ensiling fermentation on biogas formation and silage quality of wheat straw. *Bioresour. Technol.* <https://doi.org/10.1016/j.biortech.2017.08.137>
- Garlapalli, R.K., Wirth, B., Reza, M.T., 2016. Pyrolysis of hydrochar from digestate : Effect of hydrothermal carbonization and pyrolysis temperatures on pyrochar formation. *Bioresour. Technol.* 220, 168–174. <https://doi.org/10.1016/j.biortech.2016.08.071>
- Garuti, M., Langone, M., Fabbri, C., Piccinini, S., 2018. Monitoring of full-scale hydrodynamic cavitation pretreatment in agricultural biogas plant. *Bioresour. Technol.* 247, 599–609.
<https://doi.org/10.1016/j.biortech.2017.09.100>
- Gas for Climate, 2019a. Gas for Climate. The optimal role for gas in a net-zero emissions energy system.
- Gas for Climate, 2019b. Action Plan 2030.
- Gerhardt, M., Pelenc, V., Bäuml, M., 2007. Application of hydrolytic enzymes in the agricultural biogas production: Results from practical applications in Germany. *Biotechnol. J.* 2, 1481–1484.
<https://doi.org/10.1002/biot.200700220>
- Gil-carrera, L., Browne, J.D., Kilgallon, I., Murphy, J.D., 2019. Feasibility study of an off-grid biomethane mobile solution for agri-waste. *Appl. Energy* 239, 471–481.
<https://doi.org/10.1016/j.apenergy.2019.01.141>
- Giroto, F., Peng, W., Rafieenia, R., Cossu, R., 2016. Effect of Aeration Applied During Different Phases of Anaerobic Digestion. *Waste and Biomass Valorization.* <https://doi.org/10.1007/s12649-016-9785-9>
- Godon, J.-J., 2016. Aspects biochimiques et microbiologiques de la méthanisation, in: *La Méthanisation.* pp. 11–37.
- Guan, R., Li, X., Wachemo, A.C., Yuan, H., Liu, Y., Zou, D., Zuo, X., Gu, J., 2018. Enhancing anaerobic digestion performance and degradation of lignocellulosic components of rice straw by combined biological and chemical pretreatment. *Sci. Total Environ.* 637–638, 9–17.
<https://doi.org/10.1016/j.scitotenv.2018.04.366>
- Guilayn, F., Jimenez, J., Martel, J., Rouez, M., Crest, M., Patureau, D., 2019a. First fertilizing-value typology of digestates : A decision-making tool for regulation. *Waste Manag.* 86, 67–79.

- <https://doi.org/10.1016/j.wasman.2019.01.032>
- Guilayn, F., Jimenez, J., Rouez, M., Crest, M., Patureau, D., 2019b. Digestate mechanical separation : Efficiency profiles based on anaerobic digestion feedstock and equipment choice. *Bioresour. Technol.* 274, 180–189. <https://doi.org/10.1016/j.biortech.2018.11.090>
- H2020, 2016. Demeter H2020 project [WWW Document]. URL <http://www.demeter-eu-project.eu/>
- Hadziavdic, K., Lekang, K., Lanzen, A., Jonassen, I., Thompson, E.M., Troedsson, C., 2014. Characterization of the 18s rRNA gene for designing universal eukaryote specific primers. *PLoS One* 9. <https://doi.org/10.1371/journal.pone.0087624>
- Haegeman, B., Hamelin, J., Moriarty, J., Neal, P., Dushoff, J., Weitz, J.S., 2013. Robust estimation of microbial diversity in theory and in practice. *ISME J.* 7, 1092–1101. <https://doi.org/10.1038/ismej.2013.10>
- Hagos, K., Zong, J., Li, D., Liu, C., Lu, X., 2017. Anaerobic co-digestion process for biogas production : Progress , challenges and perspectives. *Renew. Sustain. Energy Rev.* 76, 1485–1496. <https://doi.org/10.1016/j.rser.2016.11.184>
- Healy, J.B., Young, L.Y., 1979. Anaerobic biodegradation of eleven aromatic compounds to methane. *Appl. Environ. Microbiol.* 38, 84–89.
- Henske, J.K., Gilmore, S.P., Knop, D., Cunningham, F.J., Sexton, J.A., Smallwood, C.R., Shutthanandan, V., Evans, J.E., Theodorou, M.K., O'Malley, M.A., 2017. Transcriptomic characterization of *Caecomyces churrovis*: A novel, non-rhizoid-forming lignocellulolytic anaerobic fungus. *Biotechnol. Biofuels* 10, 1–12. <https://doi.org/10.1186/s13068-017-0997-4>
- Hernandez, D., Sanchez, J.E., Yamasaki, K., 2003. A simple procedure for preparing substrate for *Pleurotus ostreatus* cultivation. *Bioresour. Technol.* 90, 145–150. [https://doi.org/10.1016/S0960-8524\(03\)00118-4](https://doi.org/10.1016/S0960-8524(03)00118-4)
- Herrero Garcia, N., Benedetti, M., Bolzonella, D., 2019. Effects of Enzymes Addition on Biogas Production From Anaerobic Digestion of Agricultural Biomasses. *Waste and Biomass Valorization*. <https://doi.org/10.1007/s12649-019-00698-7>
- Herrmann, C., Heiermann, M., Idler, C., 2011. Effects of ensiling , silage additives and storage period on methane formation of biogas crops. *Bioresour. Technol.* 102, 5153–5161. <https://doi.org/10.1016/j.biortech.2011.01.012>
- Hijazi, O., Munro, S., Zerhusen, B., Effenberger, M., 2016. Review of life cycle assessment for biogas production in Europe. *Renew. Sustain. Energy Rev.* 54, 1291–1300. <https://doi.org/10.1016/j.rser.2015.10.013>
- Hill, J.S., 2019. Solar Dominates Latest German Renewable Auction [WWW Document]. *Clean Tech*. URL <https://cleantechnica.com/2019/04/23/solar-dominates-latest-german-renewable-auction/>

- Hills, D.J., 1979. Effects of carbon: Nitrogen ratio on anaerobic digestion of dairy manure. *Agric. Wastes* 1, 267–278. [https://doi.org/10.1016/0141-4607\(79\)90011-8](https://doi.org/10.1016/0141-4607(79)90011-8)
- Holliger, C., Alves, M., Andrade, D., Angelidaki, I., Astals, S., Baier, U., Bougrier, C., Buf, P., Carballa, M., Wilde, V. De, Ebertseder, F., Fernández, B., Ficara, E., Fotidis, I., Frigon, J., Lacos, H.F. De, Ghasimi, S.M., Hack, G., Hartel, M., Heerenklage, J., Horvath, I.S., Jenicek, P., Koch, K., Krautwald, J., Lizasoain, J., Liu, J., Mosberger, L., Nistor, M., Oechsner, H., Oliveira, J.V., Paterson, M., Pauss, A., Pommier, S., Porqueddu, I., Raposo, F., Ribeiro, T., Pfund, F.R., Strömberg, S., Torrijos, M., Hack, G., Andrade, D., Bougrier, C., Koch, K., 2016. Towards a standardization of biomethane potential tests. *Water Sci. Technol.* 74, 2515–2522. <https://doi.org/10.2166/wst.2016.336>
- Holliger, C., Fruteau de Lacos, H., Hack, G., 2017. Methane Production of Full-Scale Anaerobic Digestion Plants Calculated from Substrate's Biomethane Potentials Compares Well with the One Measured On-Site. *Front. Energy Res.* 5, 1–9. <https://doi.org/10.3389/fenrg.2017.00012>
- Hönig, V., Prochazka, P., Obergruber, M., Smutka, L., Kucerová, V., 2019. Economic and Technological Analysis of Commercial LNG Production in the EU. *Energies* 12, 1–17.
- Hreiz, R., Adouani, N., Fünfschilling, D., Marchal, P., Pons, M.N., 2017. Rheological characterization of raw and anaerobically digested cow slurry. *Chem. Eng. Res. Des.* 119, 47–57. <https://doi.org/10.1016/j.cherd.2017.01.005>
- Hua, B., Dai, J., Liu, B., Zhang, H., Yuan, X., Wang, X., Cui, Z., 2016. Pretreatment of non-sterile, rotted silage maize straw by the microbial community MC1 increases biogas production. *Bioresour. Technol.* 216, 699–705. <https://doi.org/10.1016/j.biortech.2016.06.001>
- Ince, O., Ozbayram, E.G., Akyol, Ç., Erdem, E.I., Gunel, G., Ince, B., 2018. Bacterial Succession in the Thermophilic Phase of Composting of Anaerobic Digestates. *Waste and Biomass Valorization* 0, 0. <https://doi.org/10.1007/s12649-018-0531-3>
- IPCC, 2018. Global Warming of 1.5°C - Special Report.
- IRENA, 2019. Renewable Capacity Statistics 2019.
- IRENA, 2018. Power Generation Costs in 2017.
- IWA, 2002. Anaerobic Digestion Model No.1 (ADM1).
- Jagadabhi, P.S., Lehtomäki, A., Rintala, J., 2008. Co-digestion of grass silage and cow manure in a CSTR by re-circulation of alkali treated solids of the digestate. *Environ. Technol.* 29, 1085–1093. <https://doi.org/10.1080/09593330802180385>
- Jamaludin, Z., Rollings-Scattergood, S., Lutes, K., Vaneckhaute, C., 2018. Evaluation of sustainable scrubbing agents for ammonia recovery from anaerobic digestate. *Bioresour. Technol.* 270, 596–602. <https://doi.org/10.1016/j.biortech.2018.09.007>
- Jasmina, Ć., Gali, M., Vukojevi, J., Br, I., Staji, M., 2017. Potential of selected fungal species to degrade

- wheat straw , the most abundant plant raw material in Europe. *BMC Plant Biol.* 17, 249.
<https://doi.org/10.1186/s12870-017-1196-y>
- Jimenez, J., Gonidec, E., Cacho Rivero, J.A., Latrille, E., Vedrenne, F., Steyer, J.P., 2014. Prediction of anaerobic biodegradability and bioaccessibility of municipal sludge by coupling sequential extractions with fluorescence spectroscopy: Towards ADM1 variables characterization. *Water Res.* 50, 359–372. <https://doi.org/10.1016/j.watres.2013.10.048>
- Jimenez, J., Grelier, P., Meinhold, J., Tazi-Pain, A., 2010. Biological modelling of MBR and impact of primary sedimentation. *Desalination* 250, 562–567.
<https://doi.org/10.1016/j.desal.2009.09.024>
- Jimenez, J., Lei, H., Steyer, J.P., Houot, S., Patureau, D., 2017. Methane production and fertilizing value of organic waste: Organic matter characterization for a better prediction of valorization pathways. *Bioresour. Technol.* 241, 1012–1021. <https://doi.org/10.1016/j.biortech.2017.05.176>
- Jost, L., 2007. Partitioning diversity into independent alpha and beta components. *Ecology* 88, 2427–2439.
- Jurado, E., Skiadas, I. V., Gavala, H.N., 2013. Enhanced methane productivity from manure fibers by aqueous ammonia soaking pretreatment. *Appl. Energy* 109, 104–111.
<https://doi.org/10.1016/j.apenergy.2013.03.075>
- Kamarád, L., Pohn, S., Bochmann, G., Harasek, M., 2013. Determination of mixing quality in biogas plant digesters using tracer tests and computational fluid dynamics. *Acta Univ. Agric. Silvic. Mendelianae Brun.* 61, 1269–1278. <https://doi.org/10.11118/actaun201361051269>
- Kamimura, N., Sakamoto, S., Mitsuda, N., Masai, E., Kajita, S., 2019. Advances in microbial lignin degradation and its applications. *Curr. Opin. Biotechnol.* 56, 179–186.
<https://doi.org/10.1016/j.copbio.2018.11.011>
- Kamimura, N., Takahashi, K., Mori, K., Araki, T., Fujita, M., Higuchi, Y., Masai, E., 2017. Bacterial catabolism of lignin-derived aromatics: New findings in a recent decade. *Environ. Microbiol. Rep.* 9, 679–705. <https://doi.org/10.1111/1758-2229.12597>
- Kampman, B., Leguijt, C., Scholten, T., Tallat-Kelpsaite, J., Brückmann, R., Maroulis, G., Lesschen, J.P., Meesters, K., Sikirica, N., Elbersen, B., 2016. Optimal use of biogas from waste streams - An assessment of the potential of biogas from digestion in the EU beyond 2020.
- Kaparaju, P.L.N., Rintala, J.A., 2005. The Effects of Post-Treatments and Temperature on Recovering the Methane Potential of > 2 MM Solid Fraction of Digested Cow Manure. *Environ. Technol.* 26, 625–632. <https://doi.org/10.1080/09593330.2005.9619502>
- Kim, J.S., Lee, Y.Y., Kim, T.H., 2016. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresour. Technol.* 199, 42–48.
<https://doi.org/10.1016/j.biortech.2015.08.085>

- Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. rrndb: the Ribosomal RNA Operon Copy Number Database. *Nucleic Acids Res.* 29, 181–184. <https://doi.org/10.1093/nar/29.1.181>
- Knop, D., Yarden, O., Hadar, Y., 2015. The ligninolytic peroxidases in the genus *Pleurotus* : divergence in activities, expression, and potential applications. *Appl. Microbiol. Biotechnol.* 99, 1025–1038. <https://doi.org/10.1007/s00253-014-6256-8>
- Kratky, L., Jirout, T., 2011. Biomass Size Reduction Machines for Enhancing Biogas Production. *Chem. Eng. Technol.* 34, 391–399. <https://doi.org/10.1002/ceat.201000357>
- Kratzeisen, M., Starcevic, N., Martinov, M., Maurer, C., Müller, J., 2010. Applicability of biogas digestate as solid fuel. *Fuel* 89, 2544–2548. <https://doi.org/10.1016/j.fuel.2010.02.008>
- Kress, P., Nägele, H.J., Oechsner, H., Ruile, S., 2018. Effect of agitation time on nutrient distribution in full-scale CSTR biogas digesters. *Bioresour. Technol.* 247, 1–6. <https://doi.org/10.1016/j.biortech.2017.09.054>
- Kreuger, E., Nges, I.A., Björnsson, L., 2011. Ensiling of crops for biogas production: effects on methane yield and total solids determination. *Biotechnol. Biofuels* 4, 1–8. <https://doi.org/doi:10.1186/1754-6834-4-44>
- Lalak, J., Kasprzycka, A., Martyniak, D., Tys, J., 2016. Effect of biological pretreatment of *Agropyron elongatum* “BAMAR” on biogas production by anaerobic digestion. *Bioresour. Technol.* 200, 194–200. <https://doi.org/10.1016/j.biortech.2015.10.022>
- Lantz, M., 2012. The economic performance of combined heat and power from biogas produced from manure in Sweden – A comparison of different CHP technologies. *Appl. Energy* 98, 502–511. <https://doi.org/10.1016/j.apenergy.2012.04.015>
- Larsson, M., Gronkvist, S., Alvfors, P., 2016. Upgraded biogas for transport in Sweden e effects of policy instruments on production, infrastructure deployment and vehicle sales. *J. Clean. Prod.* 112, 3774–3784. <https://doi.org/10.1016/j.jclepro.2015.08.056>
- Lauven, L., Geldermann, J., Desideri, U., 2019. Estimating the revenue potential of flexible biogas plants in the power sector. *Energy Policy* 128, 402–410. <https://doi.org/10.1016/j.enpol.2019.01.007>
- Lazuka, A., Auer, L., Bozonnet, S., Morgavi, D.P., O’Donohue, M., Hernandez-Raquet, G., 2015. Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. *Bioresour. Technol.* 196, 241–249. <https://doi.org/10.1016/j.biortech.2015.07.084>
- Lee, D., 2017. Evaluation the financial feasibility of biogas upgrading to biomethane , heat , CHP and AwR. *Int. J. Hydrogen Energy* 42, 27718–27731. <https://doi.org/10.1016/j.ijhydene.2017.07.030>
- Legendre, P., Legendre, L., 2012. *Numerical Ecology*, Third Engl. ed.
- Leonowicz, A., Cho, N., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A.,

- Hofrichter, M., Wesenberg, D., Rogalski, J., 2001. Fungal laccase: Properties and activity on lignin. *J. Basic Microbiol.* 41, 185–227. [https://doi.org/10.1002/1521-4028\(200107\)41:3/4<185::AID-JOBM185>3.0.CO;2-T](https://doi.org/10.1002/1521-4028(200107)41:3/4<185::AID-JOBM185>3.0.CO;2-T)
- Li, F., Zhang, P., Zhang, G., Tang, X., Wang, S., 2016. Enhancement of corn stover hydrolysis with rumen fluid pretreatment at different solid contents : Effect , structural changes and enzymes participation. *Int. Biodeterior. Biodegradation* 1–8. <https://doi.org/10.1016/j.ibiod.2016.10.038>
- Li, W., Guo, J., Cheng, H., Wang, W., Dong, R., 2017. Two-phase anaerobic digestion of municipal solid wastes enhanced by hydrothermal pretreatment : Viability, performance and microbial community evaluation. *Appl. Energy* 189, 613–622. <https://doi.org/10.1016/j.apenergy.2016.12.101>
- Li, Y., Luo, W., Lu, J., Zhang, X., Li, S., Wu, Y., Li, G., 2018. Effects of digestion time in anaerobic digestion on subsequent digestate composting. *Bioresour. Technol.* 267, 117–125. <https://doi.org/10.1016/j.biortech.2018.04.098>
- Liang, J., Fang, X., Lin, Y., Wang, D., 2018. A new screened microbial consortium OEM2 for lignocellulosic biomass deconstruction and chlorophenols detoxification. *J. Hazard. Mater.* 347, 341–348. <https://doi.org/10.1016/j.jhazmat.2018.01.023>
- Lijó, L., Lorenzo-toja, Y., González-garcía, S., Bacenetti, J., Negri, M., Teresa, M., 2017. Eco-efficiency assessment of farm-scaled biogas plants. *Bioresour. Technol.* 237, 146–155. <https://doi.org/10.1016/j.biortech.2017.01.055>
- Lin, L., Xu, F., Ge, X., Li, Y., 2018. Improving the sustainability of organic waste management practices in the food-energy-water nexus: A comparative review of anaerobic digestion and composting. *Renew. Sustain. Energy Rev.* 89, 151–167. <https://doi.org/10.1016/j.rser.2018.03.025>
- Lindner, J., Zielonka, S., Oechsner, H., Lemmer, A., 2016. Is the continuous two-stage anaerobic digestion process well suited for all substrates? *Bioresour. Technol.* 200, 470–476. <https://doi.org/10.1016/j.biortech.2015.10.052>
- Lindner, J., Zielonka, S., Oechsner, H., Lemmer, A., 2015. Effects of mechanical treatment of digestate after anaerobic digestion on the degree of degradation. *Bioresour. Technol.* 178, 194–200. <https://doi.org/10.1016/j.biortech.2014.09.117>
- Liu, S., Xu, F., Ge, X., Li, Y., 2016. Comparison between ensilage and fungal pretreatment for storage of giant reed and subsequent methane production. *Bioresour. Technol.* 209, 246–253. <https://doi.org/10.1016/j.biortech.2016.02.129>
- Liu, T., Zhou, X., Li, Z., Wang, X., Sun, J., 2019. Effects of liquid digestate pretreatment on biogas production for anaerobic digestion of wheat straw. *Bioresour. Technol.* 280, 345–351. <https://doi.org/10.1016/j.biortech.2019.01.147>
- Liu, X., Hilgsmann, S., Gourdon, R., Bayard, R., 2017. Anaerobic digestion of lignocellulosic biomasses

- pretreated with *Ceriporiopsis subvermispora*. *J. Environ. Manage.* 193, 154–162.
<https://doi.org/10.1016/j.jenvman.2017.01.075>
- Liu, X., Zicari, S.M., Liu, G., Li, Y., Zhang, R., 2015. Pretreatment of wheat straw with potassium hydroxide for increasing enzymatic and microbial degradability. *Bioresour. Technol.* 185, 150–157. <https://doi.org/10.1016/j.biortech.2015.02.047>
- Liu, Y., Chen, J., Song, J., Hai, Z., Lu, X., Ji, X., Wang, C., 2019. Adjusting the rheological properties of corn-straw slurry to reduce the agitation power consumption in anaerobic digestion. *Bioresour. Technol.* 272, 360–369. <https://doi.org/10.1016/j.egypro.2019.01.314>
- Lozupone, C., Knight, R., 2005. UniFrac : a New Phylogenetic Method for Comparing Microbial Communities. *Appl. Environ. Microbiol.* 71, 8228–8235.
<https://doi.org/10.1128/AEM.71.12.8228>
- Maldaner, L., Wagner-riddle, C., Vanderzaag, A.C., Gordon, R., Duke, C., 2018. Methane emissions from storage of digestate at a dairy manure biogas facility. *Agric. For. Meteorol.* 258, 96–107.
<https://doi.org/10.1016/j.agrformet.2017.12.184>
- Mao, C., Feng, Y., Wang, X., Ren, G., 2015. Review on research achievements of biogas from anaerobic digestion. *Renew. Sustain. Energy Rev.* 45, 540–555.
<https://doi.org/10.1016/j.rser.2015.02.032>
- Markard, J., Wirth, S., Truffer, B., 2016. Institutional dynamics and technology legitimacy - A framework and a case study on biogas technology. *Res. Policy* 45, 330–344.
<https://doi.org/10.1016/j.respol.2015.10.009>
- Martin-ryals, A., Schideman, L., Li, P., Wilkinson, H., Wagner, R., 2015. Improving anaerobic digestion of a cellulosic waste via routine bioaugmentation with cellulolytic microorganisms. *Bioresour. Technol.* 189, 62–70. <https://doi.org/10.1016/j.biortech.2015.03.069>
- Mathworks, C., 2019. MATLAB and Optimization Toolbox™ Release R2019.
- Maynaud, G., Druilhe, C., Daumoin, M., Jimenez, J., Patureau, D., Torrijos, M., Pourcher, A.M., Wéry, N., 2017. Characterisation of the biodegradability of post-treated digestates via the chemical accessibility and complexity of organic matter. *Bioresour. Technol.* 231, 65–74.
<https://doi.org/10.1016/j.biortech.2017.01.057>
- Mbaye, S., Dieudé-Fauvel, E., Baudez, J.C., 2014. Comparative analysis of anaerobically digested wastes flow properties. *Waste Manag.* 34, 2057–2062.
<https://doi.org/10.1016/j.wasman.2014.06.021>
- McCabe, B.K., Schmidt, T., 2018. Integrated biogas systems - Local applications of anaerobic digestion towards integrated sustainable solutions.
- Mckee, L.S., Martínez-Abad, A., Ruthes, A.C., Vilaplana, F., Brumer, H., 2019. Focused Metabolism of Beta-Glucans by the Soil Bacteroidetes Species *Chitinophaga pinensis*. *Appl. Environ. Microbiol.*

85, 1–15.

- McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* 8.
<https://doi.org/10.1371/journal.pone.0061217>
- Meadows, D.H., Meadows, D.L., Randers, J., Behrens III, W.W., 1972. *The Limits to Growth - A Report for THE CLUB OF ROME'S Project on the Predicament of Mankind*. Universe Books.
- Mejias, L., Cerda, A., Barrena, R., Gea, T., 2018. Microbial Strategies for Cellulase and Xylanase Production through Solid-State Fermentation of Digestate from Biowaste. *Sustainability* 10, 2433. <https://doi.org/10.3390/su10072433>
- Menardo, S., Balsari, P., Dinuccio, E., Gioelli, F., 2011a. Thermal pre-treatment of solid fraction from mechanically-separated raw and digested slurry to increase methane yield. *Bioresour. Technol.* 102, 2026–2032. <https://doi.org/10.1016/j.biortech.2010.09.067>
- Menardo, S., Gioelli, F., Balsari, P., 2011b. The methane yield of digestate: Effect of organic loading rate, hydraulic retention time, and plant feeding. *Bioresour. Technol.* 102, 2348–2351. <https://doi.org/10.1016/j.biortech.2010.10.094>
- Merlin Christy, P., Gopinath, L.R., Divya, D., 2014. A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. *Renew. Sustain. Energy Rev.* 34, 167–173. <https://doi.org/10.1016/j.rser.2014.03.010>
- Meyer, A.K.P., Ehimen, E.A., Holm-nielsen, J.B., 2018. Future European biogas : Animal manure, straw and grass potentials for a sustainable European biogas production. *Biomass and Bioenergy* 111, 154–164. <https://doi.org/10.1016/j.biombioe.2017.05.013>
- Michalska, K., Bizukojć, M., Ledakowicz, S., 2015. Pretreatment of energy crops with sodium hydroxide and cellulolytic enzymes to increase biogas production. *Biomass and Bioenergy* 80, 213–221. <https://doi.org/10.1016/j.biombioe.2015.05.022>
- Ministère de la transition écologique et solidaire, 2019. *Stratégie Française pour l'énergie et le climat - Programmation pluriannuelle de l'énergie - Synthèse*.
- Ministry of Climate Energy and Utilities, 2018. *Energy Agreement - Denmark*.
- Moletta, R., Berge, S., Bernet, N., Bouchez, T., Braumann, A., Buffière, P., Bultel, Y., Camacho, P., Carrère, H., Chatain, V., Couturier, C., Fonty, G., Fouletier, J., Germain, P., Godon, J.J., Guiot, S., Hernandez-Raquet, G., Hirtzberger, P., Klein, J.M., Latrille, E., Molleta-Denat, M., Noyola, A., Ohanessian, A., Patureau-Steyer, D., Prévot, D., Roger, C., Servais, C., Steyer, J.P., Verstaete, W., 2008. *La Méthanisation*.
- Mönch-Tegeder, M., Lemmer, A., Hinrichs, J., Oechsner, H., 2015. Development of an in-line process viscometer for the full-scale biogas process. *Bioresour. Technol.* 178, 278–284. <https://doi.org/10.1016/j.biortech.2014.08.041>

- Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P., Carrère, H., 2013. Lignocellulosic Materials Into Biohydrogen and Biomethane: Impact of Structural Features and Pretreatment. *Crit. Rev. Environ. Sci. Technol.* 43, 260–322. <https://doi.org/10.1080/10643389.2011.604258>
- Monlau, F., Sambusiti, C., Antoniou, N., Barakat, A., Zabaniotou, A., 2015. A new concept for enhancing energy recovery from agricultural residues by coupling anaerobic digestion and pyrolysis process. *Appl. Energy* 148, 32–38. <https://doi.org/10.1016/j.apenergy.2015.03.024>
- Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J.P., Carrère, H., 2014. Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. *Biotechnol. Adv.* 32, 934–951. <https://doi.org/10.1016/j.biotechadv.2014.04.007>
- Monlau, F., Sambusiti, C., Ficara, E., Aboulkas, A., Barakat, A., Carrère, H., 2015. New opportunities for agricultural digestate valorization : current situation and perspectives. *Energy Environ. Sci.* 8, 2600–2621. <https://doi.org/10.1039/C5EE01633A>
- Montgomery, L.F.R., Bochmann, G., 2014. Pretreatment of feedstock for enhanced biogas production.
- Montgomery, L.F.R., Schopp, T., Fuchs, W., Bochmann, G., 2016. Design, calibration and validation of a large lab-scale system for measuring viscosity in fermenting substrate from agricultural anaerobic digesters. *Biochem. Eng. J.* 115, 72–79. <https://doi.org/10.1016/j.bej.2016.08.009>
- Moscoviz, R., Trably, E., Bernet, N., Carrère, H., 2018. The environmental biorefinery: State-of-the-art on the production of hydrogen and value-added biomolecules in mixed-culture fermentation. *Green Chem.* 20, 3159–3179. <https://doi.org/10.1039/c8gc00572a>
- Mshandete, A., Björnsson, L., Kivaisi, A.K., Rubindamayugi, S.T., Mattiasson, B., 2005. Enhancement of anaerobic batch digestion of sisal pulp waste by mesophilic aerobic pre-treatment. *Water Res.* 39, 1569–1575. <https://doi.org/10.1016/j.watres.2004.11.037>
- Muha, I., Linke, B., Wittum, G., 2015. A dynamic model for calculating methane emissions from digestate based on co-digestion of animal manure and biogas crops in full scale German biogas plants. *Bioresour. Technol.* 178, 350–358. <https://doi.org/10.1016/j.biortech.2014.08.060>
- Müller, L., Kretzschmar, J., Pröter, J., Liebetrau, J., Nelles, M., Scholwin, F., 2016. Does the addition of proteases affect the biogas yield from organic material in anaerobic digestion? *Bioresour. Technol.* 203, 267–271. <https://doi.org/10.1016/j.biortech.2015.12.038>
- Mumme, J., Eckervogt, L., Pielert, J., Diakité, M., Rupp, F., Kern, J., 2011. Hydrothermal carbonization of anaerobically digested maize silage. *Bioresour. Technol.* 102, 9255–9260. <https://doi.org/10.1016/j.biortech.2011.06.099>
- Musatti, A., Ficara, E., Mapelli, C., Sambusiti, C., Rollini, M., 2017. Use of solid digestate for lignocellulolytic enzymes production through submerged fungal fermentation. *J. Environ.*

- Manage. 199, 1–6. <https://doi.org/10.1016/j.jenvman.2017.05.022>
- Mustafa, A.M., Li, H., Radwan, A.A., Sheng, K., Chen, X., 2018. Effect of hydrothermal and Ca(OH)₂ pretreatments on anaerobic digestion of sugarcane bagasse for biogas production. *Bioresour. Technol.* 259, 54–60. <https://doi.org/10.1016/j.biortech.2018.03.028>
- Mustafa, A.M., Poulsen, T.G., Sheng, K., 2016a. Fungal pretreatment of rice straw with *Pleurotus ostreatus* and *Trichoderma reesei* to enhance methane production under solid-state anaerobic digestion. *Appl. Energy* 180, 661–671. <https://doi.org/10.1016/j.apenergy.2016.07.135>
- Mustafa, A.M., Poulsen, T.G., Xia, Y., Sheng, K., 2016b. Combinations of fungal and milling pretreatments for enhancing rice straw biogas production during solid-state anaerobic digestion. *Bioresour. Technol.* <https://doi.org/10.1016/j.biortech.2016.11.028>
- Naegele, H.J., Lemmer, A., Oechsner, H., Jungbluth, T., 2012. Electric energy consumption of the full scale research biogas plant “unterer lindenhof”: Results of longterm and full detail measurements. *Energies* 5, 5198–5214. <https://doi.org/10.3390/en5125198>
- Nielsen, H.B., Mladenovska, Z., Ahring, B.K., 2007. Bioaugmentation of a two-stage thermophilic (68°C/55°C) anaerobic digestion concept for improvement of the methane yield from cattle manure. *Biotechnol. Bioeng.* 97, 1638–1643. <https://doi.org/10.1002/bit.21342>
- Nkemka, V.N., Gilroyed, B., Yanke, J., Gruninger, R., Vedres, D., Mcallister, T., Hao, X., 2015. Bioaugmentation with an anaerobic fungus in a two-stage process for biohydrogen and biogas production using corn silage and cattail. *Bioresour. Technol.* 185, 79–88. <https://doi.org/10.1016/j.biortech.2015.02.100>
- Nkoa, R., 2014. Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: A review. *Agron. Sustain. Dev.* 34, 473–492. <https://doi.org/10.1007/s13593-013-0196-z>
- Nsair, A., Önen Cinar, S., Abu Qdais, H., Kuchta, K., 2019. Optimizing the performance of a large scale biogas plant by controlling stirring process: A case study. *Energy Convers. Manag.* 198, 111931. <https://doi.org/10.1016/j.enconman.2019.111931>
- Nzila, A., 2016. Anaerobe Mini review : Update on bioaugmentation in anaerobic processes for biogas production. *Anaerobe.* <https://doi.org/10.1016/j.anaerobe.2016.11.007>
- Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R., O’hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Szoecs, E., Wagner, H., 2019. *Vegan - Community Ecology Package.*
- Olczak, M., Piebalgs, A., 2019. What to Expect from the 2020 Gas Package. *Polit. Gov.* 7, 165–169. <https://doi.org/10.17645/pag.v7i1.1747>
- Ondov, B.D., Bergman, N.H., Phillippy, A.M., 2011. Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics* 12.

- Paavola, T., Rintala, J., 2008. Effects of storage on characteristics and hygienic quality of digestates from four co-digestion concepts of manure and biowaste 99, 7041–7050.
<https://doi.org/10.1016/j.biortech.2008.01.005>
- Pakarinen, A., Maijala, P., Jaakkola, S., Stoddard, F.L., Kymäläinen, M., Viikari, L., 2011. Evaluation of preservation methods for improving biogas production and enzymatic conversion yields of annual crops. *Biotechnol. Biofuels* 4, 20. <https://doi.org/10.1186/1754-6834-4-20>
- Parawira, W., 2012. Enzyme research and applications in biotechnological intensification of biogas production. *Crit. Rev. Biotechnol.* 32, 172–186. <https://doi.org/10.3109/07388551.2011.595384>
- Passos, F., Ortega, V., Donoso-bravo, A., 2017. Thermochemical pretreatment and anaerobic digestion of dairy cow manure : Experimental and economic evaluation. *Bioresour. Technol.* 227, 239–246. <https://doi.org/10.1016/j.biortech.2016.12.034>
- Pedrazzi, S., Allesina, G., Belló, T., Alberto, C., Tartarini, P., 2015. Digestate as bio-fuel in domestic furnaces. *Fuel Process. Technol.* 130, 172–178. <https://doi.org/10.1016/j.fuproc.2014.10.006>
- Pelaez-samaniego, M.R., Hummel, R.L., Liao, W., Ma, J., Jensen, J., Kruger, C., Frear, C., 2017. Approaches for adding value to anaerobically digested dairy fiber. *Renew. Sustain. Energy Rev.* 72, 254–268. <https://doi.org/10.1016/j.rser.2017.01.054>
- Pérez-Rodríguez, N., García-bernet, D., Domínguez, J.M., 2017. Extrusion and enzymatic hydrolysis as pretreatments on corn cob for biogas production. *Renew. Energy* 107, 597–603.
<https://doi.org/10.1016/j.renene.2017.02.030>
- Pikaar, I., Matassa, S., Rabaey, K., Bodirsky, B.L., Popp, A., Herrero, M., Verstraete, W., 2017. Microbes and the Next Nitrogen Revolution. *Environ. Sci. Technol.* 51, 7297–7303.
<https://doi.org/10.1021/acs.est.7b00916>
- Pikaar, I., Vrieze, J. De, Rabaey, K., Herrero, M., Smith, P., Verstraete, W., 2018. Carbon emission avoidance and capture by producing in-reactor microbial biomass based food , feed and slow release fertilizer : Potentials and limitations. *Sci. Total Environ.* 644, 1525–1530.
<https://doi.org/10.1016/j.scitotenv.2018.07.089>
- Plouchart, D., Milferstedt, K., Guizard, G., Latrille, E., Hamelin, J., 2018. Multiplexed chemostat system for quantification of biodiversity and ecosystem functioning in anaerobic digestion. *PLoS One* 13, 1–15. <https://doi.org/10.1371/journal.pone.0193748>
- Pöschl, M., Ward, S., Owende, P., 2010. Evaluation of energy efficiency of various biogas production and utilization pathways. *Appl. Energy* 87, 3305–3321.
<https://doi.org/10.1016/j.apenergy.2010.05.011>
- Poszytek, K., Ciezowska, M., Sklodowska, A., Drewniak, L., 2016. Microbial Consortium with High Cellulolytic Activity (MCHCA) for enhanced biogas production. *Front. Microbiol.* 7, 1–11.
<https://doi.org/10.3389/fmicb.2016.00324>

- Prussi, M., Padella, M., Conton, M., Postma, E.D., Lonza, L., 2019. Review of technologies for biomethane production and assessment of Eu transport share in 2030. *J. Clean. Prod.* 222, 565–572. <https://doi.org/10.1016/j.jclepro.2019.02.271>
- R Core Team, 2019. R: A language and environment for statistical computing.
- Ramos-suárez, J.L., Gómez, D., Regueiro, L., Baeza, A., Hansen, F., 2017. Alkaline and oxidative pretreatments for the anaerobic digestion of cow manure and maize straw : Factors influencing the process and preliminary economic viability of an industrial application. *Bioresour. Technol.* 241, 10–20. <https://doi.org/10.1016/j.biortech.2017.05.054>
- Ratkovich, N., Horn, W., Helmus, F.P., Rosenberger, S., Naessens, W., Nopens, I., Bentzen, T.R., 2013. Activated sludge rheology: A critical review on data collection and modelling. *Water Res.* 47, 463–482. <https://doi.org/10.1016/j.watres.2012.11.021>
- Raynaud, X., Nunan, N., 2014. Spatial Ecology of Bacteria at the Microscale in Soil. *PLoS One* 9. <https://doi.org/10.1371/journal.pone.0087217>
- Rincón, C.A., De Guardia, A., Couvert, A., Le Roux, S., Soutrel, I., Daumoin, M., Benoist, J.C., 2019. Chemical and odor characterization of gas emissions released during composting of solid wastes and digestates. *J. Environ. Manage.* 233, 39–53. <https://doi.org/10.1016/j.jenvman.2018.12.009>
- Romano, R.T., Zhang, R., Teter, S., McGarvey, J.A., 2009. The effect of enzyme addition on anaerobic digestion of Jose Tall Wheat Grass. *Bioresour. Technol.* 100, 4564–4571. <https://doi.org/10.1016/j.biortech.2008.12.065>
- Romero-arenas, O., Huato, M.A.D., Treviño, I.H., Lezama, J.F.C.P., García, A.A., Arellano, A.D.V., 2012. Effect of pH on growth of the mycelium of *Trichoderma viride* and *Pleurotus ostreatus* in solid cultivation mediums. *African J. Agric. Res.* 7, 4724–4730. <https://doi.org/10.5897/AJAR12.568>
- Rosenberg, E., 2014. The Family Chitinophagaceae, in: *The Prokaryotes*.
- Rouches, E., Escudié, R., Latrille, E., Carrère, H., 2019. Solid-state anaerobic digestion of wheat straw : Impact of S/I ratio and pilot-scale fungal pretreatment. *Waste Manag.* 85, 464–476. <https://doi.org/10.1016/j.wasman.2019.01.006>
- Rouches, E., Herpoël-Gimbert, I., Steyer, J.P., Carrere, H., 2016. Improvement of anaerobic degradation by white-rot fungi pretreatment of lignocellulosic biomass: A review. *Renew. Sustain. Energy Rev.* 59, 179–198. <https://doi.org/10.1016/j.rser.2015.12.317>
- Rouches, E., Zhou, S., Steyer, J.P., Carrere, H., 2015. White-Rot Fungi pretreatment of lignocellulosic biomass for anaerobic digestion: Impact of glucose supplementation. *Process Biochem.* 51, 1784–1792. <https://doi.org/10.1016/j.procbio.2016.02.003>
- Ruile, S., Schmitz, S., Mönch-Tegeder, M., Oechsner, H., 2015. Degradation efficiency of agricultural biogas plants - A full-scale study. *Bioresour. Technol.* 178, 341–349. <https://doi.org/10.1016/j.biortech.2014.10.053>

- Sambusiti, C., Monlau, F., Barakat, A., 2016. Bioethanol fermentation as alternative valorization route of agricultural digestate according to a biorefinery approach. *Bioresour. Technol.* 212, 289–295. <https://doi.org/10.1016/j.biortech.2016.04.056>
- Sambusiti, C., Monlau, F., Ficara, E., Musatti, A., Rollini, M., Barakat, A., Malpei, F., 2015. Comparison of various post-treatments for recovering methane from agricultural digestate. *Fuel Process. Technol.* 137, 359–365. <https://doi.org/10.1016/j.fuproc.2015.04.028>
- Sánchez, C., 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnol. Adv.* 27, 185–194. <https://doi.org/10.1016/j.biotechadv.2008.11.001>
- Sauer, M., Marx, H., Mattanovich, D., 2012. From rumen to industry. *Microb. Cell Fact.* 11, 121. <https://doi.org/10.1186/1475-2859-11-121>
- Scarlat, N., Dallemand, J., Fahl, F., 2018a. Biogas : Developments and perspectives in Europe. *Renew. Energy* 129, 457–472. <https://doi.org/10.1016/j.renene.2018.03.006>
- Scarlat, N., Fahl, F., Dallemand, J.F., Monforti, F., Motola, V., 2018b. A spatial analysis of biogas potential from manure in Europe. *Renew. Sustain. Energy Rev.* 94, 915–930. <https://doi.org/10.1016/j.rser.2018.06.035>
- Scarlat, N., Fahl, F., Lugato, E., Monforti-Ferrario, F., Dallemand, J.F., 2019. Integrated and spatially explicit assessment of sustainable crop residues potential in Europe. *Biomass and Bioenergy* 122, 257–269. <https://doi.org/10.1016/j.biombioe.2019.01.021>
- Schimpf, U., Hanreich, A., Mähnert, P., Unmack, T., Junne, S., Renpenning, J., Lopez-Ulibarri, R., 2013. Improving the Efficiency of Large-Scale Biogas Processes: Pectinolytic Enzymes Accelerate the Lignocellulose Degradation. *J. Sustain. Energy Environ.* 4, 53–60.
- Schloss, P.D., 2009. A high-throughput DNA sequence aligner for microbial ecology studies. *PLoS One* 4. <https://doi.org/10.1371/journal.pone.0008230>
- Schneider, N., 2018. Density and Viscosity of Biomass from Agricultural Biogas Plants. Ruhr Universität Bochum.
- Schroyen, M., Hulle, S.W.H. Van, Holemans, S., Vervaeren, H., Raes, K., 2017. Laccase enzyme detoxifies hydrolysates and improves biogas production from hemp straw and miscanthus. *Bioresour. Technol.* 244, 597–604. <https://doi.org/10.1016/j.biortech.2017.07.137>
- Schroyen, M., Vervaeren, H., Van Hulle, S.W.H., Raes, K., 2014. Impact of enzymatic pretreatment on corn stover degradation and biogas production. *Bioresour. Technol.* 173, 59–66. <https://doi.org/10.1016/j.biortech.2014.09.030>
- Schroyen, M., Vervaeren, H., Vandepitte, H., Van Hulle, S.W.H., Raes, K., 2015. Effect of enzymatic pretreatment of various lignocellulosic substrates on production of phenolic compounds and biomethane potential. *Bioresour. Technol.* 192, 696–702. <https://doi.org/10.1016/j.biortech.2015.06.051>

- Sebastian N. B. Villadsen, P.L.F., Angelidaki, I., Woodley, J.M., Nielsen, L.P., Møller, P., Abstract, 2019. The Potential of Biogas; The Solution to Energy Storage. *ChemSusChem*.
<https://doi.org/10.1002/cssc.201900100>
- Shen, J., Zheng, Q., Zhang, R., Chen, C., Liu, G., 2019. Co-pretreatment of wheat straw by potassium hydroxide and calcium hydroxide : Methane production , economics , and energy potential analysis. *J. Environ. Manage.* 236, 720–726. <https://doi.org/10.1016/j.jenvman.2019.01.046>
- Sia Partners - Energy Lab, 2019. Observatoire du biométhane.
- Sindhu, R., Binod, P., Pandey, A., 2016. Biological pretreatment of lignocellulosic biomass – An overview. *Bioresour. Technol.* 199, 76–82. <https://doi.org/10.1016/j.biortech.2015.08.030>
- Singh, B., Szamosi, Z., Siménfalvi, Z., 2019. State of the art on mixing in an anaerobic digester: A review. *Renew. Energy* 141, 922–936. <https://doi.org/10.1016/j.renene.2019.04.072>
- Sobac, n.d. L'amélioration de l'accessibilité de la matière organique et de la production d'énergie sur les installations de méthanisation grâce au Bactériométhà® [WWW Document]. URL <http://www.bacteriosol-sobac.com/omm/methanisation/methanisation-essai-amelioration-accessibilite-mo.pdf>
- Solarte-toro, J.C., Romero-garcía, J.M., Martínez-patiño, J.C., Ruiz-Ramos, E., Castro-Galiano, E., Cardona-Alzatea, C.A., 2019. Acid pretreatment of lignocellulosic biomass for energy vectors production: A review focused on operational conditions and techno-economic assessment for bioethanol production. *Renew. Sustain. Energy Rev.* 107, 587–601.
<https://doi.org/10.1016/j.rser.2019.02.024>
- Speda, J., Johansson, M.A., Odnell, A., Karlsson, M., 2017. Enhanced biomethane production rate and yield from lignocellulosic ensiled forage ley by in situ anaerobic digestion treatment with endogenous cellulolytic enzymes. *Biotechnol. Biofuels* 10, 1–13.
<https://doi.org/10.1186/s13068-017-0814-0>
- Strauch, S., Krassowski, J., Singhal, A., 2013. Biomethane Guide for Decision Makers.
- Sutaryo, S., Ward, A.J., Moller, H.B., 2014. The effect of mixed-enzyme addition in anaerobic digestion on methane yield of dairy cattle manure. *Environ. Technol.* 35, 2476–2482.
<https://doi.org/10.1080/09593330.2014.911356>
- Sverige Energigas, 2018. National Biogas Strategy 2.0.
- Tambone, F., Orzi, V., D'Imporzano, G., Adani, F., 2017. Solid and liquid fractionation of digestate: Mass balance, chemical characterization, and agronomic and environmental value. *Bioresour. Technol.* 243, 1251–1256. <https://doi.org/10.1016/j.biortech.2017.07.130>
- Tambone, F., Terruzzi, L., Scaglia, B., Adani, F., 2015. Composting of the solid fraction of digestate derived from pig slurry: Biological processes and compost properties. *Waste Manag.* 35, 55–61.
<https://doi.org/10.1016/j.wasman.2014.10.014>

- Teixeira Franco, R., Buffière, P., Bayard, R., 2016. Ensiling for biogas production: Critical parameters. A review. *Biomass and Bioenergy* 94, 94–104. <https://doi.org/10.1016/j.biombioe.2016.08.014>
- Theoleyre, M.-A., 2014. La méthanisation des fumiers, le procédé Ducelier-Isman. L'eau, l'industrie, les nuisances - Hors Série Méthanisation 106–110.
- Theuerl, S., Herrmann, C., Heiermann, M., Grundmann, P., Landwehr, N., Kreidenweis, U., Prochnow, A., 2019. The Future Agricultural Biogas Plant in Germany : A Vision. *Energies* 12, 1–32. <https://doi.org/10.3390/en12030396>
- Thomas, F., Hehemann, J.H., Rebuffet, E., Czejek, M., Michel, G., 2011. Environmental and gut Bacteroidetes: The food connection. *Front. Microbiol.* 2, 1–16. <https://doi.org/10.3389/fmicb.2011.00093>
- Thomas, H.L., Seira, J., Escudie, R., Carrère, H., 2018. Lime pretreatment of miscanthus: Impact on BMP and batch dry co-digestion with cattle manure. *Molecules* 23. <https://doi.org/10.3390/molecules23071608>
- Thomsen, S.T., Londoño, J.E.G., Ambye-Jensen, M., Heiske, S., Kádár, Z., Meyer, A.S., 2016. Combination of ensiling and fungal delignification as effective wheat straw pretreatment. *Biotechnol. Biofuels* 9, 16. <https://doi.org/10.1186/s13068-016-0437-x>
- Thygesen, O., Sommer, S.G., Shin, S.G., Triolo, J.M., 2014. Residual biochemical methane potential (BMP) of concentrated digestate from full-scale biogas plants. *Fuel* 132, 44–46. <https://doi.org/10.1016/j.fuel.2014.04.062>
- Tian, J., Pourcher, A., Bize, A., Wazeri, A., Peu, P., 2017. Impact of wet aerobic pretreatments on cellulose accessibility and bacterial communities in rape straw. *Bioresour. Technol.* 237, 31–38. <https://doi.org/10.1016/j.biortech.2017.03.142>
- Tian, L., Shen, F., Yuan, H., Zou, D., Liu, Y., Zhu, B., Li, X., 2014. Reducing agitation energy-consumption by improving rheological properties of corn stover substrate in anaerobic digestion. *Bioresour. Technol.* 168, 86–91. <https://doi.org/10.1016/j.biortech.2014.03.023>
- Treichel, H., Fongaro, G., Challenges, T., Developments, F., 2019. Improving Biogas Production.
- Tsanova, T., 2018. Low interest, 76.5 MW of awards in 2nd German biomass tender [WWW Document]. *Renew. Now*. URL <https://renewablesnow.com/news/low-interest-765-mw-of-awards-in-2nd-german-biomass-tender-627207/>
- Tsapekos, P., Kougias, P.G., Vasileiou, S.A., Lyberatos, G., Angelidaki, I., 2016. Effect of micro-aeration and inoculum type on the biodegradation of lignocellulosic substrate. *Bioresour. Technol.* <https://doi.org/10.1016/j.biortech.2016.11.081>
- Tuomela, M., Vikman, M., Hatakka, A., Itävaara, M., 2000. Biodegradation of lignin in a compost environment: A review. *Bioresour. Technol.* 72, 169–183. [https://doi.org/10.1016/S0960-8524\(99\)00104-2](https://doi.org/10.1016/S0960-8524(99)00104-2)

- Uçkun Kiran, E., Trzcinski, A.P., Liu, Y., 2015. Enhancing the hydrolysis and methane production potential of mixed food waste by an effective enzymatic pretreatment. *Bioresour. Technol.* 183, 47–52. <https://doi.org/10.1016/j.biortech.2015.02.033>
- Válková, H., Novotný, Č., Malachová, K., Šlosarčíková, P., Fojtík, J., 2017. Effect of bacteria on the degradation ability of *Pleurotus ostreatus*. *Sci. Total Environ.* 585, 1114–1120. <https://doi.org/10.1016/j.scitotenv.2017.01.171>
- Valli, L., Rossi, L., Fabbri, C., Sibilla, F., Gattoni, P., Dale, B.E., Kim, S., Ong, R.G., Bozzetto, S., 2017. Greenhouse gas emissions of electricity and biomethane produced using the Biogasdoneright™ system : four case studies from Italy. *Biofuels, Bioprod. Biorefining* 11, 847–860. <https://doi.org/10.1002/bbb>
- Vaneckhaute, C., Lebuf, V., Michels, E., Belia, E., Vanrolleghem, P.A., Tack, F.M.G., Meers, E., 2017. Nutrient Recovery from Digestate : Systematic Technology Review and Product Classification. *Waste and Biomass Valorization* 8, 21–40. <https://doi.org/10.1007/s12649-016-9642-x>
- Vasco-correa, J., Ge, X., Li, Y., 2016. Fungal pretreatment of non-sterile miscanthus for enhanced enzymatic hydrolysis. *Bioresour. Technol.* 203, 118–123. <https://doi.org/10.1016/j.biortech.2015.12.018>
- Vasco-correa, J., Khanal, S., Manandhar, A., Shah, A., 2018. Anaerobic digestion for bioenergy production : Global status , environmental and techno-economic implications , and government policies. *Bioresour. Technol.* 247, 1015–1026. <https://doi.org/10.1016/j.biortech.2017.09.004>
- Venkiteswaran, K., Milferstedt, K., Hamelin, J., Zitomer, D.H., 2016. Anaerobic digester bioaugmentation influences quasi steady state performance and microbial community. *Water Res.* 104, 128–136. <https://doi.org/10.1016/j.watres.2016.08.012>
- Venturin, B., Frumi Camargo, A., Scapini, T., Mulinari, J., Bonatto, C., Bazoti, S., Pereira Siqueira, D., Maria Colla, L., Alves, S.L., Paulo Bender, J., Luís Radis Steinmetz, R., Kunz, A., Fongaro, G., Treichel, H., 2018. Effect of pretreatments on corn stalk chemical properties for biogas production purposes. *Bioresour. Technol.* 266, 116–124. <https://doi.org/10.1016/j.biortech.2018.06.069>
- Verbeeck, K., Buelens, L.C., Galvita, V. V., Marin, G.B., Geem, K.M. Van, Rabaey, K., 2018. Upgrading the value of anaerobic digestion via chemical production from grid injected biomethane. *Energy Environ. Sci.* <https://doi.org/10.1039/c8ee01059e>
- Wagner, A.O., Janetschek, J., Illmer, P., 2018. Using Digestate Compost as a Substrate for Anaerobic Digestion. *Chem. Eng. Technol.* 41, 747–754. <https://doi.org/10.1002/ceat.201700386>
- Wang, D., Sakoda, A., Suzuki, M., 2001. Biological efficiency and nutritional value of *Pleurotus ostreatus* cultivated on spent beer grain. *Bioresour. Technol.* 78, 293–300.
- Wang, J., Peng, S., Wan, Z., Yue, Z., Wu, J., Chen, T., 2013. Feasibility of anaerobic digested corn

- stover as biosorbent for heavy metal. *Bioresour. Technol.* 132, 453–456.
<https://doi.org/10.1016/j.biortech.2013.01.160>
- Wang, X., Li, Z., Zhou, X., Wang, Q., Wu, Y., Saino, M., Bai, X., 2016. Study on the bio-methane yield and microbial community structure in enzyme enhanced anaerobic co-digestion of cow manure and corn straw. *Bioresour. Technol.* 219, 150–157.
<https://doi.org/10.1016/j.biortech.2016.07.116>
- Wang, X., Lu, X., Li, F., Yang, G., 2014. Effects of temperature and Carbon-Nitrogen (C/N) ratio on the performance of anaerobic co-digestion of dairy manure, chicken manure and rice straw: Focusing on ammonia inhibition. *PLoS One* 9, 1–7.
<https://doi.org/10.1371/journal.pone.0097265>
- Wang, Y., Liu, Q., Yan, L., Gao, Y., Wang, Yanjie, Wang, W., 2013. A novel lignin degradation bacterial consortium for efficient pulping. *Bioresour. Technol.* 139, 113–119.
<https://doi.org/10.1016/j.biortech.2013.04.033>
- Wang, Y., Qian, P.Y., 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One* 4.
<https://doi.org/10.1371/journal.pone.0007401>
- Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L., 2008. Optimisation of the anaerobic digestion of agricultural resources. *Bioresour. Technol.* 99, 7928–7940.
<https://doi.org/10.1016/j.biortech.2008.02.044>
- Wei, S., 2016. The application of biotechnology on the enhancing of biogas production from lignocellulosic waste. *Appl. Microbiol. Biotechnol.* 100, 9821–9836.
<https://doi.org/10.1007/s00253-016-7926-5>
- Wellinger, A., Murphy, J.D., Baxter, D., 2013. The biogas handbook Science, production and applications, IEA Bioenergy. <https://doi.org/10.1533/9780857097415>
- Wéry, N., Pourcher, A.-M., Fuchs, J.G., 2018. Devenir des pathogènes lors du compostage, in: Tec&Doc, L. (Ed.), *Compostage et Composts - Avancées Scientifiques et Techniques*. pp. 253–274.
- Whitman, W.B., 2009. *Bergey's Manual of Systematic Bacteriology - Volume Three the Firmicutes*, Springer. <https://doi.org/10.1007/978-0-387-68489-5>
- Wood, D.A., Leatham, G.F., 1983. Lignocellulose degradation during the life cycle of *Agaricus bisporus*. *FEMS Microbiol. Lett.* 20, 421–424.
- World Bioenergy Association, 2018. *WBA Global Bioenergy Statistics 2018*.
- Wyman, V., Henríquez, J., Palma, C., Carvajal, A., 2017. Lignocellulosic waste valorisation strategy through enzyme and biogas production. *Bioresour. Technol.*
<https://doi.org/10.1016/j.biortech.2017.09.055>

- Xergi, n.d. Nix® technology [WWW Document]. URL <https://www.xergi.com/biogas-plant/nix.html> (accessed 10.1.19).
- Xia, A., Murphy, J.D., 2016. Microalgal Cultivation in Treating Liquid Digestate from Biogas Systems. *Trends Biotechnol.* 34, 264–275. <https://doi.org/10.1016/j.tibtech.2015.12.010>
- Xie, X., Feng, X., Chi, S., Zhang, Y., Yu, G., Liu, C., Li, Z., Li, B., Peng, H., 2018. A sustainable and effective potassium hydroxide pretreatment of wheat straw for the production of fermentable sugars. *Bioresour. Technol. Reports* 3, 169–176. <https://doi.org/10.1016/j.biteb.2018.07.014>
- Xu, R., Zhang, K., Liu, P., Han, H., Zhao, S., Kakade, A., Khan, A., Du, D., Li, X., 2018. Lignin depolymerization and utilization by bacteria. *Bioresour. Technol.* 269, 557–566. <https://doi.org/10.1016/j.biortech.2018.08.118>
- Yan, Z., Song, Z., Li, D., Yuan, Y., Liu, X., Zheng, T., 2015. The effects of initial substrate concentration, C/N ratio, and temperature on solid-state anaerobic digestion from composting rice straw. *Bioresour. Technol.* 177, 266–273. <https://doi.org/10.1016/j.biortech.2014.11.089>
- Young, D., Dollhofer, V., Callaghan, T.M., Reitberger, S., Lebuhn, M., Benz, J.P., 2018. Isolation, identification and characterization of lignocellulolytic aerobic and anaerobic fungi in one- and two-phase biogas plants. *Bioresour. Technol.* 268, 470–479. <https://doi.org/10.1016/j.biortech.2018.07.103>
- Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol. Bioeng.* 89, 670–679. <https://doi.org/10.1002/bit.20347>
- Yue, Z., Li, W., Yu, H., 2013. Application of rumen microorganisms for anaerobic bioconversion of lignocellulosic biomass. *Bioresour. Technol.* 128, 738–744. <https://doi.org/10.1016/j.biortech.2012.11.073>
- Zadrazil, F., Puniya, A.K., 1994. Influence of Carbon Dioxide on Lignin Degradation and Digestibility of Lignocellulosics Treated with *Pleurotus sajor-caju*. *Int. Biodeterior. Biodegradation* 237–244.
- Zeng, Y., De Guardia, A., Dabert, P., 2016. Improving composting as a post-treatment of anaerobic digestate. *Bioresour. Technol.* 201, 293–303. <https://doi.org/http://dx.doi.org/10.1016/j.biortech.2015.11.013>
- Zhang, H., Zhang, P., Ye, J., Wu, Y., Fang, W., Gou, X., Zeng, G., 2016. Improvement of methane production from rice straw with rumen fluid pretreatment: A feasibility study. *Int. Biodeterior. Biodegradation* 113, 9–16. <https://doi.org/10.1016/j.ibiod.2016.03.022>
- Zhang, N., Castlebury, L.A., Miller, A.N., Huhndorf, S.M., Schoch, C.L., Seifert, K.A., Rossman, A.Y., Rogers, J.D., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Sung, G.H., 2006. An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. *Mycologia* 98, 1076–1087. <https://doi.org/10.3852/mycologia.98.6.1076>

- Zhang, Q., He, J., Tian, M., Mao, Z., Tang, L., Zhang, J., Zhang, H., 2011. Enhancement of methane production from cassava residues by biological pretreatment using a constructed microbial consortium. *Bioresour. Technol.* 102, 8899–8906.
<https://doi.org/10.1016/j.biortech.2011.06.061>
- Zhao, X., Liu, Jinhuan, Liu, Jingjing, Yang, F., Zhu, W., Yuan, X., Hu, Y., Cui, Z., Wang, X., 2017. Effect of ensiling and silage additives on biogas production and microbial community dynamics during anaerobic digestion of switchgrass. *Bioresour. Technol.* 241, 349–359.
<https://doi.org/10.1016/j.biortech.2017.03.183>
- Zheng, Y., Zhao, J., Xu, F., Li, Y., 2014. Pretreatment of lignocellulosic biomass for enhanced biogas production. *Prog. Energy Combust. Sci.* 42, 35–53. <https://doi.org/10.1016/j.peccs.2014.01.001>
- Zhong, C., Wang, C., Wang, F., Jia, H., Wei, P., Zhao, Y., 2016. Enhanced biogas production from wheat straw with the application of synergistic microbial consortium pretreatment. *RSC Adv.* 6, 60187–60195. <https://doi.org/10.1039/C5RA27393E>
- Zhong, X.Z., Ma, S.C., Wang, S.P., Wang, T.T., Sun, Z.Y., Tang, Y.Q., Deng, Y., Kida, K., 2018. A comparative study of composting the solid fraction of dairy manure with or without bulking material: Performance and microbial community dynamics. *Bioresour. Technol.* 247, 443–452.
<https://doi.org/10.1016/j.biortech.2017.09.116>
- Zhou, J., Song, S., Huang, Z., Yang, L., Jiao, A., Liu, Y., Wang, S., 2018. Cultivation of *Pleurotus Ostreatus*, a potential candidate for biogas residues degradation. *BioResources* 13, 5432–5449.
- Zhou, S., Zhang, Y., Dong, Y., 2012. Pretreatment for biogas production by anaerobic fermentation of mixed corn stover and cow dung. *Energy* 46, 644–648.
<https://doi.org/10.1016/j.energy.2012.07.017>
- Zhou, Y., Li, C., Achu, I., Liu, J., 2017. The effects of pre-aeration and inoculation on solid-state anaerobic digestion of rice straw. *Bioresour. Technol.* 224, 78–86.
<https://doi.org/10.1016/j.biortech.2016.11.104>
- Zhu, S., Feng, S., Xu, Z., Qin, L., Shang, C., Fenga, P., Wang, Z., Yuan, Z., 2019. Cultivation of *Chlorella vulgaris* on unsterilized dairy-derived liquid digestate for simultaneous biofuels feedstock production and pollutant removal. *Bioresour. Technol.* 285, 121353.
<https://doi.org/10.1016/j.biortech.2019.121353>
- Ziemiński, K., Kowalska-Wentel, M., 2015. Effect of enzymatic pretreatment on anaerobic co-digestion of sugar beet pulp silage and vinasse. *Bioresour. Technol.* 180, 274–280.
<https://doi.org/10.1016/j.biortech.2014.12.035>