Universitat de Girona

TOWARDS THE IMPLEMENTATION OF A BIOTECHNOLOGY FOR BIOGAS UPGRADING: ROLE OF BACTERIA IN SILOXANE REMOVAL

Ellana Boada Cahueñas

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OF A BIOTECHNOLOGY
FOR BIOGAS UPGRADING:

Role of Bacteria in Siloxane Removal

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DOCTORAL THESIS

Towards the implementation of a biotechnology for biogas

upgrading:

role of bacteria in siloxane removal

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2020

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2020

Supervised by:

Dr. Lluis Bañeras Vives   Dr. Frederic Gich Batlle   Dr. Alba Cabrera Codony.

Thesis submitted in fulfilment of the requirements for the degree of doctor from the

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뜨겁게 지져봐라

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부서지고 재가되고 찍어버리는

섭리마저 거부하리

살아남은 나

나 는 다이아몬드

Soy una roca
Adelante, búscame.
No me moveré ni una pulgada porque soy una roca.
Adelante, golpéame
Soy una roca sólida
Adelante, déjame en la oscuridad.
Soy una roca que brillará sola.
No me rompo, ni me descompongo...
Yo sobrevivo.
Soy un diamante".

Adaptado de un poema original de Jo *Kwang Jin*

*Towards the implementation of
a biotechnology for biogas upgrading:
role of bacteria in siloxane removal*

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Professor titular del Departament de Biologia de la Universitat de Girona i, Investigador Sènior de l'Institut d'Ecologia Aquàtica (IEA) de la Universitat de Girona

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Certifiquem:

Que la graduada en Biotecnologia Ellana Boada Cahueñas ha dut a terme sota la nostra direcció el treball titulat **Towards the implementation of a biotechnology for biogas upgrading: role of bacteria in siloxane removal** que es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al Grau de Doctor per la Universitat de Girona.

A Girona, 07 de Abril de 2020

Dr. Lluis Bañeras Vives   Dr. Frederic Gich Batlle   Dr. Alba Cabrera i Codony.

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Don't be afraid of what makes you different. Your own special "madness" is what leads you to greatness.

Alice in Wonderland, by Lewis Carroll

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Gracias a Dios y que toda la gloria sea para Él…

Romanos 16:27

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Symbols and Non-Conventional Abbreviations.

Abbreviation	Description
1-D	Simpson's index of diversity.
16S rRNA	16S ribosomal ribonucleic acid.
AC	Activated carbon.
ACA	Catalan Water Agency (Agència Catalana de l'Aigua).
AD	Anaerobic digestion.
BEC	Batch enrichment cultures.
bcm	Billion cubic metres.
BEA-38	BEA-type zeolite.
BLAST	Basic local alignment search tool.
bp	Base pair.
BTF	Biotrickling filter.
BTFas	Biotrickling filter inoculated with activated sludge.
BTFpi	Biotrickling filter inoculated with potential isolates.
CH_4	Methane.
CHP	Combined heat and power.
CO_2	Carbon dioxide.
Cq	Quantification cycles.
CTAB	Cetyltrimethylammonium bromide.
DST-2	Commercial activated carbons.
DMSD	Dimethylsilanediol.
D3	Hexamethylcyclotrisiloxane.
D4	Octamethylcyclotetrasiloxane.
D5	Decamethylcyclopentasiloxane.
D6	Dodecamethylcyclohexasiloxane.
DNA	Deoxyribonucleic acid.
EBA	European Biogas Association.
EC	Elimination capacity.
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
$LMH \cdot bar^{-1}$	$(liters \cdot m^{-2} \cdot hour^{-1}) \cdot bar^{-1}$
L2 (MM)	Hexamethyldisiloxane.
L3 (MDM)	Octamethyltrisiloxane.
L4 (MD2M)	Decamethyltetrasiloxane.
L5 (MD3M)	Dodecamethylpentasiloxane.
MBR	Membrane bioreactor.
MGD	Million gallons per day.
Mio	Million.

Abbreviation	Description
MW	Megawatts.
MST	Methylsilanetriol.
NCBI	National Centre for Biotechnology Information.
NRT-2	Commercial activated carbons.
OTU	Operational Taxonomic Unit.
OD	Optical density.
PSA	Pressure swing adsorption.
PCoA	Principal coordinate analysis.
PCR	Polymerase chain reaction.
PTFE	Polytetrafluoroethylene.
qPCR	Quantitative polymerase chain reaction.
RE	Removal efficiency.
RNA	Ribonucleic acid.
RT	Room temperature.
T	Temperature.
TSB	Trypticase soy broth.
TMSol	Trimethylsilanol.
VOCs	Volatile organic compounds.
VMS	Volatile methyl-siloxanes.
VOSiC	Volatile organic silicon compounds.
WWTP	Wastewater treatment plant.

Summary

Summary

During the past decades, most energy has been provided by burning oil and only a very small percentage has been generated from renewable resources. Biogas has been fully recognized as an effective way to ease the predicted energy crisis based on fossil fuel burning. Biogas offers a wide range of possible applications. In fact, the European Biogas Association, in its Statistical report 2018, showed that the number of European biogas plants has increased steadily over the past decade.

Typical composition of biogas contain CO_2, H_2S, NH_3, H_2Ov, Dust, O_2, Cl^- F^- and volatile silicon compounds, in addition to methane (CH4) as the main component. These contaminants presence and quantities depend largely on feedstocks for biogas generation. The presence of volatile silicon compounds in the biogas can cause severe problems in the energy recovery systems, inducing costly damages. Among these silicon compounds, siloxanes are of great concern since they have been extensively used in industrial applications and accumulate in biogas streams. During the combustion of biogas, the presence of siloxanes causes serious damage to the gas processing equipment because of their oxidation and deposition as silica particles. In fact, organosilicon compounds are highly undesirable compounds for the energy recovery that have remained reluctant to biodegradation. Reasons for the low biodegradability are the low energy content of these molecules to be used by microorganisms and the mass transfer effect that decreases their availability for degradation in physiologically relevant conditions. Decamethylcyclopentasiloxane (D5) and Octamethylcyclotetrasiloxane (D4) are the siloxanes primarily found in biogas.

In this thesis, we aimed at exploring the role of bacteria in siloxane removal and optimized operational conditions to minimize mass transfer limitation. We have used a basic "biotechnological approach" for a poorly studied process that has progressively moved from enrichment-isolation of new bacteria, screening of new isolates, and applications in lab-scale bioreactors, such as biotrickling filters to select the most promising siloxane degraders.

The first part of this dissertation is focused to define a steady and specialized bacterial community present in anoxic enrichments obtained from activated sludge as inoculum. Approaches to elucidate the microbial community structure of siloxane removing biofilms were established in **chapter 4**, in which D4 was used as carbon and energy source. Up to 19 phylotypes were defined as the core microbial community regardless of the substrate material (activated carbon or zeolite). Most of them

belonged to the *Rhizobiales* and *Betaproteobacteriales*. Furthermore, the results confirmed that packing carriers could enrich some specific bacteria, thus improving the richness and diversity of microorganisms in enrichments saturated with D4 as sole carbon source. The larger surface area of AC and the reticular structure of zeolite were suspected to be key determinants that favoured the transfer D4, which, in turn, promoted selection of specific microorganisms on their surface and minimized the mass transfer problem.

In **chapter 5**, isolated strains showing potential for D4 degradation were inoculated and tested to evaluate their capacity to grow with siloxane as sole carbon source or in the presence of a multicomponent mixture of organics. Herein we isolated 58 bacterial species. Among those, *Methylibium* sp. and *Pseudomonas aeruginosa* showed the highest capacity to remove D4 (53.04% ± 0.03 and 24.42% ± 0.02, respectively). A co-culture with a mixture of 10 of the most promising bacteria was evaluated. Contrarily to what was expected, biodegradation of siloxanes together with volatile organic compounds poorly removed D4 compared to other substrates, such as toluene and limonene, which were completely removed. Remarkably, the siloxane D5 proved to be more effectively degraded than D4.

In **chapter 6,** a lab-scale BTF was inoculated with the same inoculum of enrichment: activated sludge. The microbial community of the lab-scale BTF was studied while testing its biodegradation capacity on D4 and D5 in the presence of toluene, limonene and hexane, and at different empty bed residence times (EBRT), and packing materials. Microbial diversity and richness were higher in the inoculum and progressively decreased during BTF operation (Simpson's diversity index changing from 0.98 to 0.90 and Richness from 900 to 200 OTUs). Minimum diversity was found when reactor was operated at relatively low EBRT (7.3 min). The core community was composed of 36 OTUs (accounting for 55% of total sequences). The packing material played a key role in the community structure. *Betaproteobacteriales* were dominant in the presence of lava rock and were partially substituted by *Corynebacteriales* and *Rhizobiales* when activated carbon was added to the BTF. Despite these changes, the stable and resilient core microbiome was selected, defining a set of potentially degrading bacteria as alternatives to siloxane bioremoval.

The importance of these chapters reside in that microorganisms are rarely encountered as single species populations and their use as pure cultures for biotechnological applications has some drawbacks in up-scaling. Moreover, the study of the community may help deciphering microbial

interactions, which can induce the activation of otherwise silent biosynthetic pathways leading to the degradation of recalcitrant substrates and production of new and easy degradable products.

In addition, this is the first report about *Methylibium* sp. associated to siloxane degradation. In order to evaluate its potential as siloxane degrader, a BTF was inoculated with *Methylibium* sp. iso58 with the intention of analyse their performance in pure culture under oxic and anoxic (in the presence of nitrate) conditions. Results are presented in **chapter 7.** A versatile metabolism for *Methylibium* sp. iso58 was confirmed and contributed two interesting highpoints. First, its ability to grow in anaerobic and oxic conditions. Second, its capacity to use nitrate as an alternative electron acceptor in respiration. These two properties could be beneficial when the bioreactor has an anoxic performance, or higher nitrate concentrations are supplied.

Therefore, the implementation of hybrid technologies that combine absorption technologies and biological technologies is an economically viable alternative. This dissertation offers preliminary view about the advantages of biological methods as potential biogas purification technology. We are confident the results of the thesis will be considered in future management strategies in biogas upgrading as well as in biological technologies systems to achieve a good gas quality.

Resum

Durant les últimes dècades, la major part de l'energia que produïm s'ha obtingut mitjançant combustibles fòssil, mentre que una petita part es genera de fonts renovables. En un context on es preveu una crisi energètica a causa de l'esgotament dels combustibles no renovables, el biogàs es reconeix com un eficient font energètica renovable. En aquest sentit, la *European Biogas Association* va mostrar en el seu informe estadístic publicat el 2018 com el nombre de plantes de generació de biogàs ha seguit incrementant any rere any durant la passada dècada.

La composició típica del biogàs conté diferents components com CO_2, H_2S, NH_3, H_2Ov, O_2, Cl^- F^- i compostos volàtils de silici, siloxans, a més del metà (CH_4), que n'és el component majoritari i que posseeix el poder calorífic del gas. Els contaminants presents, així com la seva concentració, depenen de la varietat de substàncies orgàniques a partir de les quals s'ha produït el biogàs per digestió anaeròbia. La presència de siloxans causa severs problemes en els sistemes de recuperació energètica que provoquen grans costos econòmics quan cal reemplaçar-ne parts malmeses. Durant la combustió del biogàs, els siloxans presents es converteixen en òxid de silici que es diposita impedint la conducció calorífica i la lubricació, que malmet les parts mòbils els sistemes de recuperació energètica. Entre les diferents espècies de siloxans, el decametilciclopentasiloxà (D5) i l'octametilciclotetrasiloxà (D4) són els que generalment es troben a concentracions més elevades, de l'ordre de 10 a 100 mg m^{-3} procedents de la disposició tant de productes d'higiene personal com lubricants i altres productes industrials que contenen silicones.

Les concentracions de siloxans tolerables per la majoria de sistemes de recuperació energètica són inferiors a 1 mg m^{-3}, per aquest motiu es necessària la seva eliminació en un procés de purificació del biogàs abans de ser utilitzat. L'adsorció en carbó actiu és la tecnologia més àmpliament utilitzada arreu, però les tècniques biològiques de tractament de gasos es plantegen com a alternativa per millorar la sostenibilitat del tractament de biogàs, ja que el reemplaçament dels adsorbents exhaurits suposa la generació d'un gran volum de residu a l'hora de suposar importants costos econòmics.

Fins ara, els siloxans s'han considerat compostos poc biodegradables degut al baix contingut energètic d'aquestes molècules per ser usat pels microorganismes, mentre que les limitacions el la transferència de massa han limitat la biodisponibilitat per la seva degradació en condicions fisiològicament rellevants.

En aquests context, aquesta tesi presenta com a objectius explorar el rol dels bacteris en l'eliminació de siloxans i optimitzar les condicions operacionals per minimitzar les limitacions existents en la transferència del biogàs a la biomassa. Hem usat una aproximació biotecnològica per estudiar un procés escassament investigat, des de l'enriquiment i aïllament de bacteris, la selecció de les soques més eficients i estudiar la seva aplicació en biofiltres a escala de laboratori utilitzant mescles sintètiques de composició similar al biogàs de digestors anaerobis de depuradores d'aigües residuals.

La primera part d'aquesta dissertació s'enfoca en la definició d'una comunitat bacteriana estable i especialitzada a partir d'enriquiments anòxics obtinguts de fangs actius utilitzats com a inòcul. En el **capítol 4**, utilitzem D4 com a única font de carboni i energia per estudiar el desenvolupament de la comunitat bacteriana capaç de créixer amb aquest substrat. Per tal de subministrar aquesta substància hidrofòbica es van utilitzar diferents materials porosos, carbó actiu i zeolita, i depenent de l'adsorbent es van identificar fins a 19 fílums, la majoria dels quals pertanyents a *Rhizobiales* i *Betaproteobacteriales*. A més, els resultats van confirmar que els materials utilitzats per subministrar el D4 poden enriquir algunes espècies bacterianes, millorant la riquesa i diversitat dels enriquiments. Per tant, l'ús de materials adsorbents millora la transferència de massa del D4 a l'hora que promou el creixement bacterià a la seva superfície.

En el **capítol 5** d'aquesta dissertació es van aïllar les soques que mostraven potencial per la biodegradació de D4 per tal d'estudiar separadament la seva capacitat de créixer amb D4 com a única font de carboni i d'altra banda la seva capacitat de degradar siloxans (D4 i D5) quan hi ha presència d'altres compostos orgànics que són presents en el biogàs. En total es van aïllar 58 espècies bacterianes, de les quals *Methylibium* sp. i *Pseudomonas aeruginosa* van mostrar la major capacitat d'eliminació de D4 ($53.04\% \pm 0.03$ and $24.42\% \pm 0.02$, respectivament). Finalment, es va avaluar l'eficiència d'un co-cultiu format per la mescla de les 10 espècies més prometedores per l'eliminació de siloxans. Contràriament al que esperàvem, la presència d'altres compostos orgànics volàtils va disminuir la biodegradació de siloxans mentre que compostos com el toluè i el limonè s'eliminaven completament. En termes generals, el D4 va resultar menys biodegradable que el D5, l'altre siloxà avaluat de major massa molecular amb un monòmer més en la seva estructura circular.

Per tal d'investigar la biodegradació de siloxans en un corrent gasós, en el **capítol 6** vam utilitzar fangs actius obtinguts d'una depuradora d'aigües residuals urbanes per fer la inoculació inicial d'un biofiltre (BTF) a escala de laboratori. L'objectiu d'aquest capítol és estudiar el desenvolupament de la comunitat

bacteriana a l'hora que l'avaluació de la seva capacitat per eliminar els siloxans D4 i D5 presents en un cabal gasós on també hi a toluè, limonè i hexà. Durant 210 dies el BTF s'opera en diferents períodes on reduïm progressivament el temps de contacte del gas (EBRT) i posteriorment la utilització de carbó actiu com a rebliment per tal de millorar la transferència de massa i reduir la mida del llit necessari.

Tant la diversitat com la riquesa de la comunitat microbiana van disminuir progressivament durant l'operació del BTF: l'índex de diversitat de Simpson va variar de 0.98 fins a 0.90 i la riquesa des de 900 fins a 200 OTUs i vam determinar que el material utilitzat pel rebliment del biofiltre juga un paper clau en l'estructura de la comunitat. *Betaproteobacteriales* tenien una presència dominant durant l'operació amb pedra volcànica, i van ser parcialment substituïts per *Corynebacteriales* i *Rhizobiales* després de l'addició de carbó actiu en el BTF. Tot i els canvis observats, el nucli de la comunitat es va mantenir estable i resilent, demostrant el potencial de les principals espècies detectades com alternatives al tractament fisicoquímic del biogàs per l'eliminació biològica de siloxans.

La importància d'aquests capítols resideix en el fet que les aplicacions biotecnològiques rarament consisteixen es espècies úniques de microorganismes, ja que mantenir cultius purs suposa un problema per l'escalat del reactors. A més, l'estudi de la comunitat en conjunt ajuda a desxifrar les interaccions microbianes, les quals poden induir l'activació de vies diferents processos biològics que d'altra manera restarien silenciats.

En aquest context, aquest treball suposa la primera publicació identificant *Methylibium* sp. associat a la degradació de siloxans. Per tal d'avaluar el potencial d'aquesta espècie, en el **capítol 7** vam inocular un BTF a escala de laboratori amb un cultiu de *Methylibium* sp. iso58 amb la intenció d'analitzar la seva capacitat de degradar siloxans en un corrent de gas composat a més de siloxans per compostos orgànics volàtils en condicions anòxiques, en presència de nitrat, i en presència d'oxigen a una baixa concentració, similar a la del biogàs de digestors anaerobis (1%). Vam confirmar que *Methylibium* sp. té un metabolisme versàtil, que pot utilitzar tant el nitrat com l'oxigen com a acceptadors d'electrons alternatius, reduint el requeriment de nitrat subministrat al BTF.

Les eficiències d'eliminació biològica de siloxans en els BTFs operats al laboratori van arribar a l'ordre el 50%, tot i que els altres compostos presents en el biogàs sintètic utilitzat, toluè, limonè i hexà, van ser eliminats completament tot i tenir una concentració d'un ordre de magnitud més alt. Aquests compostos volàtils, que competeixen amb els siloxans reduint l'eficàcia i la duració del carbó actiu en

els processos d'adsorció permetran allargar d'una manera molt significativa la vida útils dels adsorbents, que encara seran requerits per l'eliminació completa de siloxans després del procés biològic.

La implementació de tecnologies híbrides que combinin processos biològics amb un pas final d'adsorció suposarà una alternativa més sostenible. Aquest estudi ofereix una visió preliminar dels avantatges i el potencial dels mètode biològics per la purificació del biogàs. Tanmateix, en els propers anys caldrà seguir desenvolupant la recerca en aquest àmbit per tal de poder implementar solucions biotecnològiques per l'eliminació de siloxans del biogàs.

Resumen

Durante las últimas décadas, la mayor parte de la energía que producimos se ha obtenido mediante combustibles fósiles, mientras que una pequeña parte se genera de fuentes renovables. En un contexto donde se prevé una crisis energética debido al agotamiento de los combustibles no renovables, el biogas se reconoce como una eficiente fuente energética renovable. Es así como el número de plantas de generación de biogás ha seguido incrementando año tras año durante la década pasada, como se declara en el informe estadístico emitido por la Asociación Europea de Biogás publicado en 2018.

El biogás típicamente está compuesto de CO_2, H_2S, NH_3, H_2Ov, O_2, Cl^- F^-, compuestos volátiles de silicio (siloxanos), y metano (CH_4), siendo este último, el componente mayoritario y que otorga el poder calorífico del gas. Dentro de esta composición, los compuestos catalogados como contaminantes del biogás juegan un papel fundamental en la determinación de calidad energética del biogás y la eficiencia y rentabilidad del proceso de producción del mismo. Los contaminantes presentes, así como su concentración, dependen de la variedad de sustancias orgánicas a partir de las que se ha producido el biogás por digestión anaerobia. La presencia de siloxanos causa severos problemas en los sistemas de recuperación energética y provocando grandes costes económicos cuando hay reemplazar partes dañadas. Durante la combustión del biogás, los siloxanos presentes se convierten en óxido de silicio que se deposita en las partes móviles de los sistemas de recuperación energética impidiendo la conducción calorífica y la lubricación. Entre las diferentes especies de siloxanos, el decametilciclopentasiloxano (D5) y el octametilciclotetrasiloxano (D4), son los que generalmente se encuentran en concentraciones más elevadas, del orden de 10 a 100 mg·m^{-3} procedentes de la disposición residual tanto de productos de higiene personal como lubricantes y otros productos industriales que contienen siliconas.

Las concentraciones de siloxanos tolerables para la mayoría de sistemas de recuperación energética son inferiores a 1 mg·m^{-3}, por este motivo es necesaria su eliminación en un proceso de purificación del biogás antes de ser utilizado. La adsorción en carbón activo es la tecnología más ampliamente utilizada, pero las técnicas biológicas de tratamiento de gases se plantean como alternativa para mejorar la sostenibilidad del tratamiento de biogás, optimizando costes económicos con la disminución de la cantidad de residuos dados por el reemplazo de los materiales adsorbentes y la renovación de otros insumos y equipos afines al proceso.

Hasta ahora, los siloxanos han sido considerados compuestos poco biodegradables, principalmente debido al coste energético que supone su metabolismo y a las subsecuentes limitaciones de biodisponibilidad ligadas a sus características fisicoquímicas. En este contexto, esta tesis presenta como objetivos explorar el rol de las bacterias en la eliminación de siloxanos y optimizar las condiciones operacionales para minimizar las limitaciones existentes ligadas a su degradación biológica. Hemos usado una aproximación biotecnológica para estudiar un proceso escasamente investigado, desde el enriquecimiento y aislamiento de bacterias, la selección de las cepas más eficientes y su evaluación en bio-filtros a escala de laboratorio utilizando mezclas sintéticas de composición similar al biogás proveniente de digestores anaerobios de depuradoras de aguas residuales.

La primera parte de esta disertación se enfoca en la definición de una comunidad bacteriana estable y especializada a partir de enriquecimientos anóxicos obtenidos de fangos activos utilizados como inóculo. En el capítulo 4, utilizamos D4 como única fuente de carbono y energía para estudiar el desarrollo de la comunidad bacteriana capaz de crecer con este sustrato. Dado que los siloxanos son compuestos hidrofóbicos, se utilizaron diferentes materiales porosos, como carbón activado y zeolita, que aseguraron mejor disposición y mayor accesibilidad del sustrato para los microorganismos. Dependiendo del adsorbente se identificaron hasta 19 *phyla*, la mayoría de los cuales pertenecientes a los órdenes *Rhizobiales* y *Betaproteobacteriales*. Además, los resultados confirmaron que los materiales utilizados para suministrar el D4 pueden enriquecer algunas especies bacterianas, mejorando la riqueza y diversidad de los enriquecimientos. Por lo tanto, el uso de materiales adsorbentes mejora la transferencia de masa del D4 a la vez que promueve el crecimiento bacteriano en su superficie.

En el capítulo 5 de esta disertación se aislaron las cepas que mostraban potencial para la biodegradación de D4 para estudiar separadamente su capacidad de crecer con D4 como única fuente de carbono y por otra parte su capacidad de degradar siloxanos (D4 y D5) cuando hay presencia de otros compuestos orgánicos que están presentes en el biogas. En total se aislaron 58 especies bacterianas, de las que *Methylibium* sp. iso58 y *Pseudomonas aeruginosa* iso07 mostraron la mayor capacidad de eliminación de D4 (53.04% ± 12:03 and 24.42% ± 12:02, respectivamente). Finalmente, se evaluó la eficiencia de un co-cultivo formado por la mezcla de las 10 especies más prometedoras para la eliminación de siloxanos. Contrariamente a lo que esperábamos, la presencia de otros compuestos orgánicos volátiles disminuyó la biodegradación de siloxanos mientras que compuestos como el tolueno y el limoneno se eliminaban completamente. En términos generales, el D4 resultó

menos biodegradable que el D5, el otro siloxano evaluado de mayor masa molecular con un monómero más en su estructura circular.

Con el fin de investigar la biodegradación de siloxanos en una corriente gaseosa, en el capítulo 6 utilizamos fangos activos obtenidos de una depuradora de aguas residuales urbanas para hacer la inoculación inicial de un bio-filtro (BTF) a escala de laboratorio. El objetivo de este capítulo fue estudiar el desarrollo de la comunidad bacteriana a la vez que la evaluación de su capacidad para eliminar los siloxanos D4 y D5 presentes en un caudal gaseoso donde también a tolueno, limoneno y hexano. Durante 210 días el BTF se operó en diferentes períodos donde se redujo progresivamente el tiempo de contacto del gas (EBRT) y posteriormente se incluyó la utilización de carbón activo como relleno para mejorar la transferencia de masa y reducir el tamaño de la cama del reactor.

Tanto la diversidad como la riqueza de la comunidad microbiana disminuyeron progresivamente durante la operación del BTF: el índice de diversidad Simpson varió de 0.98 hasta 0.90 y la riqueza fue desde 900 hasta 200 OTUs. Determinándose que el material utilizado por el relleno del bio-filtro juega un papel clave en la estructura de la comunidad. La menor diversidad microbiana fue determinada cuando el BTF fue operado al tiempo más bajo EBRT (7.3min). la comunidad central bacteriana (*core community*) estuvo compuesta de 36 OTUs, los cuales correspondían al 55% del total de las secuencias evaluadas. Representantes del orden *Betaproteobacteriales* tenían una presencia dominante durante la operación con piedra volcánica, y fueron parcialmente sustituidos por los órdenes *Corynebacteriales* y *Rhizobiales* tras la adición de carbón activo en el BTF. A pesar de los cambios observados, el núcleo de la comunidad se mantuvo estable y permanente, demostrando el potencial de las principales especies detectadas como alternativas al tratamiento fisicoquímico del biogás para la eliminación biológica de siloxanos.

La importancia de estos capítulos reside en el hecho de que los microorganismos raramente son encontrados como especies aisladas o poblaciones puras, por lo tanto, su uso como cultivos puros en aplicaciones biotecnológicas se aleja de las condiciones reales y supone una limitación operacional e incluso económica. Haciendo prioritario su estudio a nivel de comunidad. Además, el estudio de la comunidad en conjunto ayuda a descifrar las interacciones microbianas, las cuales pueden inducir la activación de vías diferentes procesos biológicos que de otra manera quedarían silenciados.

En este contexto, este trabajo supone la primera publicación identificando *Methylibium* sp. asociado a la degradación de siloxanos. Para evaluar el potencial de esta especie, en el capítulo 7 se inoculó un BTF a escala de laboratorio con un cultivo de *Methylibium* sp. iso58 con la intención de analizar su capacidad de degradar siloxanos en una corriente de gas compuesto además con otros compuestos orgánicos volátiles en condiciones anóxicas, en presencia de nitrato, y en presencia de oxígeno a una baja concentración, similar a la del biogás de digestores anaerobios (1%). Con esto, se confirmó que *Methylibium* sp. tiene un metabolismo versátil, que puede utilizar tanto el nitrato como el oxígeno como aceptadores de electrones alternativos, reduciendo el requerimiento de nitrato suministrado al BTF.

En general, las eficiencias de eliminación biológica de siloxanos en los BTFs operados en el laboratorio llegaron a valores cercanos al 50%, mientras que los otros compuestos presentes en el biogás sintético utilizado, como tolueno, limoneno y hexano, fueron eliminados completamente a pesar de tener una concentración de un orden de magnitud más alto. Esto sugiere una ventaja, ya que, con la degradación de estos compuestos volátiles que compiten con los siloxanos reduciendo la eficacia y la duración del carbón activo en los típicos procesos de adsorción, se podrá alargar de una manera muy significativa la vida útil de los adsorbentes, que posteriormente todavía serán requeridos por la eliminación completa de siloxanos.

La implementación de tecnologías híbridas que combinen procesos biológicos con un paso final de adsorción supondrá una alternativa más sostenible. Este estudio ofrece una visión preliminar de las ventajas y el potencial de los métodos biológicos para la purificación del biogás. Sin embargo, en los próximos años habrá que seguir desarrollando la investigación en este ámbito con el fin de poder implementar soluciones biotecnológicas más completas para la eliminación de siloxanos del biogás.

Towards the implementation of
a biotechnology for biogas upgrading:
role of bacteria in siloxane removal

CHAPTER 1

Introduction

1.1 Biogas

Natural degradation of organic material results in the production of biogas (Kirakosyan & Kaufman, 2009). Evidence indicates that biogas was used in Assyria during the 17th century and in Persia in the 16th century (Steinhauser & Deublein, 2011). Initially, two types of biogas were defined: (1) biogas produced by anaerobic digestion or fermentation of biological materials by means of anaerobic bacteria; and (2) synthesis gas or syngas, which is created by gasification of wood, wood chips or other carbon-rich biomass. The former is mainly composed of carbon dioxide and methane, while the latter is a mixture of nitrogen, hydrogen, carbon monoxide and trace amounts of methane, this gas requires a gasifier or wood gas generator (Accettola, Guebitz, & Schoeftner, 2008; Kirakosyan & Kaufman, 2009). Nowadays the origin of biogas as a "renewable energy source" is produced by the anaerobic digestion of organic matter from many sources, including manure, sewage, and agricultural organic wastes (Kajolinna, Aakko-Saksa, Roine, & Kåll, 2015).

In the early stages of waste treatment, produced biogas was considered as an unusable residue. Now, biogas is a highly valorised product and is used to: (1) reduce operational costs for disposal of the produced sludge from wastewater treatment plants, and (2) the stabilization under legal requirements of the organic matter and others compounds (M. Shen, Zhang, Hu, Fan, & Zeng, 2018). Likewise, the development and utilization of biogas can reduce nasty odours, and reduce emissions of methane and other dangerous gases emissions to the atmosphere, improving the overall quality of air.

1.1.1 Biogas as renewable energy source

During the past decades, globally most energy was provided by burning oil and only a very small percentage was generated by contribution of energy from renewable resources. However, as with all fossil resources, the quantity of oil is limited and will not last forever. This situation leaves "renewable energies" with the greatest potential for securing the availability of energy in the future. In this context, the use of renewable energy sources has experienced a significant growth worldwide. Unlike natural gas derived from fossil fuels, biogas is a renewable "natural gas" that is produced from organic/biological materials (Kirakosyan & Kaufman, 2009). Thus, its use remains one of the most sustainable energy sources. Due to its use, energy and climate policies have been implemented and several support schemes for promoting the utilization of renewable resources have been encouraged, ameliorating the development of new biogas plants for energy production (Scarlat, Dallemand, & Fahl, 2018). Even, some countries use different technologies, depending on their climatic and geographic

location. Likewise, researchers have deepened their knowledge about the chemical and microbial processes contributing to efficient biogas production. Further, by 2025 the technologies around renewable resources are expected to have reached the potential for full economic use.

Biogas offers a wide range of possible applications. In agriculture, biogas production facilitates the effective recovery of nutrients that can be used as bio-fertilizers. In the energy sector, biogas can be used in combined heat and power production and is especially valuable for local and decentralized energy production (European Biogas Association (EBA), 2018). In transport, biogas upgraded to bio-methane can serve as a vehicle fuel, which can be distributed using existing natural gas grids.

1.1.2 Perspectives for Biogas in Europe

Biogas production plants for the treatment of wet-waste biomass, from wastewater treatment plants and landfill gas recovery is expanding in a number of countries. Biogas upgrading to higher-quality bio-methane is also increasing, for use as a vehicle fuel or for injection into the natural gas grid. Biogas production has faced a significant growth in the last years in Europe, mainly driven by the favourable support schemes in place in European Union Member States (Torrijos, 2016). The European Biogas Association (EBA) (2019), in its Statistical report 2018 showed that the number of European biogas plants has increased steadily over the past dwecade (**Figure 1.1.**), showing that national biogas markets are well established and strong enough to overcome the political uncertainty which has affected certain countries (Scarlat et al., 2018; Y. Shen, Linville, Urgun-Demirtas, Mintz, & Snyder, 2015).
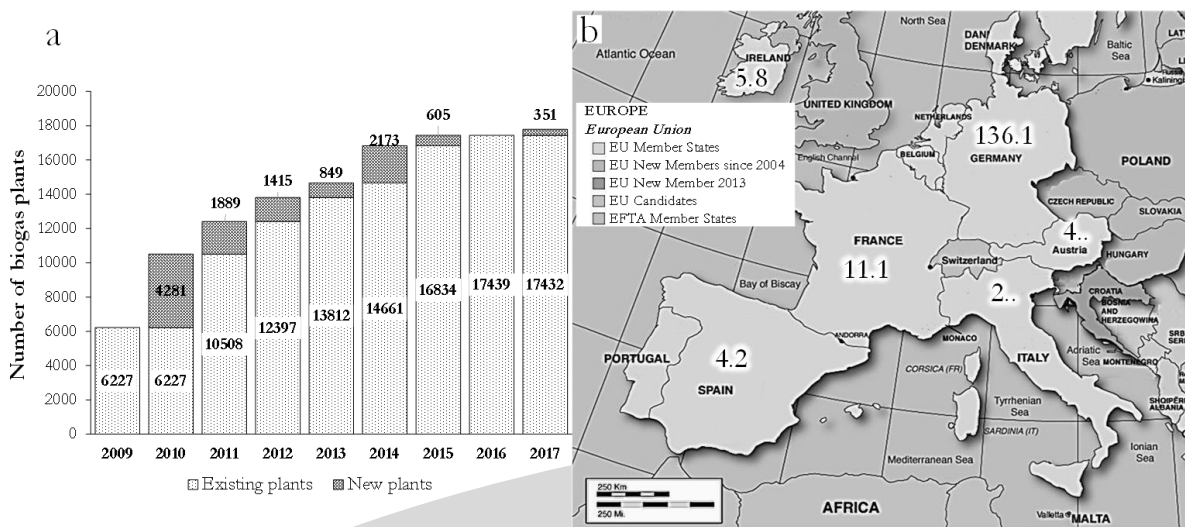
Figure 1.1. **a.** Development of number of biogas plants in Europe. **b.** Number of biogas plants per 1 Mio capita in European countries 2017 (European Biogas Association (EBA), 2018).

The rate of increase in the number of European biomethane plants from 2016 to 2017 was half that between 2014 and 2015, but biomethane production has nevertheless continued to grow, rising by 12% in 2017, a total of 19,352 GWh was reached in 2017 (**Figure 1.2.a.**). For biomethane as for biogas, there is a trend towards larger installations. Most of the biogas in the European Union is used as a fuel for electricity generation, in electricity only or combined heat and power plants with the effort toward the maximum use of heat aiming to increase the income and to improve the economics of the biogas plants (**Figure 1.2.b.**) (Scarlat et al., 2018).
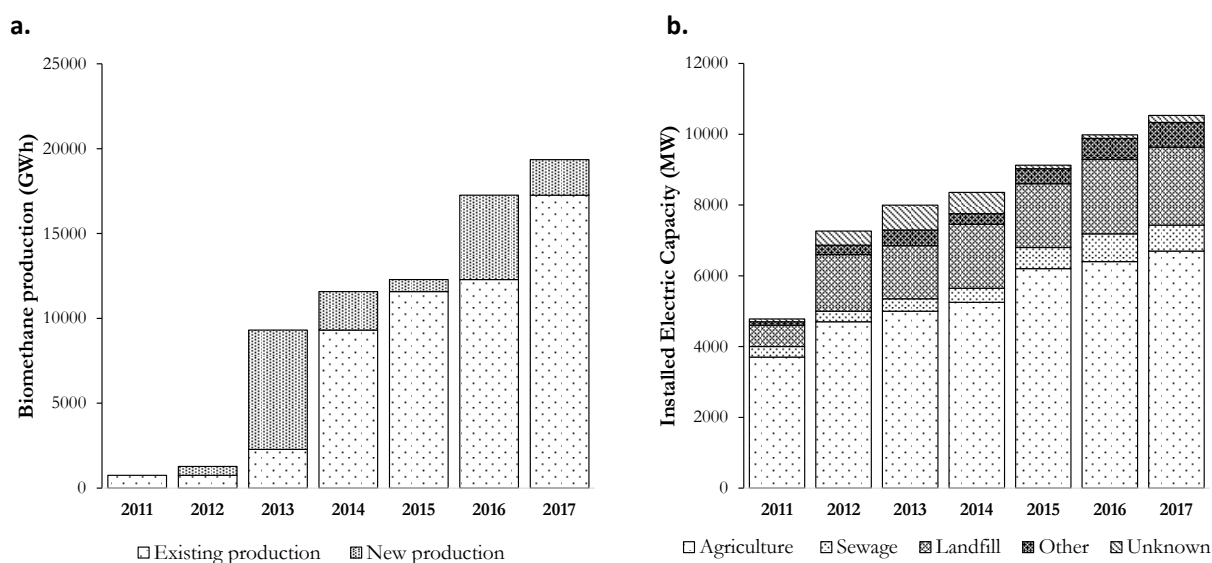
Figure 1.2. a. Development of European biomethane production in GWh. **b.** Development of the biogas IED (MW) by feedstock in European Countries (European Biogas Association (EBA), 2019).

1.1.3 Synthesis (anaerobic digestion)

Anaerobic digestion is, technically, the biodegradation of any organic compound in the absence of oxygen, usually catalysed by microorganisms or their products. Biogas production process is carried out in four successive stages (**Figure 1.3.**): hydrolysis, acidogenesis, acetogenesis and methanogenesis. Hydrolysis (*step 1*) is the degradation of both insoluble organic material and high molecular weight compounds, into soluble organic substances. Hydrolytic bacteria excrete extracellular enzymes and convert carbohydrates, lipids and proteins into sugars, long chain fatty acids (LCFAs) and amino acids, respectively. Hydrolysis has an optimum temperature range between 30 to 50 °C, and with an optimum pH between 5 and 7. It is important to note, that the hydrolysis rate is a determining step in the overall kinetics of anaerobic digestion. This step is especially relevant for the digestion of heavy substrates, such as lignin or cellulose, which are difficult to degrade (Meegoda, Li, Patel, & Wang, 2018).
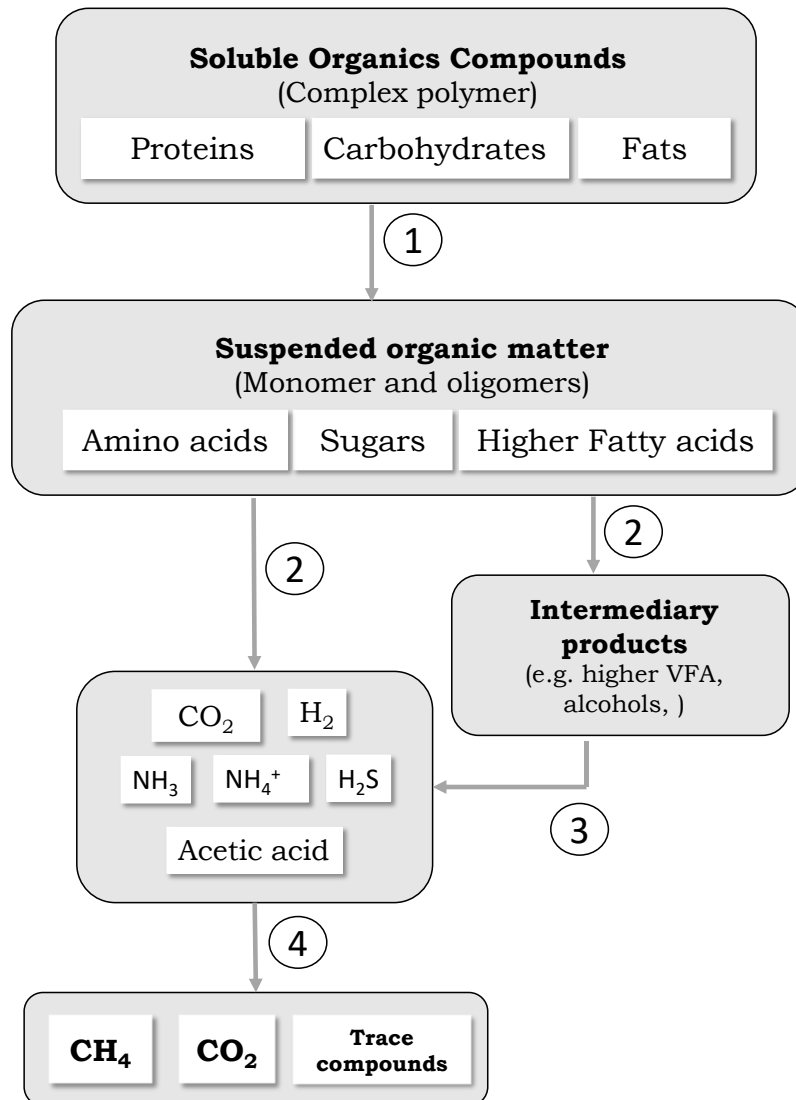
Figure 1.3. Diagram depicting a simplified sequence steps in the process of anaerobic digestion: 1) Hydrolysis, 2) Acidogenesis, 3) Acetogenesis and 4) Methanogenesis.

Simple monomers formed after the hydrolysis step, are further split into low molecular weight compounds during acidogenesis (*step 2*); Acidogenic microorganisms, typically have shorter generation times, usually lower than 36 hours. Products formed are mainly volatile fatty acids (VFA) such as acetates, butyrate and propionate, a small amount of long C chain organic acids, along with ammonia (NH_3), CO_2, hydrogen sulfide (H_2S), and other by-products such as ethanol and lactate. VFA concentrations can fluctuate due to the conditions of the digester, and sometimes their over-production can cause a dramatic failure in the process, constituting a second process-control step (Meegoda et al., 2018; Scarlat et al., 2018).

In the Acetogenesis process (*step 3*), higher VFA and alcohols are further digested by acetogens to produce mainly acetic acid as well as CO_2 and H_2. The hydrogen broaches the discussion of an interesting syntrophic relationship that is present in the anaerobic digestion-hydrogen interspecies transfer. An excessive hydrogen partial pressure can be deleterious to acetogenic microorganisms. However, hydrogenotrophic methanogens can create an exergonic reaction and maintain hydrogen partial pressure at lower values. It should be noted that propionate has its particularity and inevitably requires a specific acetogenesis process because its odd number of carbons (Kirakosyan & Kaufman, 2009).

Final stage is methanogenesis per se (*Step 4*). Accessible intermediates (acetate, CO_2 and H_2) are consumed to produce methane. Two groups of obligate anaerobic archaea (methanogenic) are involved in methanogenesis; the first group splits acetate into methane and CO_2 and the second group uses hydrogen as electron donor and CO_2 as acceptor to produce methane. Methanogens likely constitute the most sensitive microbial groups in anaerobic digestion due to their tendency to require a higher pH than previous stages to have a significantly slower regeneration time (5 to 16 days) compared to other microorganisms in anaerobic digestion, upwards of 5 to 16 days (Kirakosyan & Kaufman, 2009; Meegoda et al., 2018).

Overall, within the anaerobic environment, various important parameters affect the rate at which different steps in the process are going to occur, i.e. pH and alkalinity, temperature, and sludge retention time. However, hydrolysis is generally considered as rate limiting (Rasi, Läntelä, Veijanen, & Rintala, 2008). In addition, a variety of materials can become toxic to anaerobic bacteria and inhibit digestion, e.g. salts, heavy metals, ammonia and antibiotics.

1.1.4 Biogas production feedstocks

In comparison with fossil fuels, anaerobic digestion technology can utilize locally available substrates. For instance, historically some ancient Chinese literature suggests that biogas was generated from sewage 2,000 to 3,000 years ago. Nowadays, the main substrates used for the production of biogas are sewage sludge from wastewater treatment plants (WWTPs), manure, waste from the agri-food industry and the organic fraction of municipal solid waste (usually from landfill sites). Different feedstocks that can be utilized for biogas production and their comparative production amount and energy potential are given in **Table 1.1.**

Table 1.1. Comparison of biogas yields and electricity produced from different potential sources (Bharathiraja et al., 2018).

Source Type	Biogas production [m³ ton⁻¹ fresh matter]	Produced kilowatt-hour [kW h ton⁻¹ fresh matter]*
Manure		
Cattle dung	55-68	122.5
Chicken litter/dung	126	257.3
Horse manure	56	114.3
Landfills		
Fat	826-1200	1,687.4
Food waste	110	224.6
Fruit wastes	74	151.6
Municipal solid waste	101.5	207.2
WWTPs		
Sewage sludge	47	96

*35% electrical efficiency CHP, heating value 21MJ·m⁻³, 55% Methane content, 3.6 MJ·kWh

During the biogas production process, relevant decisions on using one or either substrate are their sustainability, the expected energy yield, and additional environmental and economic aspects of their use. In fact, sewage sludge from WWTP, manure and landfill are the most important sources with higher production values of biogas.

Manure

Anaerobic digestion of animal manure has been extensively researched for biogas production, due to the manure contains a portion of volatile (organic) solids that are available as substrate to growth and reproduction of anaerobic bacteria. The four main gases produced from decomposing manure are hydrogen sulphide, methane, ammonia and carbon dioxide. The energy value of the gas itself is the methane production. Average values of potential gas production for the various animal species are given in **Table 1.2.** Considering that, during the digestion process, only a fraction of the volatile solids contained in the raw manure are broken down or destroyed by the bacteria (Fulhage, Sievers, Fischer, & Extension University of Missouri, 2018).

Table 1.2. Potential gas production of manure animals (Fulhage et al., 2018).

	Gas yield [ft³·lb⁻¹]ᵃ	Volatile solids voided [lb·day⁻¹]	Volatile solids reduction [%]	Potential gas production [ft³ animal unit⁻¹·day⁻¹]	Energy production rate [BTU·h⁻¹ animal unit⁻¹]	Available energy [BTU·h⁻¹]ᵇ
Swine (150 lb)	12	0.7	49	4.1	103	70
Dairy (12,000 lb)	7.7	9.5	31	22.7	568	380
Poultry (4 lb)	8.6	0.0044	56	0.21	5.25	3.5
Beef (1,000lb)	15	5	41	31	775	520

ᵃ volatile solids destroyed
ᵇ 1000 *BTU·h⁻¹ = 0.2930 kW* *Recollected data from process in 68º F, 1 atm (Fulhage et al., 2018)

Landfills

Landfills gas production is dependent, on the degradation status of the waste material as well as landfill age, and climatic conditions (moisture, temperature). Gas composition in landfills can vary substantially both spatially and temporally. Landfill gas typically has methane concentrations around 50%; it may also have a high sulphur compound concentration, and volatile organic compound (VOC) concentrations in landfill gas vary from few to hundreds mg·m⁻³. In addition, halogenated and VOCs contained within landfill gas contribute to the formation of unhealthy photochemical smog. **Table 1.3.**, shows the concentrations of halogenated and organic silicon compounds in landfill gas in different landfill sites.

Table 1.3. Concentrations of halogenated and organic silicon compounds in landfill gas in different landfill sites (Rasi, Läntelä, & Rintala, 2011).

Site	Halogenated compounds [mg m⁻³]	Organic silicon compounds [mg m⁻³]	Hydrogen Sulphide [ppm]
Jyväskylä, Finland	0.3-1.4[a]	<4	90-230
Nokia, Finland	n.r.	<0.3	27-32
Tampere, Finland	n.r	<1.7	53-125
Balanca, France	224.6	7.8	n.r
Germany	n.r	28.5 - 35.8	230 - 280
Augsburg, Germany	n.r	9.3 - 10.3	n.r
Berlin, Germany	n.r	36.3	n.r
Vienna, Austria	n.r	9.3	n.r
Midlands, UK	327 – 739[b]	n.r	n.r
Pianezza, Italy	17.4 – 32 mg Cl m⁻³ 1.2-6.6 mg F m⁻³	<0.2	114-205

n.r: not reported. [a] Measured five halogenated compounds. [b] Chlorinated compounds.

Sewage sludge from wastewater treatment plants (WWTPs)

The disposal of sludge is a problem of growing importance, representing up to 50% of the current operating costs of a wastewater treatment plant (Appels, Baeyens, Degrève, & Dewil, 2008). Anaerobic digestion (AD) is a common technology for sludge treatment at WWTPs. The US Environmental Protection Agency (USEPA) reports that 1484 WWTPs digest sludge to produce biogas (Y. Shen et al., 2015). Anaerobic digestion plays an important role in their treatment; this process is commonly used to stabilize the primary and secondary sludge until disposal. Primary sludge contains most of the solids discharged in the sewage system. Secondary sludge is mainly microbial biomass, which is generated in the biological aerobic treatment of the wastewater. Nowadays, sewage sludge is one of the top biomass energy resources in Europe in terms of biogas like fuel (Appels et al., 2008; Matsui & Imamura, 2010). Digester biogas typically contains 55–65% methane and 35–44% carbon dioxide.

The amount of organic silicon compounds may be high because of the various uses of silicon containing compounds in households and industry. As with landfills, more wide use of silicon containing materials ending up in WWTPs leads in higher amounts of siloxanes in biogas and it is likely that they are found in most WWTP biogas (Rasi et al., 2011). **Table 1.4.** shows the overall potential of WWTP sludge-derived biogas production in the US, based on different feedstock resource investigations.

Table 1.4. Summary of reported data on energy content of biosolids generated from WWTPs in the US (Y. Shen et al., 2015).

Resource	Basis	Thermal energy [MMBtu year^{-1}]	Electric power [kWh year^{-1}]	Total energy potential [MMBtu year^{-1}]
40 billion gallons of wastewater per day	1MGD wastewater equates 26kW of electric capacity and 2.4 MMBtu day^{-1} of thermal energy	3.52×10^7	9.11×10^9	6.65×10^7
6.5 million dry tons of biosolids per year	Sludge energy content = 8000 Btu dry lb CHP electric efficiency = 30% CHO thermal efficiency = 40%	4.59×10^7	1.01×10^{10}	9.86×10^7
WWTPs with average flow rate > 1 MGD (CHP available in 133 WWTPs, feasible for additional 1351 sites)	Available: 190MWBtu day^{-1} Thermal energy Potential: 40MW Electric power 38,000 MMBtu day^{-1} Thermal energy	2.04×10^7	5.17×10^9	3.81×10^7

MGD: million gallons per day. CHP: combined heat and power

1.1.5 Variations in BioGas Composition

According to its source, biogas compositions can be variable but usually contains 50 to 70 % methane, 30 to 40 % carbon dioxide and < 1 % nitrogen. These percentages can change and other compounds can appear when the primary source contain particular components like silicon or sulphide and halogenated compounds (**Table 1.5.**).

Methane

It is normally released into the atmosphere at most waste treatment facilities and landfills (Kirakosyan & Kaufman, 2009). The methane can be combusted more cleanly than coal, and can provide the desired energy with limited levels of carbon dioxide emission in the atmosphere. Methane in biogas burns with a clean blue flame, which is much hotter than fire that is used as traditional resource for cooking.

Table 1.5. Average composition of biogas and some their properties according their source (Kajolinna et al, 2015; Steinhauser & Deublein, 2011).

	Units	General Sewage gas	Agricultural gas	Landfill gas	Waste water treatment digester	House hold waste digester	Industrial waste	Animal manure digester
Gas composites								
Methane (CH$_4$)	[Vol.%]	65-75	45-75	45-55	65	65	60-80	50-70
Ethane (C$_2$H$_6$)	[Vol.%]	<300 mg· Nm-3	n.r	n.r	n.r	n.r	n.r	n.r
Carbon dioxide (CO$_2$)	[Vol.%]	20-35	25-55	25-30	33.5	29	20-40	30-50
Carbon monoxide (CO)	[Vol.%]	<0.2	<0.2	<0.2	<5	n.r	n.r	n.r
Nitrogen (N$_2$)	[Vol.%]	<0.2	<0.2	<0.2	<5		n.d	<5
Oxygen (O$_2$)	[Vol.%]	0.5	0.01-2.00	1-5	<1		n.d	<1
Hydrogen (H$_2$)	[Vol.%]	Traces	0.5	0.00	Traces	n.r	n.r	n.r
Hydrogen sulphide (H$_2$S)	[ppm]	0-4,000	<15,000	10-1,000	150-3,000	n.r	<30,000	<5,000
Sulphur (S)	[mg·m^{-3}]	n.s	n.s	29-900	<25	30	n.r	n.r
Ammonia (NH$_3$)	[ppm]	100	<0.1	<5	<5	<5	<5	<5
Halogenated compounds (F-; Cl)	[mg·m^{-3}]	0	<500	0-7	n.d	n.d	<0.1	<0.1
Benzene (C$_6$H$_6$)	[mg·m^{-3}]	n.r	0	<36	<0.3	n.r	n.r	n.r
Toluene (C$_7$H$_8$)	[mg·m^{-3}]	n.r	n.r	<287	<12	n.r	n.r	n.r
Siloxanes (L2-L4, D2-D4)	*Siloxanes, treated separately and extensively in Section 1.2*							
Features								
Net calorific value	[kWh·Nm^{-3}]	6.0-7.5	5.0-7.5	4.5-5.5				
Normal density	[kg·Nm^{-3}]	1.16	1.16	1.27				
Relative humidity	[%]	100	100	<100				
Temperature	[°C]	35-(60)	35-(60)	0-25				

n.d: Not determined, n.s: Not specified, n.r: Not reported

Sulphur components

The main sulphur compound in biogases is hydrogen sulphide (H_2S). However, other reduced sulphur compounds, as sulphides, disulphides and thiols, can also be found in biogases. Their effects both water and biogas can be undesirable because in water, can cause corrosion to compressors, gas storage tanks and engines (Persson, Jonsson, & Wellinger, 2007). Whereas their effects in biogas, in anaerobic conditions, for instance methanethiol and dimethyl sulphide (DMS) are formed from the degradation of sulphur-containing amino acids and from the anaerobic methylation of sulphide (Lomans, Pol, & Op den Camp, 2002; Rasi et al., 2011).

Nitrogen Compounds

High concentrations of ammonia are problematic for gas engines, and are often limited by manufacturers of gas engines. If oxygen or nitrogen is present in the biogas this is a sign that air has been entered the system. According to Persson et al., (2007) concentrations of ammonia are in landfill gas typically 5 ppm and in anaerobic digester plants >100ppm. In some upgrading processes like PSA and membranes the oxygen and nitrogen content is also reduced to some extent (Rasi, 2009; Y. Shen et al., 2015).

Volatile Organic Compounds

Natural origins of volatile organic compounds (VOCs) include wetlands, forests, oceans and volcanoes. However, nowadays, a majority of VOCs are created from anthropogenic activities consisting of manufacturing industries, petrochemical industries and vehicular emissions (Berenjian, Chan, & Malmiri, 2012). These compounds are classified according to molecular structure or functional group like: aliphatic and aromatic hydrocarbons, alcohols, ethers, esters, aldehydes (Anand, Philip, & Mehendale, 2014).

These organic chemical compounds of variable lipophilicity and volatility; evaporate easily at room temperature and by its overuse, now, are among the most toxic chemicals which are detrimental to humans and environment. VOCs are not only outdoor pollutants as high concentrations have been recorded indoors as well. Considered among the most toxic chemical compounds, which are detrimental to humans and environment (Anand et al., 2014; Berenjian et al., 2012). Its main receptor is the biogas. VOCs like: hydrocarbons and some silicon compounds may cause severe problems in

the energy producing engines, potentially affecting the performance and inducing costly problems (Gaj & Pakuluk, 2015; Kajolinna et al., 2015). For example, biogas from landfill usually has aromatics, heterocyclic compounds (siloxanes includes), ketones, aliphatic acids (either halogenated or not), terpenes and alcohols, which, due to their vapour pressure and low solubility, are harmful to the environment cause engine failure if the gas is used as an energy source (Rasi et al., 2011; M. Shen et al., 2018). An interesting member of these compounds are the siloxanes, the main topic of this thesis, which are being treated separately and extensively in Section 1.2.

Others

Specific halogenated compounds are often found in landfill gases and only rarely in biogases produced from sewage sludge or organic wastes. Compounds that containing organochloride contribute to corrosion in combustion engines and formation of some kind of dioxins and furans (Persson et al., 2007). For instance, previous studies (Larkin, Allen, Kulakov, & Lipscomb, 1999; Rasi et al., 2011) on halocarbons in landfill gases, were detected total chloride in amounts from 118 to 735 and 169 mg·m^{-3} and total fluorine in amounts from 63 to 256 and 25.9 mg·m^{-3}, respectively.

1.1.6 Problematic and Harmful pollutants in biogas

Typical compositions of different kinds of biogas, which are comparable with natural gas and the possible impact of the contaminants, are showed in **Table 1.6.** These contaminants presence and quantities depend largely on the biogas source. The removal of these contaminants will significantly improve the quality of the biogas for its further uses (M. Shen et al., 2018).

Biogas cleaning primarily involves removal of contaminants in order to increase the calorific value and quality of the gas (Hepburn, Vale, Brown, Simms, & McAdam, 2015). Current removal technologies are based on physical and chemical methods although biological treatments are gaining importance as environmentally friendly biotechnological approaches since the physicochemical methods require high amounts of consumables and are associated to operating and safety points that induce higher costs (Berenjian et al., 2012; Gaj & Pakuluk, 2015).

Table 1.6. Effects of typical components and impurities of raw compounds during biogas conversion. Modified of Shen et al., (2018).

Component		Effect
Carbon dioxide (CO_2)	-	Reduces caloric value
	-	Promotes corrosion because of having weak carbonic acid
	-	Damages alkaline fuel cells
Hydrogen sulphide (H_2S)	-	Corrosion in equipment (toxic concentrations of $H_2S > 5$ mL m^{-3}
	-	Due to furiously combustion, sulphur dioxide (SO_2) and sulphur trioxide (SO_3) are generated in the energy conversion process, which are more toxic than H_2S and can form H_2SO_4 causing more serious corrosion than H_2S (stress corrosion)
	-	Catalytic converter poison
Ammonia (NH_3)	-	Nitrogen: low caloric value
	-	Corrosion in equipment due to reaction with H_2O to form base
	-	Emissions NOx after combustion
	-	Harmful for fuel cells
	-	Increases the anti-knock properties of engines
Water vapour (H_2O)	-	Corrosion in equipment because of reaction with H_2S and CO_2 to from acid
	-	Accumulation of water in pipes
	-	Condensation and/or freezing owing to high pressure
Particulate matter (Dust)	-	Clogging equipment and engine because of deposition
	-	Blocks nozzles and fuel cells
Oxygen (O_2)	-	Risk of explosion while greatly enriched in biogas during the process
Chloride ion (Cl^-) and Fluoride ion (F^-)	-	Corrosion in equipment/combustion engine
Siloxanes	-	Forming silica (SiO_2) and microcrystalline quartz during combustion process and deposition at valves, spark plugs and cylinder heads abrading the surface

1.1.7 Technologies for pollutant removal from biogas

Traditionally, the focus has always been the use of chemical or physical methods to pollutant removal, and the current knowledge on pollutants bioremoval is limited due to the chemical characteristics of contaminants, namely their hydrophobicity, volatility, biodegradability and biocompatibility. **Figure 1.4.** shows an indicative overview about the path of impurities through the purifying treatments of biogas.

Despite the effectiveness of physical or chemical methods in destroying pollutants, the VOCs pollution have not an efficient or complete elimination, and the use of conventional methods may not be economical, mainly for silicon compounds (G. Wang, Zhang, & Hao, 2019).

Product gas
Make up H₂O
O₂ N₂
VOC, H₂S — Water scrubber — Stripper air
NH₃
H₂S   H₂O bleed
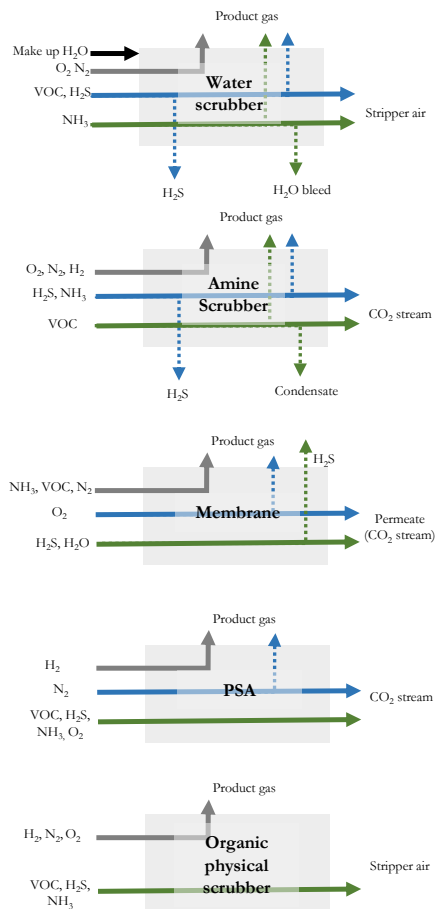
High pressure water scrubbing

CO₂ and H₂S removal Methane emissions are avoided by thermal oxidation in the process air	CH_4 (%)	CO_2 (%)	H_2S (%)	Methane loss (%)	Estimated Costs
	>97	<2	<2 ppm$_v$	<2	0.105 € m⁻³ (250 Nm³ h⁻¹)

Product gas
O₂, N₂, H₂
H₂S, NH₃ — Amine Scrubber — CO₂ stream
VOC
H₂S   Condensate

Chemical scrubbing

CO₂ and H₂S removal Absorption by amines Atmospheric pressure Exhaust-gas treatment is not necessary	CH_4 (%)	CO_2 (%)	H_2S (%)	Methane loss (%)	Estimated Costs
	>99	<1	<4 ppm$_v$	<0.1	0.01 € kwh⁻¹ of raw biogas

Product gas   H₂S
NH₃, VOC, N₂
O₂ — Membrane — Permeate (CO₂ stream)
H₂S, H₂O

Membrane separation

CO₂ removal Previous H₂S and water vapour removal	CH_4 (%)	CO_2 (%)	H_2S (%)	Methane loss (%)	Estimated Costs
	96-99	1-3		<1	0.432 ct Nm³ (50 Nm³ h⁻¹)

Product gas
H₂
N₂ — PSA — CO₂ stream
VOC, H₂S, NH₃, O₂

Pressure swing adsorption (PSA)

Previous H₂S removal Regeneration under vacuum pressure Removal CO₂ and water vapour	CH_4 (%)	CO_2 (%)	H_2S (%)	Methane loss (%)	Estimated Costs
	98	1-2			0.01 € (250 Nm³ h⁻¹)

Product gas
H₂, N₂, O₂ — Organic physical scrubber — Stripper air
VOC, H₂S, NH₃

Organic physical scrubbing

Absorption by polyethylene glycol Capacity: 200-1600 Nm³ h⁻¹	CH_4 (%)	CO_2 (%)	H_2S (%)	Methane loss (%)	Estimated Costs
	98		<1		2 ct (310 Nm³ h⁻¹)

Figure 1.4. Path detail of pollutants present in biogas though different biogas upgrading processes. Modified from (Energiforsk, 2016; Muñoz, Meier, Diaz, & Jeison, 2015).

1.2. Siloxanes

1.2.1 Siloxanes as pollutants in biogas

Siloxanes are a subgroup of silicones, containing Si–O bonds with organic radicals (mostly methyl-groups bound to Si). These compounds are insoluble in water and have a high adsorption coefficient (M. Shen et al., 2018). **Table 1. 7.**, shows some properties of the most representative siloxanes. Volatile methyl-siloxanes (VMS) can be divided into two categories considering their physical properties and structures, i.e., linear and cyclic compounds. The corresponding chemical formulas can be abbreviated as Ln and Dn, respectively, n representing the number of silicon atoms (G. Wang et al., 2019). They are widely used in various industrial processes and consumer products, especially in personal care products such as detergents, shampoos, cosmetics, paper-coatings and textile or as softener and wetting agents (Dewil, Appels, Baeyens, Buczynska, & Van Vaeck, 2007; Rasi, Lehtinen, & Rintala, 2010).

Table 1.7. Chemical-physical characteristics and SiO2 yield of principal volatile methyl siloxanes present in biogas modified of Dewil et al., (2007), Rasi et al., (2010), Soreanu et al., (2011b) and Tansel and Surita, (2014).

a. Linear compounds

Name compound	Hexamethyldisiloxane	Octamethyltrisiloxane	Decamethyltetrasiloxane	Dodecamethylpentasiloxane
Abbreviation	L2 (MM)	L3 (MDM)	L4 (MD2M)	L5 (MD3M)
Chemical formula	$C_6H_{18}OSi_2$	$C_8H_{24}O_2Si_3$	$C_{10}H_{30}O_3Si_4$	$C_{12}H_{36}O_4Si_5$
Molecular weigth [g·mol-1]	162.4	236.54	310.7	384.8
Boiling point [°C]	101 ± 5.9	153	194	230
Melting point [°C]	-59	-82	-76	-81
Vapour pressure at 25 °C [KPa]	4.12 ± 1.2	0.52	0.07	0.009
Water solubility 25 °C [mg·L-1]	0.93	0.034	2.17·10-8 M[c]	7.04·10-5 [c]
Log P_{ow}[a]	4.2	4.8	8.21	4.72
Henry's Law constant dimensionless	2.4 ± 0.2 (27 °C)	121 ± 12 (27 °C)	17	324
Vapour density (air =1)	5.5	8.16		
Viscosity, cP at 25°C	0.86	1.2		
SiO2 yield [mg·mg-1]	0.740	0.762	0.773	0.781
O2 required [b] [mol·mol-1]	12	15	20	24
Physical state, appearance, odour	Liquid, clear, colourless, odourless	Liquid, clear, colourless, odourless	Liquid	Liquid, colourless

b. Cyclic compounds

Name compound	Hexamethylcyclotrisiloxane	Octamethylcyclotetrasiloxane	Decamethylcyclopentasiloxane	Dodecamethylcyclohexasiloxane
Abbreviation	D3	D4	D5	D6
Chemical formula	$C_6H_{18}O_3Si_3$	$C_8H_{24}O_4Si_4$	$C_{10}H_{30}O_5Si_5$	$C_{12}H_{36}O_6Si_6$
Molecular weigth [g·mol-1]	222.5	296.6	370.8	444.9
Boiling point [°C]	135.2	175.7	211.2	245.1
Melting point [°C]	64	17.5	7.5	-3
Vapour pressure at 25 °C [KPa]	1.14	0.13	0.02	0.003
Water solubility 25 °C [mg·L-1]	1.56	0.056	0.017	0.005
Log P_{ow}[a]	4.47	5.1	5.2	5.86
Henry's Law constant dimensionless	-	24 ± 3 (28 °C)	12 ± 2 (26 °C)	5.9 ± 2.9 (26 °C)
Vapour density (air =1)	8	>1	-	-
Viscosity, cP at 25°C	-	2.6		
SiO2 yield [mg·mg-1]	0.810	0.810	0.810	0.810
O2 required [b] [mol·mol-1]	12	16	20	22
Physical state, appearance, odour	Solid, white, hydrocarbon odour	Liquid, oily, clear, colourless, odourless	Liquid, clear, oily	Liquid, clear, colourless, faint odour

[a] P_{ow} = octanol water partition coefficient-1. [b] O2 requirement for complete combustion to CO_2 and H_2O. [c] practically insoluble in water.

The use of siloxanes is increasing because VMS solvents are aroma-free, and widely available, and because they are not included in volatile organic compound (VOC) regulations, they are not considered a health risk to humans. Thus due to their widespread use, siloxanes are commonly found in air, water, sediment, sludge, and biota, and the variation in concentrations can be high (B. Li et al., 2016; G. Wang et al., 2019). The most of these compounds enter into the atmosphere via volatilization, where they are decomposed into silanols, which are eventually oxidised into carbon dioxide, and the rest are discharged into receiving waste water treatment (M. Shen et al., 2018). For instance, small molecules D3, (hexamethylcyclotrisiloxane), volatilise rapidly and are only present in wastewater in small amounts, while the larger molecules, D6, (dodecamethylcyclohexasiloxane) do not volatilise and adsorbed onto extracellular polymeric substances (EPS) of sludge flocks (Tansel & Surita, 2014).

Likewise, some siloxanes molecules are not decomposed in the waste activated sludge process due to have a significantly lower solubility in water and are more adsorptive than many organic compounds. During the anaerobic digestion, most siloxanes are released from the sludge flocs due to the breakdown of organic matter and the increased temperature (up to 37 °C for mesophilic digestion).
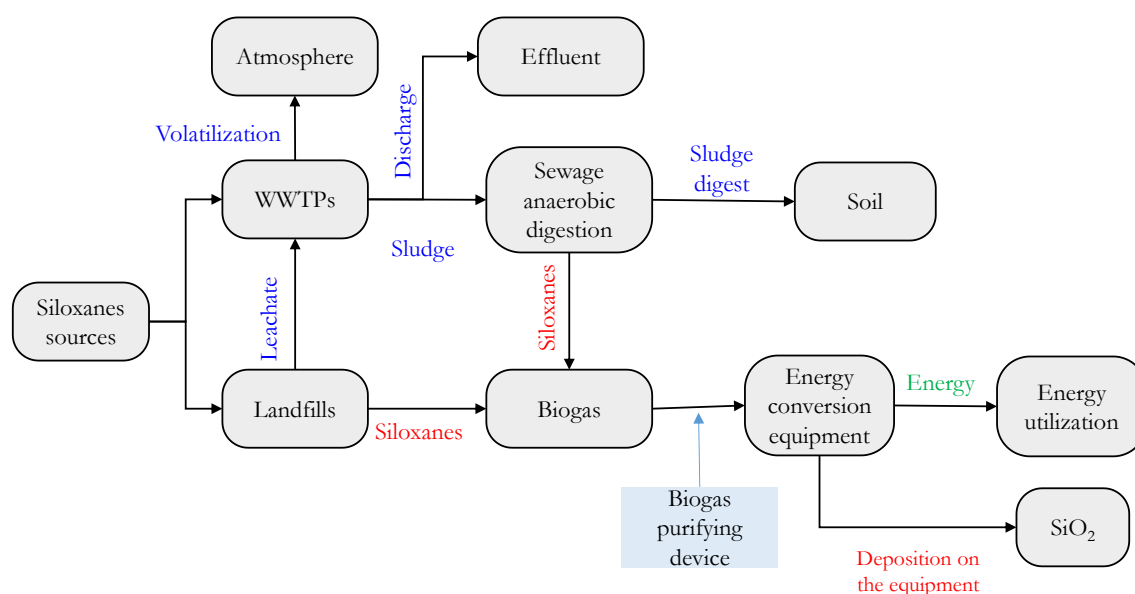
Figure 1.5. Environmental fates of siloxane in WWTPs and landfills and their occurrence in biogas (M. Shen et al., 2018).

They then end up in the biogas, especially the cyclic siloxanes octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) which are detected in significant amounts in the biogas produced during sludge digestion (M. Shen et al., 2018). The main linear Volatile organic silicon compounds

(VOSiC) are the trimethylsilanol (TMSol), the hexamethyldisiloxane (L2), the octamethyltrisiloxane (L3), and the barely present decamethyltetrasiloxane (L4) (Y. Li, Zhang, & Xu, 2014). Environment fate of siloxanes in WWTPs and landfills and their occurrence in biogas are presented in **Figure 1.5.**

Depending upon the type, origin, and quality of organic waste landfilling, sewage sludge digestion, or sorted biowaste digestion processes, relative proportions of siloxanes can fluctuate. For instance, usually the contents of siloxanes in biogas from new landfills are greater than those from older landfills. Similarly, siloxanes from closed landfills occur at greater proportion compared to operative landfills. **Figure 1.6.** shows distribution of siloxane concentration from landfills and WWTP digesters.
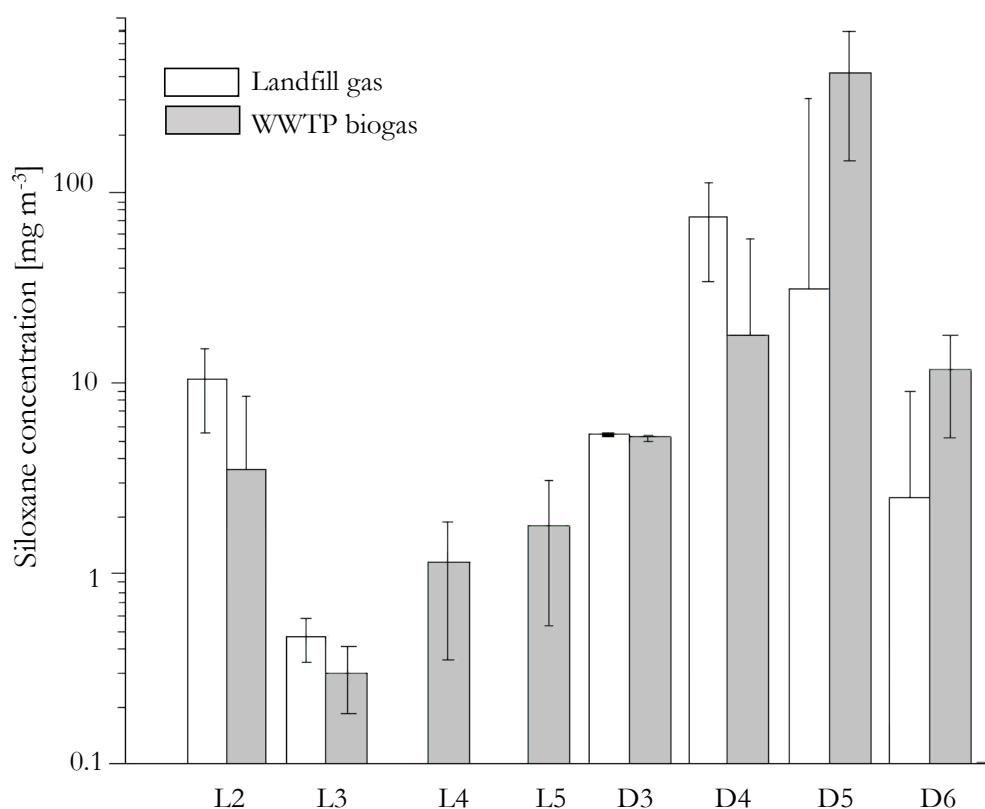
Figure 1.6. Distribution of siloxane average concentrations in biogas from at some selected landfills and WWTP digesters located in Europe. ***Landifill gas plants:*** Sint-truiden, Belgium, Mustankorkea, Finland; Tampere, Finland, Espoo, Finland, Aachen, Germany; Ihlenberg, Germany; Munich, Germany, Torún Poland; Zurich, Switzerland; Trecatti, UK. ***WWTP biogas:*** Wolfgangsee-Ishl, Austria; Prague Central, Czech Republic; Hämeenlinna, Finlad; Treviso, Italy; Viareggio Italy; Velenje, Slovenia; Tarragona, Spain; Boden, Sweden. Modififed from: (Ajhar, 2011; B. Li et al., 2016; Soreanu et al., 2011a).

The predominant chemical species in landfill gas is D4, whereas in WWTP digester gas, D5 is the most abundant. Additional examples of siloxane concentrations in biogas are given in **Table 1.8.** The reason is that different siloxanes possess different water solubilities. In anaerobic digestion process,

due to its low vapour pressure, dodecamethylcyclohexasiloxane (D6) is less volatile in to digestion biogas and still remains in sludge. The concentration of TMSol in landfill biogas is much higher reaching 3500 mg·L⁻¹, but less occurred in digestion biogas because of its volatility and excellent water solubility. Moreover, siloxane concentrations in digestion gas are relatively higher than in landfill biogas, because the temperature of anaerobic digestion process is higher than that in landfills.

Table 1.8. Concentrations of siloxanes in biogas (average values of samples from several sewage treatment plants and landfills[a]).

Compounds	% Silicon	Gas concentration [mg·m⁻³]	Silicates generation (as SiO_2)[b] [lb·Year⁻¹]
Tetramethylsilane	31.84	3.66	5.2
Trimethylsilanol	31.14	3.74	5.2
Tetramethyldisiloxane	41.82	5.57	10.4
Pentametrhyldisiloxane	37.87	6.15	10.4
MM (L2)	36.40	6.73	10.4
Hexamethyltrisiloxane	40.42	8.64	15.6
MDM (L3)	35.63	9.80	15.6
MD2M (L4)	36.17	12.88	20.7
MD3M (L5)	36.50	15.95	25.9
D3	37.88	9.22	15.6
D4	37.88	12.29	20.7
D5	37.88	15.37	25.9
D6	37.88	18.44	31.1

[a] Sampling site (Total siloxane concentration, mg·m⁻³): **Sewage treatment**: Zurich Switserland (25.1), Neuburg, Germany (59.8), Sint-Truiden, Belgium (20.0), Minworth, UK (>16). **Landfill**:Berlin, Germany (36.3), Augsburg, Germany (4.8), Vienna, Austria (9.3) Modified from (Accettola et al., 2008; G. Wang et al., 2019)
[b] Conditions: silicates generated based on 238 Nm³·h for a 1 MW Generator Engine

Organic silicon compounds present in biogas are oxidized during biogas combustion into microcrystalline silicon dioxide, a residue with chemical and physical properties similar to glass. Silicon dioxide forms silica layer and collects in deposits on valves, cylinder walls, and liners, causing abrasion and blockage of pistons, cylinder heads, and valves. The damages caused by siloxanes are easily visible: white-greyish deposits, partly over several millimetres thick. The engine oil, naturally meant to lubricate contact points between moving and non-moving engine parts, gradually becomes enriched with particulate during operation, abrading the engine from within. In the same way, in gas turbines, siloxane deposits usually form on the nozzles and blades, causing erosion of the turbine blades, shorten the life of the engine and subsequently lowering operating efficiency (M. Shen et al., 2018). The thermal insulator it contributes to overheating of sensitive motor parts and abrasion of gas motor surfaces. Moreover, the glassy residues of silicon dioxide can de-activate the surface of the emission control system catalyst for both pre-combustion and post-combustion gas purification, e.g. to reduce

formaldehyde concentrations in engine emissions (Anand et al., 2014; Gislon, Galli, & Monteleone, 2013; Schweigkofler & Niessner, 2001). According to Shen et al., (2018), there is a correlation between the increasing CO emissions and the build-up of silicate-based deposits from siloxane combustion in generator engines. Some organic silicon compounds can end up in the engine oil after the combustion process. In this case, the engine oil needs to be changed more frequently (Soreanu et al., 2011a). This has led some gas engine manufacturers to introduce a limit on silicon of 1 mg L^{-1} in the oil of gas engines (Accettola et al., 2008). Thus, the occurrence of siloxanes is a major barrier to use of biogas as renewable energy source, and removal of siloxanes from biogas before combustion is needed (Lee & Rittmann, 2016; Y. H. Liu, Meng, Wang, Dong, & Ma, 2019).

1.2.2 Siloxanes removal methods and technology

In recent years, with siloxane concentrations having a significant increasing due to the consumption of silicon-containing substances, the development of technology in biogas upgrading have increased too. Damages and adverse effects of siloxane in biogas utilization have been concerned in many places and many technologies have been set up to purge the siloxane in biogas. Several methods are used in industry to remove siloxanes from biogas, **Table 1.9.**, show a summary of each technology, where there are three major commercial technologies available: adsorption, absorption, and cryo-condensation.

Other technologies with better prospects for development also have made a research progress, including membrane, catalysts, biotrickling filters (M. Shen et al., 2018); however, their performance is often unsatisfactory, because they employ expensive or non-recyclable materials, involve long treatment time and high energy consumption, and only afford low saturation adsorption levels (Ajhar, 2011; Kajolinna et al., 2015). Overall, research has tended to focus on typical methods rather than newer technologies as biological methods, which research into silicon compounds treated by bioreactors are still in their experimental stages, and biological technology continues to pose challenges.

Most methods require high amounts of consumables and are associated to operating and safety points that induce higher costs. Previous work has only focused or typically based on adsorption on activated carbon or other media however, nowadays biological removal of siloxane is more increased concerned due to its more convenient, economical and environment friendly.

Table 1.9. Methods and technologies for siloxanes removal modified of Soreanu et al., (2011) and Shen et al., (2018).

Method / Technology	Siloxane removal efficiency [%]	Estimated [a] operating cost [$ m³ h⁻¹ treated gas]	Scale	Advantages	Disadvantages
Solid adsorption (activated carbon, zeolites, molecular sieves, silica gel, activated aluminium oxide, etc.)	90–99	Medium	Full	Simplicity (easy to operation and widely used in removal process). High adsorption capacity (strong adsorption ability). High degree of regeneration possible (heating, solvents). Wide raw material sources and low costs. Increased performance possible via multiple columns in parallel. Can be used for biogas dryin (except AC)	Loss of adsorption material by regeneration process. Thermal regeneration efficiency is limited (formation of polymerization products and deposition of amorphous silica). Minimum two units are necessary (i.e. 1st used, 2nd regeneration). Risk of pressure drop. Activated carbon sensitive to the relative humidity of gases and the occurrence of halogenated compounds or sulphide.
Liquid absorption (methanol, Selexol, etc.)	>97% organic solvent <95% inorganic solvent	High	Full	Efficiency increase by the type of contacting phase (counter-current, etc.). High absorption capacity. Effective for chemical absorption destroys siloxane with strong acids and bases.	High operational and investment costs. High solvent toxicity and flammability. Environmental safety and corrosion. High energy required for regeneration (heating and distillation). Only strong acids can be used and bases react with CO_2 to form carbonates. High energy required for removal process (60°C) and absorbent regeneration.
Refrigeration / Condensation	15-50 (-25°C) 95-99 (-70°C)	High	Full	Simplicity. Low reagent cost. Non-toxic. Biogas drying.	High-energy consumption. High investment and operation costs. Economically suitable for at high flow rate and high siloxane load.
Membrane	>80%	Medium	Laboratory	Simplicity and easy to operation. Large surface area and small volume.	High investment costs and moderate operation costs. Risk of fouling, blocking (pressure drop) and pollution. Membrane destruction by H_2S, halogenated compounds.
Catalytic process	-	Medium	Laboratory	High efficiency. Economical and simplicity.	High temperature required (200-400 °C). Carbonation occurs at basic oxide materials surface.
Degradation (sulphuric acid 97%)	95 –99 (60 °C) 56-70 (RT)	Medium	Laboratory	Simplicity. Low reagent cost. Non-toxic.	Risk of corrosion. Dangerous of handle and transport. Difficult to regenerate.
Biotrickling filtration	10-43	Low	Laboratory	Simplicity / Can be regenerated Economical Ecological Low operational costs	Risk of low efficiency Loss of material by regeneration Risk of fouling Risk of pressure drop
Biological technology	10 - 20% [b] 10 - 43% < 62.8%	Medium	Laboratory	Economical and simplicity. Environmental friendly. Low operation costs. Can achieve reuse.	Low removal efficiency (severely suffer mass transfer constraints and bioavailability). Risk of fouling, blocking and pollution. Long time operation process.

[a] Scale semmification: 1 – 8 (low); 8 – 20 (medium); >20 (high). [b] each author obtained in different operational conditions (e.g. aerobic, anaerobic, variable EBRT)

1.3. Biological technology in siloxane removal

1.3.1 First steps in biological siloxane removal

Current tendencies intensify the need for effective siloxane removal technology, especially an economical and efficient technology with high values of removal percentage and sustainable waste management (Bharathiraja et al., 2018).

Preliminary studies suggested that VOCs can be removed by microorganisms under aerobic and anaerobic/anoxic conditions (Meegoda et al., 2018). To date, few studies have been focused to biological siloxane removal processes (Accettola et al., 2008; Y. Li et al., 2014; Popat, Zhao, & Deshusses, 2012). Bio-treatment of siloxane include the concept of bio-filtration, because is cheaper and easy to operate compared to adsorption or cryogenic methods (M. Shen et al., 2018). Biotrickling Filters (BTFs), bioscrubbers and biomembrane reactors are popular in digestion biogas in experiments for VOCs (siloxanes including) (Berenjian et al., 2012; M. J. Miller & Allen, 2004).

Early studies performed by Popat and Deshusses (2008) and Accettola et al., (2008b) reported siloxane removal using anaerobic and aerobic BTF on lab scale. Firstly, D4 was the only carbon and energy source used and suggested that siloxane removal by biological process needs to take longer residence times, the authors inferred a linear relation between residence time and removal efficiency. In aerobic assays with a RE 43%, and EBRT 19.5 min and REs higher than 43% with an EBRT of 30-40 min; while anaerobic assays reported 15% RE at EBRT of 4 min, concluding D4 was thoroughly degraded in 3 to 4 months (Popat & Deshusses, 2008). However, biodegradation of D4 was poor, probably due to the low mass transfer coefficients between the gas phase and biological cell surface or cell membrane.

Accettola and co-workers (Accettola et al., 2008) reported on the D3 and D4 biological degradation using batch cultures and an aerobic BTF. D3 has a Removal Efficiency (RE) of 10-20% and dimethylsilanediol accumulation was observed during batch culture experiment, suggesting that microorganisms played an important role in the hydrolysis process. Subsequently, Soreanu et al., (2011a); and Șoreanu, Falletta, Béland, Edmonson, & Seto, (2009) worked in an anaerobic gas-phase biomatrices and reported the siloxane partition removal through shaken flask experiments, reporting that the siloxane removal was done mainly by physical-chemical methods (adsorption and absorption), and the microorganisms just accounted for 5–8% (total removal efficiency = 44 – 82%). Likewise, the

formation of dimethylsilanediol (DMSD) as D4 degradation product was determined. *Pseudomonas, Rhodanobacter, Zooglea, Mesorhizobium* and some *Xanthomonadacea* species were identified as major components in a mixed culture leading this formation process (Lehmann, Miller, & Collins, 1998; Singh Ningthoujam & Shovarani, 2008; Singh et al., 2000).

Other studies (B. Li et al., 2016) compared biodegradation of D3, D4, D5 and D6 using activated sludge under anaerobic conditions in an in vitro digestion system. D4 and D5 tended to be degraded more easily compared to the other contaminants. In addition, according to the silicon-mass balance, about 21.4 – 30.6 % of D4 and D5 has been degraded to DMSD. Which could also be biodegradated to form SiO_2, CO_2, and H_2O by microorganisms such as *Arthrobacter* and *Fusarium oxysporum* previously described as VOCs degraders and detected in the studied inoculum (Lehmann et al., 1998; Sabourin, Carpenter, Leib, & Spivack, 1996). Finally, the most recent studies (Y. Li et al., 2014; J. Wang, Zhang, Xu, Li, & Xu, 2014) using aerobic lab-scale biotrickling filters for D4 biodegradation reported high removal efficiencies and identified sub-products, such as rhamnolipids and biosurfactants by *Pseudomonas aeruginosa* and *Phyllobacterium myrsinacearum*. Rhamnolipids, biosurfactants produced by *P. aeruginosa* played an important role in improving D4 removal due to were able to improve the gas–liquid transfer of D4 and facilitate the D4 absorption in the liquid phase (Ma et al., 2016; He et al., 2019). However, the microorganisms present in sludge from siloxane producing facilities may help obtaining more efficient removal values, due to the microorganism will be already adapted to VMS.

1.3.2 Proposed siloxane degradation pathway

Although *Agrobacterium radiobacter, Arthrobacter* sp., *Fusarium oxysporum, Pseudomonas aeruginosa, P. fluorescens* and *P. Putida*, together with other microorganisms found in sludge, have been reported with siloxane degrading capacities (Awe et al., 2017; Rasi et al., 2011; M. Shen et al., 2018; Soreanu et al., 2011a), no clear degradation pathway has been confirmed so far, and only some proposals exist (Y. Li et al., 2014; J. Wang et al., 2014).

A tentative pathway for D4 degradation by P. aeruginosa S240 proposed by Y. Li et al., (2014) is represented in **Figure 1.7.** As degradation products of D4, dimethylsilanediol, methanol and silicic acid were identified in liquid phase and carbon dioxide in the gas phase. It was the first time the existence of methanol in the D4 degradation products was reported.
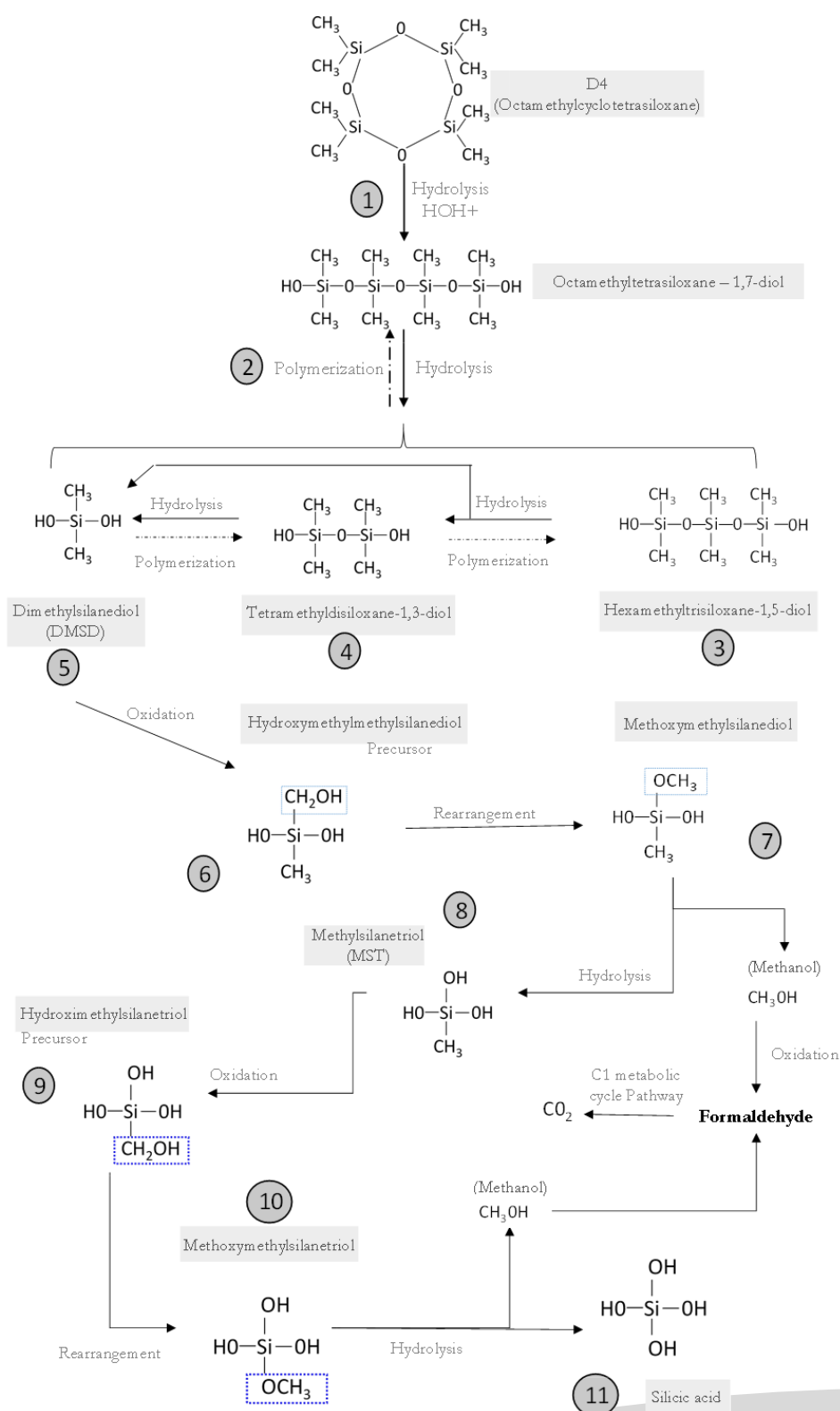
Figure 1.7. Proposed pathway for D4 degradation by *P. aeruginosa* S240 in the BTF. The solid arrow refers to hydrolysis, oxidation or rearrangement reactions, and the dashed arrow to polymerization reactions (Y. Li et al., 2014). Numbers in reactions are explained in the text and are indicated to facilitate comprehension of the figure.

The proposed degradation pathway of D4 would be as follows: D4 ring is opened after hydrolysis to produce a linear derivative (**2**), which is successively cleaved at different Si-O bonds to produce intermediates **3**, **4** and **5**, a process that can be reversed after polymerization (Y. Li et al., 2014). The intermediate **5** is oxidized to the intermediate **6** (a precursor to MST), which is prone to rearrange to the intermediate **7** and be hydrolysed to **8** (MST) and methanol. Similarly, the intermediate **8** can be oxidized to the intermediate **9** (precursor to silicic acid), which is further rearranged to the intermediate **10**. Finally, the intermediate **10** was readily hydrolysed to methanol and the final product **11** (silicic acid). Methanol could be further oxidized to formaldehyde, and the latter will be converted to CO_2 via the C1 metabolic cycle pathway. The accumulation of DMSD in medium implied that the hydroxylation of DMSD might be a rate-limiting step for D4 aerobic biodegradation (Y. Li et al., 2014).

1.3.3 Metabolism and microbial growth in presence on methylsiloxane (C1 compounds)

Overall, the previous investigations suggested that microorganisms were be able to effectively degrade siloxanes, mainly D4, although they took time to evolve and change their metabolic pathways to use siloxane (D4) as a primary carbon and energy source. Most efforts have been setting off to D4 removal, because this compound is the most abundant siloxane in the biogas pollutants group.

Some chemical-physical characteristics of D4 are presented in **Table 1.7.** These characteristics highlight the structure of D4 as a compound with an oxygen–silicon backbone (Si–O–Si) where each Si atom carries methyl groups. In other words, D4 is a multi-carbon compound lacking carbon-carbon bonds. Multiple diverse microorganisms have evolved the intriguing ability to utilize single-carbon (C1) compounds (e.g. methanol or methane) or multi-carbon compounds lacking carbon-carbon bonds (e.g. dimethyl ether, dimethylamine, etc.) as the sole energy source for their growth. Organisms that can use one-carbon compounds as energy sources are called methylotrophs.

The methylotrophic bacteria and the methylotrophic metabolism have been widely studied considering the very natural distribution of C1 compounds at all redox levels. The aerobic methylotrophs have representatives in the *Proteobacteria*, and the majority of the methylotrophs that have been isolated belong to either the *Alphaproteobacteria* or *Gammaproteobacteria*. The most prominent organisms that shaped the major concepts and assumptions within the field have been *Methylomonas*

methanica, Methylosinus trichosporium, and *Methylococcus capsulatus,* representing the three recognized classes (I, II and X) of typical obligate methane utilizers (known as methanotrophs); *Methylobacterium extorquens* and *Paracoccus denitrificans*, representing typical facultative and typical autotrophic methylotrophs, respectively; and a few species within the family *Methylophilaceae*, representing obligate or restricted facultative non-methane utilizing methylotrophs. Another interesting member of methylotrophs namely *Methylibium petroleiphilum* a potent degrader of a synthetic methylated compound, methyl tert-butyl ether (MTBE) has been described belonging to the order *Burkholderiales*: *Methylibium petroleiphilum* grows actively on methanol as well as on a wide variety of multicarbon compounds, including aromatic compounds such as VOCs and as an important member of microbial communities in many VOCs-contaminated activated sludge from WWTPs (Nakatsu et al., 2006 ;Kalyuzhnaya et al., 2006).

In aerobic methylotrophs carbon assimilation proceeds initially by conversion of three C_1 units into a C3 compound via a cyclic pathway. In bacteria three of these pathways are now firmly established, namely the ribulose bisphosphate (RuBP) or Calvin cycle (e.g. in *Paracoccus denitrificans, Xanthobacter autotrophicus*), the ribulose monophosphate (RuMP) cycle (e.g. in *Methylophilus methylotrophus*) and the serine pathway (e.g. in *Methylobacterium* AM1, *Hyphomicrobium* X). The three C1 assimilation pathways involving sugar phosphate molecules as intermediates are not only functionally but also structurally very similar. They all employ condensation of a C1 unit. CO2 or formaldehyde, with a Cs sugar phosphate as the key mechanism of C1 carbon assimilation (fixation phase) (Cheng, Chen, Chen, & Zhang, 2011; Nakatsu et al., 2006; Van Verseveld & Duine, 1987). The common terminal acceptor used during the oxidation of reduced C1 compounds to CO_2 is the oxygen in the most well-studied profiles of methylotrophs. However, the use of other electron acceptors in methylotrophy is more common than previously recognized. For instance, the methane oxidation can be linked to denitrification in the absence of oxygen, but at the moment, the mechanism of coupling methane oxidation to nitrate reduction also remains unknown due to no pure cultures have yet been isolated because they belong to a deeply divergent phylum without any cultivated representatives.

As we see, new directions in methylotrophic physiology are necessary that improve our understanding of the role of methylotrophy in several biochemical processes. For instance, the new challenges in methylotrophy will include uncovering the mechanisms to utilize multi-carbon compounds lacking carbon-carbon bonds such as siloxane D4 as the sole energy source for their growth.
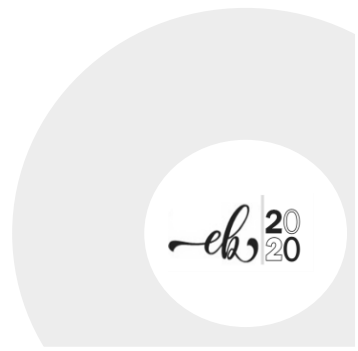
Towards the implementation of
a biotechnology for biogas upgrading:
role of bacteria in siloxane removal

CHAPTER 2

Objectives

Siloxanes are one of the major contaminants in biogas which reduce its quality and cause abrasion to engines during its combustion. Current siloxane removal technologies based on activated carbon absorption are low cost efficient and require the regeneration of activated carbon. In order to develop new environmentally friendly approaches microorganisms can be potentially used as a biotechnological tool for siloxane removal. To understand the role of bacteria in siloxane removal it is important to enrich and isolate microorganisms showing the capacity to grow on siloxane as unique carbon and energy sources and to study microbial communities developed in siloxane feeded enrichments and in biotrickling filters combining different methodological approaches, namely microbiological culture methods, molecular microbial community analysis and lab-scale biotrickling filters performance. Bacterial isolates and microbial communities will show different siloxane biodegradation capabilities depending on the organic substrates used and the operational conditions of the biotrickling filters.

Accordingly, the main goal for this thesis is to study the role of bacteria in siloxane removal focusing in biotechnological approaches for biogas upgrading.

The following specific objectives were defined:

1. To enrich a specialized bacterial community in the presence of D4 by using two porous types of packing materials as physical supports for D4 adsorption and cell attachment.
2. To characterize and define the core bacterial communities developed in the porous materials potentially involved in D4 removal and test for the effect of materials in species enrichment and D4 removal.
3. To generate a culture collection of previously uncultured bacterial isolates showing capacity for siloxane removal to select potential siloxane degraders.
4. To evaluate the removal of D4 alone and in the presence of a multicomponent gas mixture in biodegradation assays both using pure cultures and a mixed bacterial community to be used for biogas purification assays in biotrickling filters.
5. To investigate the performance of a lab-scale biotrickling filter (BTFme) inoculated with *Methylibium* sp. iso58 in order to determine the removal efficiency of siloxanes and other VOCs operating at different gas residence time to minimize the required reactor size and to infer the influence of the final electron acceptor (nitrate and oxygen) disposition in siloxane biodegradation process.

Towards the implementation of
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CHAPTER 3

Materials and Methods
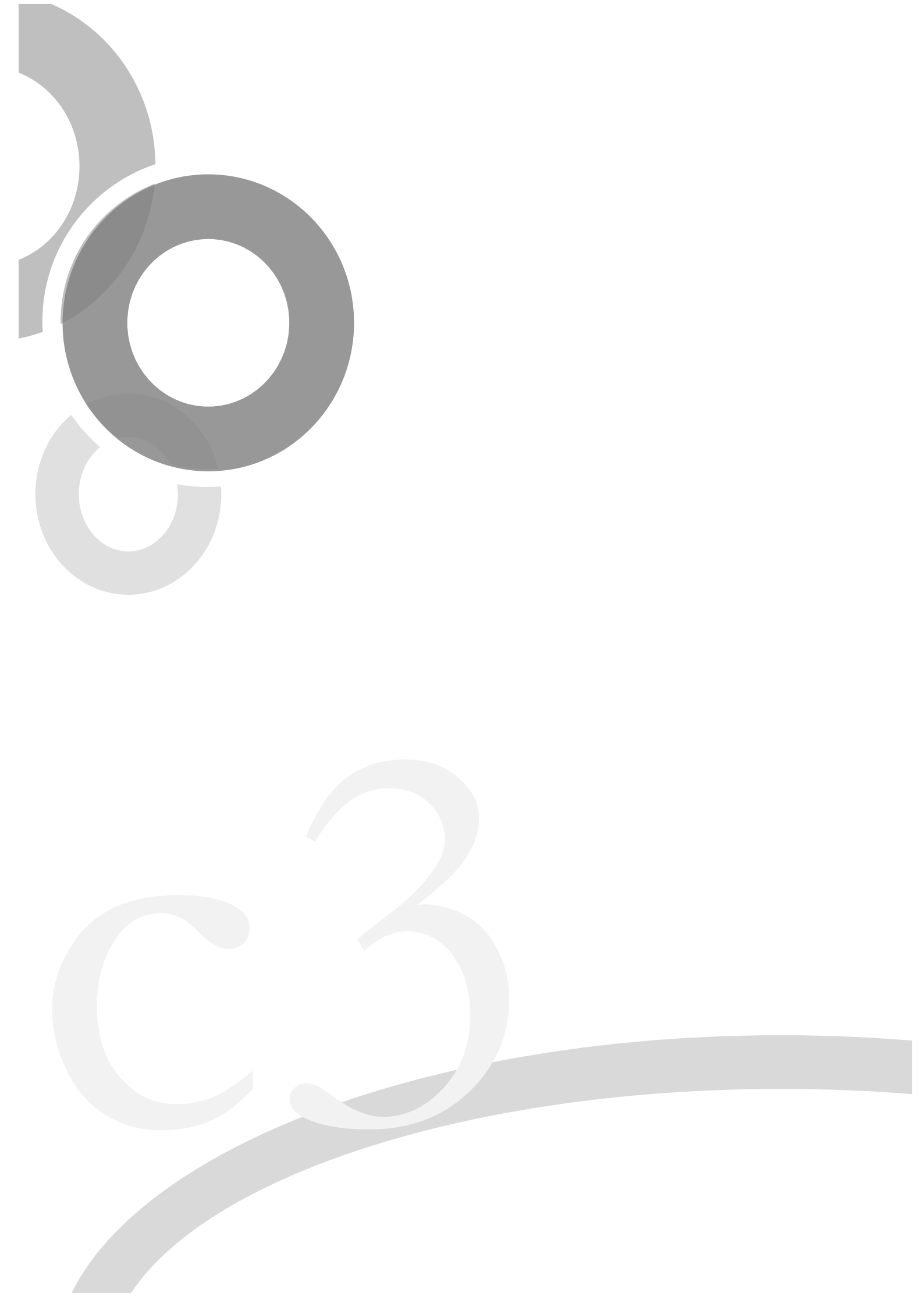
3.1 Sampling site and collection

The Rubi wastewater treatment plant (WWTP) is located at latitude: 41°27'31.1"N and longitude: 2°00'09.3"E in the province of Barcelona, Catalonia, Spain. The Rubí WWTP services the waters of 2 municipalities: Rubí and Valldoreix. The Catalan Water Agency (Agència Catalana de l'Aigua – ACA) (ACA, 2019) is responsible for its planning and management and Cadagua operates it. The WWTP of Rubí have a capacity of treatment of 27,000 m^3 day^{-1} and 195 kW cogeneration plant composed of three 65 kW microturbines, each one producing electricity and hot water at 80 °C. Main points of treatment in the plant are pre-treatment (large particle screens, fines screens, desander, sand classifier, deoiler and influent flow metre), the primary treatment (physical-chemical and sedimentation). The secondary treatment which consist in biological treatment of active sludge through a piston flow system with elimination of organic matter and nitrogen, mud recirculation and sedimentation, and finally mud treatment (thickener, sifting, digestion, dehydration and cogeneration). The plant has also a biogas recovery system. This biogas is used as fuel in a cogeneration plant (ACA, 2019).

Anaerobic sludge was collected from the anaerobic digester in 1 L sterile glass screw capped bottles and kept for two hours at 4 °C until use. The anaerobic sludge was washed to reduce the presence of soluble organic carbon content as follows and according to the protocol described by (Santos-Clotas, 2019). A subsample of 50 mL was centrifuged at 12,000 rpm for 10 minutes and the pellet was re-suspended in 50 mL of synthetic anaerobic mineral media (see below). Centrifugation and resuspension steps were repeated four times. Washed sludge samples were resuspended in 200 mL of the synthetic anaerobic mineral media and stored at 4 °C until use.

3.2 Cultivation-dependent methods: enrichment and isolation procedures

3.2.1 Culture medium and carbon source

Mineral medium used for maintenance and culture growth was prepared as follows. Synthetic anaerobic mineral media without carbon source contained (g·L^{-1}): NaCl 0.5; MgSO$_4$·7H$_2$O 0.1; CaCl$_2$·2H$_2$O 0.01; NH$_4$Cl 0.02; NaNO$_3$ 1.0; KH$_2$PO$_4$·H$_2$O 0.58; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 2.38. The pH was adjusted to 6.9 with NaOH 1 M. The solution was shortly boiled to reduce the oxygen content and degassed under a pure nitrogen gas flow until cooled

to room temperature. Finally, the mineral media was autoclaved (121 ºC, 20 min) and cooled under a constant nitrogen gas flow. Once cold, 10 mL of 10 vitamins solution (Bartscht, Cypionka, & Overmann, 1999) and 1 mL trace element solution SL-10 (Widdel F, 1983) were added per liter.

For culture in solid media with D4 as carbon source, a solution of 40 µL of D4 (99%) was homogeneously distributed onto agar mineral medium. Agar (1.5 % [w/v]) was previously washed with ethanol (99%) solution and rinsed extensively with Milli-Q water by inversion in order to eliminate traces of soluble organic matter that could serve as carbon source.

For liquid cultures performed with D4 as sole carbon source, after the sterilization process a solution of D4 (approximately 60 mg·m^{-3}) was added. Due to the difficulties in preparing stock solutions of D4, concentrations varied from 56 to 62 mg·m^{-3} for different preparations. Exact concentrations were measured for stocks. Usage and final concentrations in the various experiments are given for every specific assay section.

For liquid cultures performed with multicomponent mix as carbon source. After the sterilization of basal medium, a solution of toluene, limonene, hexane, D4 and D5 at different concentrations according to type of experiment (kinetic, removal or bioreactor assays) was added. Information about concentration details are given in specific sections.

3.2.2 Enrichment

D4 saturated porous adsorbents (activated carbons and zeolites) were used to deliver this low water-soluble compound to the anaerobic batch enrichment cultures (BEC). Two different commercial activated carbons (DST-2 and NRT-2), and a synthetic BEA-type zeolite (BEA-38) were used, whose chemical characterization and textural properties and D4 adsorption capacities have been fully described in previous works of Alves et al., (2018) and Cabrera-Codony et al.,(2017; 2014). See **Table 3.1.** for a summary of properties of the ACs used. The properties of the AC depend on the raw material, the activating agent, the carbonization and activation processes undergone (Cabrera-Codony, 2016).

Table 3.1. Commercial name, origin and activation process undergone for activated carbon. (Cabrera-Codony, 2016).

Activated carbon	NRT-2	DST-2
Commercial name	Norit ™ RB3	Airpel 10
Origin	Peat	Anthracite
Activation	Steam	Steam
Odour	Odourless	Odourless
Appearance	Powder	Coal based pelletized
Colour	Black	Black
pH	6 – 9	6 - 8
$D4$ adsorption capacity $D4$ x/M [mg·g^{-1}]	480 ± 4	322 ± 23
BET surface area, S_{BET} [m²·g^{-1}]	1183	933
Total pore volume, Vt [cm³·g^{-1}]	0.53	0.46
Total micropore volume, VDR_{N2} [cm³·g^{-1}]	0.45	0.38
Narrow micropore volume, VDR_{CO2} [cm³·g^{-1}]	0.24	0.09
Mesopore volume, V_{meso} [cm³·g^{-1}]	0.08	0.08
Height of the mass transfer zone, H_{MTZ} [cm]	0.45	0.36

Zeolites are crystalline alumina-silicates with crystalline polymers that form a three-dimensional network of tetrahedral (TO$_4$) SiO$_4$ or AlO$_4^-$ connected through their oxygen atoms (Muñoz et al., 2015; G. Wang et al., 2019). There are several zeolite structures; synthetic o natural. In this study, zeolite BEA-38 was used as packing material in enrichment cultures. BEA-38 was supplied by Zeolyst, and obtained in powder form; has 7.5 Å of maximum diameter, 25 molar ratio SiO$_2$/Al$_2$O$_3$ and NH$_4^+$ cation form (Cabrera-Codony, 2016; Cabrera-Codony et al., 2017). **Table 3.2** summarize some physicochemical properties of the BEA-38 zeolite and its D4 uptake, according to a study of Cabrera-Codony et al., (2017), which reported that BEA type zeolites performed the best for D4 removal compared to other types of zeolites such as: FE-BEA, BEA-300, Fe-MFI, USY, DAY and Clinoptilolite. As was cited, BEA belongs to zeolites with the largest pore dimensions, since they have 12-ring channels with dimensions of 7.5×3.5 Å. However, taking into account that the molecular cross-sectional size of the D4 is 10.8×10.3 Å, D4 molecules should not be able to diffuse into zeolite pores irrespective of the zeolite type used. Thus uptake of D4 (or more precisely its transformation products) into the inner pore volume of the zeolites depends on ring-opening reactions which can be catalyzed by the zeolite surface (Montanari et al., 2010). It has to be highlighted that the BEA 38 zeolite used in this work differ markedly in its channel structure. BEA type zeolites, with high content of Lewis and Brønsted sites, promote the catalytic D4 ring-opening leading on the formation of smaller a-x-silanediols, which are narrower molecules able to diffuse into the channel system. Thus, zeolites with lower SiO$_2$/Al$_2$O$_3$ ratios and higher content of Brønsted acidic sites (Si-OH-Al) as BEA-38 are suitable during an efficient D4 removal process. In addition, BEA zeolites are known to have

generally small crystallite sizes (20-50nm) and thus a high external surface area. This explains that specifically BEA zeolites have been successfully applied for catalytic conversions involving bulky molecules.

Table 3.2. physicochemical properties of the BEA 38 zeolite tested and D4 uptake (Cabrera-Codony et al., 2017)

Zeolite	S_{BET}	D4 uptake[a]	Brønsted acidic sites	Lewis acidic sites	Humidity
	$[m^2 \cdot g^{-1}]$	$[mg \cdot g^{-1}]$	$[mmol \cdot g^{-1}]$	$[mmol \cdot g^{-1}]$	[wt %]
BEA-38	710	135	0.48	3.07	9.5

[a] Tested in dynamic adsorption test, inflow concentration of D4: 3000 $mg \cdot m^{-3}$

Washed anaerobic sludge (100 mL) was inoculated in sterile glass screw capped bottles with septum which contained 1 L of synthetic anaerobic mineral media (initial $OD_{550\ nm} \sim 0.1$) and 500 $mg \cdot L^{-1}$ of D4 retained in the porous adsorbent. All enrichment included duplicates and were defined as Batch enrichment culture (BEC): BEC_DST-2_1, BEC_DST-2_2, BEC_NRT-2_1, BEC_NRT-2_2, BEC_BEA-38_1, BEC-BEA-38_2, classified according to the type of packing material and the number of replicate. The enrichment bottles were incubated at 30 ºC in the dark without agitation until growth was observed by eye inspection (c.a. 30 days). Subsequently, the enrichment bottles were gently mixed, and 20 mL were transferred into 180 mL of fresh synthetic anaerobic mineral media in 250 mL glass serum bottles. In this second enrichment step, 150 $mg \cdot L^{-1}$ of D4 were introduced using the same D4-saturated porous material for each enrichment type. All bottles were incubated at 30 ºC in an orbital shaker (125 rpm) and transferred to fresh medium every 30 days. All cultivation manipulations were done in an anaerobic chamber Coy Lab Instruments using a synthetic gas mixture (N_2:H_2:CO_2 [90:5:5]).

3.2.3 Isolation

In order to isolate D4-degrading strains from BEC, 50 µL of the culture media were transferred onto mineral medium agar plates (agar 1.5% w/v). Once plates were prepared and solidified, 40 µL of D4 [98%] was spread and distributed homogenously on the surface. Whereas, to isolate strains from biofilm of BTF samples, 100 mg of solid samples, i.e. lava rock, were washed with mineral media by gentle mixing followed by centrifugation (5,000 rpm during 5 min), in order to detach the biofilm formed. Recovered cells in the washing solution were transferred onto mineral medium agar plates supplemented with the corresponding carbon source as described.

The inoculated agar plates were incubated for 20 days at 30 ºC in anaerobic conditions by means of an Anaerocult® (Millipore, Germany). An opened tube containing 200 μL of liquid D4 was placed inside the jar during the incubation period in order to ensure the presence of D4 [98%] in the gas phase. Once grown, colonies were selected according to their different morphology and transferred onto fresh solid agar media at least five times to ensure purity.

3.3 Scanning Electron Microscopy

To check for the presence of microbial growth on the porous adsorbents support in BEC, and lava rock in BTFs, samples of the respective solid material were fixed with 2.5% [W/V] glutaraldehyde in cacodylate buffer 0.1 M (pH 7.4), washed and dehydrated successively in ethanol, dried at the critical point, and evaporated carbon. Examinations were carried out with a scanning electron microscope FE-SEM S-4100 (Hitachi, Tokyo, Japan) at the Serveis Tècnics de Recerca (STR Universitat de Girona). Digital images were collected and processed using the Quartz PCI measurement software (Quartz Imaging Corporation, Vancouver, Canada).

3.4 Molecular methods

3.4.1 DNA extraction and quantification

3.4.1.1 Activated sludge

Activated sludge samples were homogenized and 500 μL were transferred to 2 mL sterile centrifuge tubes and immediately stored at –20 °C. DNA was extracted from 500 μL (± 20 μL) of activated sludge using the FastDNA® Spin kit for soil (MP Biomedicals, Solon, OH, USA) in accordance with the manufacturer's instructions. Three replicates were performed per sample. DNA concentration and purity were determined in a Nanodrop ND-1000 UV–Vis spectrophotometer (Nanodrop, Wilmington, DE, USA). DNA extracts were stored at -20 °C.

3.4.1.2 Enrichments

Liquid samples (1 mL) were taken anaerobically from enrichment bottles by means of a sampling port and syringe. The biomass collected from each sample was used for DNA extraction through a combination of enzymatic and chemical cell lysis as follows. Sample was treated with lysozyme (final concentration 1 mg·mL^{-1}, at 37 °C for 45 minutes) and proteinase K (final concentration 0.2 mg·mL^{-}

[1] at 55 °C for 1 h), followed by a modified CTAB (cetyltrimethylammonium bromide) extraction protocol (Llirós, 2010). DNA concentration and purity were determined in a Nanodrop ND-1000 UV–Vis spectrophotometer (Nanodrop, Wilmington, DE, USA).

3.4.1.3. Isolated strains

From each strain, 3 to 5 colonies were resuspended in 10 µL of Tris-HCl buffer 10 mM (pH 7.5). Cells were lysed by six freeze-thaw cycles (3 minutes at -80 °C followed by 3 min at 100 °C). Purity of DNA extracts (presence of proteins or other contaminants) was evaluated from spectrophotometric readings using a Nanodrop ND-1000 UV–Vis spectrophotometer (Nanodrop, Wilmington, DE, USA). Concentration of highly pure extracts was measured by fluorimetry using Qubit ® 2.0 fluorometer (Invitrogen, Molecular Probes Inc, Oslo, Norway). DNA samples were stored at -20°C.

3.4.1.4 Biomass from pilot scale BTF

Packing material taken from BTF was crushed using a sterile mortar and pestle to obtain a homogeneous solid sample. DNA was extracted from 500 mg of crushed material using the FastDNA® Spin kit for soil (MP Biomedicals, Solon, OH, USA) in accordance with the manufacturer's instructions. DNA concentration was determined using Qubit ® 2.0 fluorometer (Invitrogen, Molecular Probes Inc, Oslo, Norway). DNA were stored at -20°C.

3.4.2 16S rRNA gene amplification by PCR

Aliquots of 0.2 µL of DNA extracts were directly used for PCR amplifications of the full 16S rRNA gene sequence. Primers and thermal cycling conditions were used as described in C. S. Miller et al., (2013) with minor modifications. PCR cycling conditions were as follows: (1) at 95 °C for 5 min; (2) 30 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; and (3) 72 °C for 10 min with primers forward 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse 1492R (5'-GGTTACCTTGTTACGACTT-3'). Amplified products were purified and sequenced using the BigDye Terminator Sequencing Kit (Applied Biosystems, US) and an ABI PRISM 3500 genetic analyser (Applied Biosystems) (Chen et al., 2015). PCR products were compared with previously reported sequences in the GenBank at the National Center for Biotechnology Information (NCBI) as previously described (Gich & Overmann, 2006). The sequences were aligned using ClustalW Multiple Alignment Version 2.0.12, (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997).

ClustalW and MEGA Version 7.0.21, (Kumar, Stecher, & Tamura, 2016) were used to infer the isolates phylogeny by the neighbour-joining based on the Tamura-Nei model (Tamura & Nei, 1993).

3.4.3 Quantification of gene copies using qPCR

Gene abundances were determined using quantitative PCR (qPCR). The qPCR amplification was performed for the bacterial 16S rRNA gene and used as a proxy for total bacterial abundance. All reactions were performed in a LightCycler 96 Real-Time PCR system using the LightCycler® 480 SYBR Green I Master (Roche Life Science, Basel, Switzerland). The reactions were performed with a final volume of 20 µL containing 1x LightCycler ® 480 SYBR Green I Master, 35-40 ng of DNA, and 1 µM of each primer, 341F (5'-CCTACGGGAGGCAGCAG-3') and the primer reverse 534R (5'-ATTACCGCGGCTGCTGGCA -3'). Primers and thermal cycling conditions were used as described earlier (Bru, Martin-Laurent, & Philippot, 2008; López-Gutiérrez et al., 2004) with minor modifications: 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and 35 cycles. Standard curves were obtained using serial dilutions from 10^2 to 10^8 copies·µL of linearized plasmids containing a copy of a 16S rRNA gene. PCR efficiency was 89% (r^2 0.98) and controls without template gave null or negligible values.

3.4.4 Barcoded Amplicon Massive Sequencing

Diversity analysis of the microbial community was carried out in DNA extracted from samples of enrichment and BTF. The bacterial 16S rRNA V4 region was amplified using dual indexed Illumina compatible primers Pro515F (5'-GTGCCAGCMGCCGCGGTAA-3') and Pro806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011; Walters et al., 2016). Raw sequence data from this study was deposited via the Biosample Submission Portal (NCBI) under the accession number **PRJNA554091**.

Sequencing of the 16S rRNA gene was performed at MSU Genomics Core (Michigan, USA) using an Illumina MiSeq platform 2x250 bp paired end format using a v2 500 cycle reagent cartridge (Mardis, 2008). Raw sequences were quality-filtered using a maximum expected error of 0.25 (90.4% merged sequences) and a minimum sequence length of 250 bp using Usearch v.9.1 (Edgar & Flyvbjerg, 2015). Paired reads of filtered sequences were joined and checked for the presence of chimera before being clustered into Operational Taxonomic Units (OTUs) (97% cut-off). Singletons and doubletons were removed to avoid spurious diversity. Mothur v1.35.1 was used for taxonomic classification of the

representative sequences of each OTU against the SILVA release 132 reference alignment and taxonomy database (Quast et al., 2013; Schloss et al., 2009). To deeply analyse the microbial community diversity, Simpson's index of diversity (1-D), as well as Good's coverage were calculated. A "composition approach" was used to define the core community of the siloxane degrading BTF and from enrichments (Shade & Handelsman, 2012). For enrichments, members of the core community were identified as those OTUs being present in all samples and the two packing materials, zeolite and activated carbon. Whereas for BTF, members of the core community were identified as those OTUs being present in all samples taken during the BTF operation that occurred at a relative abundance (according to number of sequences) above 2 %.

Statistical analyses were performed using SPSS Windows 25.0 (IBM SPSS, Inc) and Primer-e v6 (PRIMER-E, Ltd). Different tests were applied to compare the microbial community structure analysed in enrichment samples or constructed BTFs. Differences in abundance (16S rRNA gene copy number) were tested for significance with Kruskal-Wallis test. Differences in main diversity indices (richness, Shannon's and Simpson) among sample treatments were tested for significance with Kruskal-Wallis test or U-Mann-Whitney tests. Kruskal-Wallis test was used when more than two enrichments were compared, while U-Mann-Whitney test was chosen to determine differences between two groups.

Differences among bacterial community compositions indicators (beta diversity) were analyzed using the non-parametric multivariate statistical test PERMutational Analysis Of Variance (PERMANOVA) tests using primer-e v6. Statistical correlation between microbial communities was analysed using a Principal Coordinates Analysis (PCoA), based on the Bray-Curtis similarity matrix. Differences between relative abundances of each core community member in the type of enrichment were tested with U-Mann-Whitney tests. Significant differences were declared when $P < 0.05$.

3.5 Kinetics and Bioreactor assays with Microbial community and Bacterial Isolates to degrade Siloxane

3.5.1 Siloxane removal and kinetic assays

3.5.1.1 Culture assays preparation

D4 biodegradation was investigated through laboratory experiments with isolates obtained from both the BECs with porous materials and the BTF. Key selection criteria to choose specific isolates for additional test were double. First, the ability of isolates for organosilicon and volatile organic compounds degradation. Second, similarity, on the basis of 16S rRNA gene sequence comparison, to isolates or species previously reported to have enhanced pollutants removal attributes (Accettola et al., 2008; Grund, Denecke, & Eichenlaub, 1992; Hamme & Ward, 2001). The selected isolates were grown in liquid TSB media, centrifuged (5,000 rpm, 10 min, RT) and resuspended in mineral media. This procedure was repeated four times to ensure the removal of any residual organic compounds present in the TSB media before starting experimental with specific carbon substrates.

In assays with D4 as the sole carbon source, 10 mL of mineral media (initial cell optical density $OD_{550\ nm} \sim 0.1$) was added into 20 mL vials sealed with PTFE septa and 1 µL of liquid D4 (99%, Sigma Aldrich) was injected for each strain.

Another set of vials was similarly prepared in order to investigate the biodegradation of siloxanes (D4) in the presence of other organic compounds by supplementing 18 µL of a mix of siloxanes D4 (5.6 % V/V) and D5 (11% V/V) and a mixture of hexane (55 % V/V), toluene (2.8 % V/V) and limonene (25 % V/V). These compounds were selected based on their co-occurrence in biogas from anaerobic digesters and the reported concentrations range (Papadias, Ahmed, & Kumar, 2012).

In the same way, another set of vials was prepared in order to investigate the influence of the final electron acceptor during biodegradation of siloxane as D4, D5 and VOCs as hexane, toluene and limonene. Several concentrations of Sodium nitrate ($NaNO_3$) and 1% of oxygen were used as final electron acceptors in facultative and aerobic degradation process respectively. The evaluated concentrations of $NaNO_3$ were 1, 2.5 and 8 g·L^{-1}.

Triplicates for all assays including blanks (no bacteria added) and controls (bacteria inoculated without the addition of substrate) were prepared. All treatments were incubated at 30 ºC in a rotary shaker (125 rpm) during 60 days without additional carbon supply.

3.5.1.2 Cell growth measurements

Culture growth was estimated from optical density, cell counts and protein concentration. Optical density at 550 nm was measured using a cell density meter Ultrospec 10® (Biochrom, USA). To quantify the number of cells, equal proportions of cell cultures and buffer solution (HEPES 10 mM, pyrophosphate 10 mM, and Tween 0.08%, pH 7.2), were mixed, agitated 30 min at 150 rpm and fixed with 2% [v/v] paraformaldehyde at 4 ºC overnight. Different volumes of these suspensions were filtered through white 0.22 µm-pore-size polycarbonate filters (Millipore, Germany) and washed twice with autoclaved particle free Milli-Q water. The filters were embedded in agarose at 0.1% [w/v] to avoid cell detachment in the subsequent filter treatments as previously described (Llirós, 2010). The filters were stained with SYBR® Gold (Molecular Probes, Eugene, OR) diluted × 10,000 in HEPES buffer 10 mM (pH 7.5) for 20 min on a shaker at 125 rpm in the dark. The filters were consecutively washed with autoclaved particle free Milli-Q water and cold ethanol (70%). Finally filter sections were air dried, embedded in Citifluor antifading solution (Citifluor Ltd., United Kingdom), and examined under an Axioskop epifluorescence microscope (Zeiss, Germany). Duplicate filters were always processed. Counts were performed by triplicate by one analyst under a 40× objective according to the standard methodology. At least 40 microscopic fields were randomly selected to count SYBR ® Gold-stained cells.

Cell growth was also estimated as protein concentration. Protein extraction protocol was based on the Trizol–phenol–chloroform method as described by the provider (Invitrogen, 2016). Cell protein suspension was quantified using Qubit® Protein Assay Protocol as described by the provider (Life Technology, 2010).

3.5.1.3 Siloxane Analysis

In order to determine the siloxane consumption by biological activity, headspace concentration in the vial equilibrium was determined by means of a gas chromatograph-mass spectrometry detector (GC-MS, 5977D Agilent Technologies) equipped with a PAL automatic injector system and a headspace tool. Prior to gas analysis, all the samples were pre-heated at 60 ºC and agitated during 40 min in order

to reach the headspace equilibrium before injection to the instrument. The capillary column used was a HP-5 ms Ultra Inert operated with helium. The oven temperature ramped from 60 ºC (1 min) to 120 ºC at 30 ºC min^{-1}, then to 150 ºC at 10 ºC min^{-1}, and finally to 300 ºC at 50 ºC min^{-1}. Injector temperature was set at 150 ºC. 500 μL samples were injected at a split ratio 1:6. D4 and D5 detection limit was 500 μg·m^{-3}.

The separation of the target compounds was performed in a capillary column HP-5ms Ultra Inert (Agilent Technologies). Calibration was carried out using commercial standards of siloxanes (D4 and D5) and VOCs (hexane, toluene and limonene) and removal efficiencies were calculated considering the concentration in the blank samples in comparison with the concentration in the inoculum samples.

3.5.2 Lab- scale biotrickling filters

Lab scale biotrickling filter reactors were constructed and operated to test for siloxane removal in continuous gas streams. Briefly, the reactors consisted of a cylindrical column made in Plexiglas (inner diameter: 6 cm, height: 46 cm) operated in a counter-current configuration (**Figure 3.1.**). Inside the column, the packing material was distributed homogenously. Two types of packing material were used, lava rock (particle size: 8-12mm) and activated carbon (particle size: 2-3 mm), and a combination of both depending on the experimental conditions tested. A trickling solution of synthetic mineral medium (composition detail in next section) was continuously recirculated from an external reservoir by a peristaltic pump at a rate of 47 cm·h^{-1}. The synthetic solution was sprayed through the top of the bed column, and was renewed every 72 h.

The feed gas (N$_2$: 70 cm^3·min^{-1}) was generated by a syringe pump (Harvard Apparatus) used for infusing the target compounds to a N$_2$ gas stream regulated by means of a mass flow controller (Alicat Scientific). The average test gas composition was hexane: 375 ± 18 mg·m^{-3}, toluene: 24 ± 2 mg·m^{-3}, limonene: 220 ± 11 mg·m^{-3}, D4: 54 ± 3 mg·m^{-3}, D5: 102 ± 4 mg·m^{-3}.

The inlet and outlet composition of the gas stream was continuously monitored and analysed by GC-FID (7890B Agilent Technologies) in order to calculate both the removal efficiency (RE) and the elimination capacity (EC) following the procedures described in our previous work (Santos-Clotas, Cabrera-Codony, Boada, et al., 2019) following Eqs. (1) and (2). Where C_0 and C_F are the target compound concentrations (g·m^{-3}) in the inlet and outlet of the BTF, Q is the gas flow (m^3·h^{-1}) and V the reactor volume (m^3).

$$RE\ (\%) = \left(\frac{C_0 - C_F}{C_0}\right) \times 100 \qquad (1)$$

$$EC\ (g m^{-3} \cdot h^{-1}) = \left(\frac{(C_0 - C_F) \times Q}{V}\right) \qquad (2)$$

The BTF were initially operated under abiotic conditions (non-inoculated) for a period of 72 hours in order to rule out physicochemical removal of siloxane. Afterwards, different BTF were inoculated with i) activated sludge from anaerobic digester of an urban WWTP (BTFas) and ii) a bacterial isolate (BTFme).
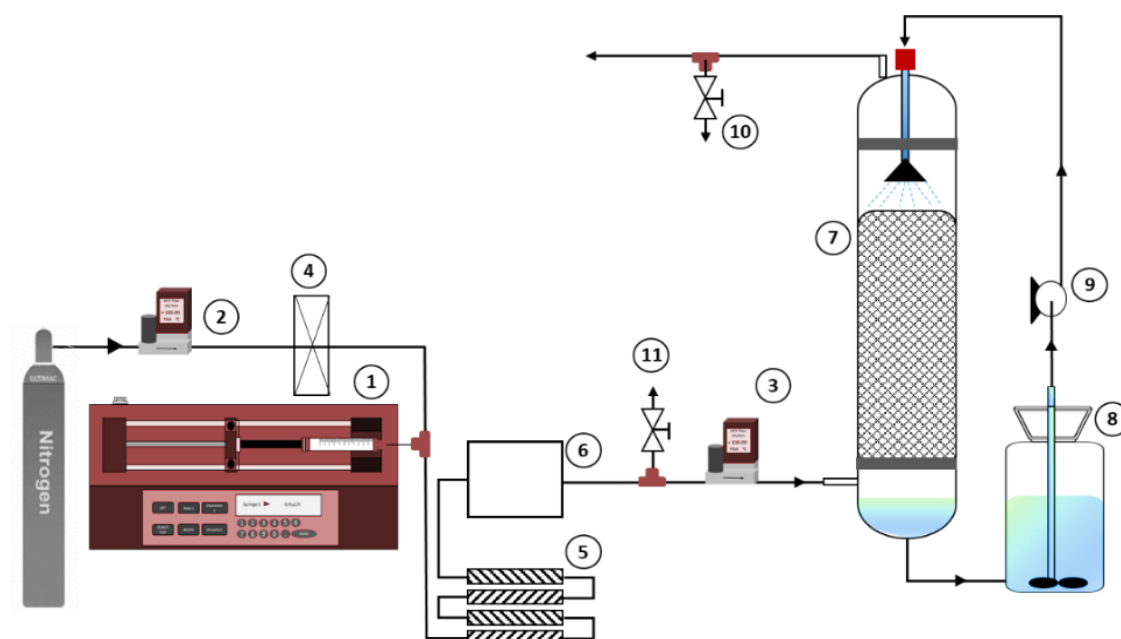
Figure 3.1. Biotrickling filter BTFas setup (1 Syringe pump; 2 and 3 Mass flow controllers; 4 Water column; 5 Static mixers; 6 Mixing chamber; 7 Reactor; 8 Mineral media tank; 9 Peristaltic pump; 10 and 11 Sampling points) (Santos-Clotas, Cabrera-Codony, Boada, et al., 2019).

3.5.2.1 BTF inoculated with activated sludge

The BTFas was inoculated with 320 mL of activated sludge obtained from an anaerobic digester of an urban wastewater treatment plant. Previously, the activated sludge was processed with four cycles of centrifugation (2 × 6,000 rpm, 10 minutes; 2 × 8,000 rpm, 100 minutes) and resuspension in synthetic mineral media with the aim to decrease the content of soluble organic matter in the inoculum.

After the inoculation, four stages of operation were stablished, whose main operational conditions are gathered in **Table 3.3.** The BTFas was operated for a short period (42 days, stage I) at constant empty bed residence time (EBRT) of 14.5 minutes with single D4 as the sole carbon substrate. During the second stage (operation days: 43 to 152), the BTFas was fed with a multicomponent mixture and EBRT was progressively decreased from 14.5 to 4 minutes. During the third stage, from 153 to 186 operation days, the packing media was supplemented with an activated carbon layer (PhAC-1 fully characterized in (Cabrera-Codony et al., 2018) aiming at increasing the mass transfer of the most hydrophobic compounds. Thus, the EBRT was increased up to 12 minutes. Finally, in the fourth stage, lava rock was removed and the activated carbon layer constituted the only packing media, decreasing the EBRT to 2 minutes.

Table 3.3. Operational conditions by each operation stage of the BTFas.

Stage	Operation period [days]	Operational conditions		
		EBRT [min]	Substrate	Packing material
I	0-42	14.5	D4 ($62 \pm$ mg m^{-3})	Lava rock
II-1	43-85	14.5	Multicomponent	Lava rock
II-2	86-106	10.0	Multicomponent	Lava rock
II-3	107-127	07.3	Multicomponent	Lava rock
II-4	127-152	04.0	Multicomponent	Lava rock
III	153-186	12.0	Multicomponent	Lava rock + AC
IV	187-220	02.0	Multicomponent	AC

Density of the initial inoculum was set to an $OD_{550nm} > 0.2$. In order to test for the stability of selected siloxane degraders in the system, approximately every 50 days, the BTFas was re-inoculated using 250 mL of a bacterial mixed culture (cell density: 4.06×10^6 cell·mL^{-1} measured by cell counting) of isolates previously obtained (Boada et al., 2020). A mixture of *Alicycliphilus denitrificans* (iso02); *Pseudomonas aeruginosa* (iso03 and iso07), *Ciceribacter lividus* (iso05), *Pseudomonas citronellolis* (iso22); *Nocardioides* sp. (iso40), *Gordonia polyisoprenivorans* (iso45), *Rhodococcus erythropolis* (iso52), *Microbacterium foliorum* (iso55) and *Methylibium* sp. (iso58) at equal densities was used. Before every re-inoculation the mix culture was previously adapted to the presence of D4 (20 mg·L^{-1}, 99%) by maintaining the cultures in closed 250 mL serum bottles. Immediately before inoculation those cultures were washed by centrifugation and resuspended with mineral media to remove residual organic compounds.

Data on absorbance values, cell numbers and protein concentrations from the assays were evaluated using Levene's test for homogeneity of variances and Shapiro–Wilk's test for normality. The data were normally distributed and homoscedastic. Comparison of growth according to incubation conditions

and strains, were done using analysis of variance (ANOVA) and post hoc Tukey test. All tests were performed using SPSS v25.0 (SPSS, Chicago, IL, USA).

Samples of 5 g of granular packing material (lava rock, activated carbon, or both) were periodically collected from the BTFas. Biofilm samples for the analyses of bacterial communities were taken from bottom section of BTF. Collected samples were stored at -20 ºC for molecular analyses until its use.

3.5.2.2 BTF inoculated with potential isolates

An additional a lab-scale anoxic BTF (BTFme) was inoculated with a specific isolates for evaluating its performance in the biodegradation of a multicomponent gaseous mixture of siloxanes and VOCs. It was inoculated with 250 mL (initial cell optical density $OD_{550nm} \sim 0.1$) of a bacterial isolate previously selected as microorganisms that exhibit great bioremediation potential because they are able to grow in presence of D4 as sole carbon and energy source. After the inoculation, three stages of operation were stablished according to **Table 3.4.** The first period (0-94 days, stage I) was operated at empty bed residence time (EBRT) of 15.5 minutes. During the second stage (operation days: 95 to 170), the EBRT was decreased from 15.5 to 7.7 minutes. During the third stage, from 171 to 235 operation days, the EBRT decreased from 7.7 to 4.4 minutes.

Until operation day 128, the NO_3^- was supplied by the periodic replacement of the mineral medium solution. From day 129 on, an automatic dosage system was installed in the set up in order to maintain stable NO- concentration of 2.5 $g \cdot L^{-1}$. Finally, since operation day 213, the test gas was supplemented with 1% of Oxygen in order to evaluate the performance in oxic conditions. NO_3^- concentration in the recycling solution of the HF-MBR was analysed by a UV–Vis Spectrophotometer (Cary3500, Agilent Technologies) following the Standard Methods 4500-NO_3^- (American Public Health Association (A.P.H.A., 1998) (UV absorption at $\lambda = 220$ nm).

Table 3.4. Time period and operational conditions related to final electron acceptor.

Stage	Operation days	EBRT [min]	e⁻ acceptor
I	0-94	15.5	NO_3^- batch loading
IIa	95-128	7.3	NO_3^- batch loading
IIb	129-170	7.3	NO_3^- continuous 2.5 mg L^{-1}
IIIa	171-212	4.0	NO_3^- continuous 2.5 mg L^{-1}
IIIb	213-235	4.0	NO_3^- continuos 2.5 mg L^{-1} + 1% O_2
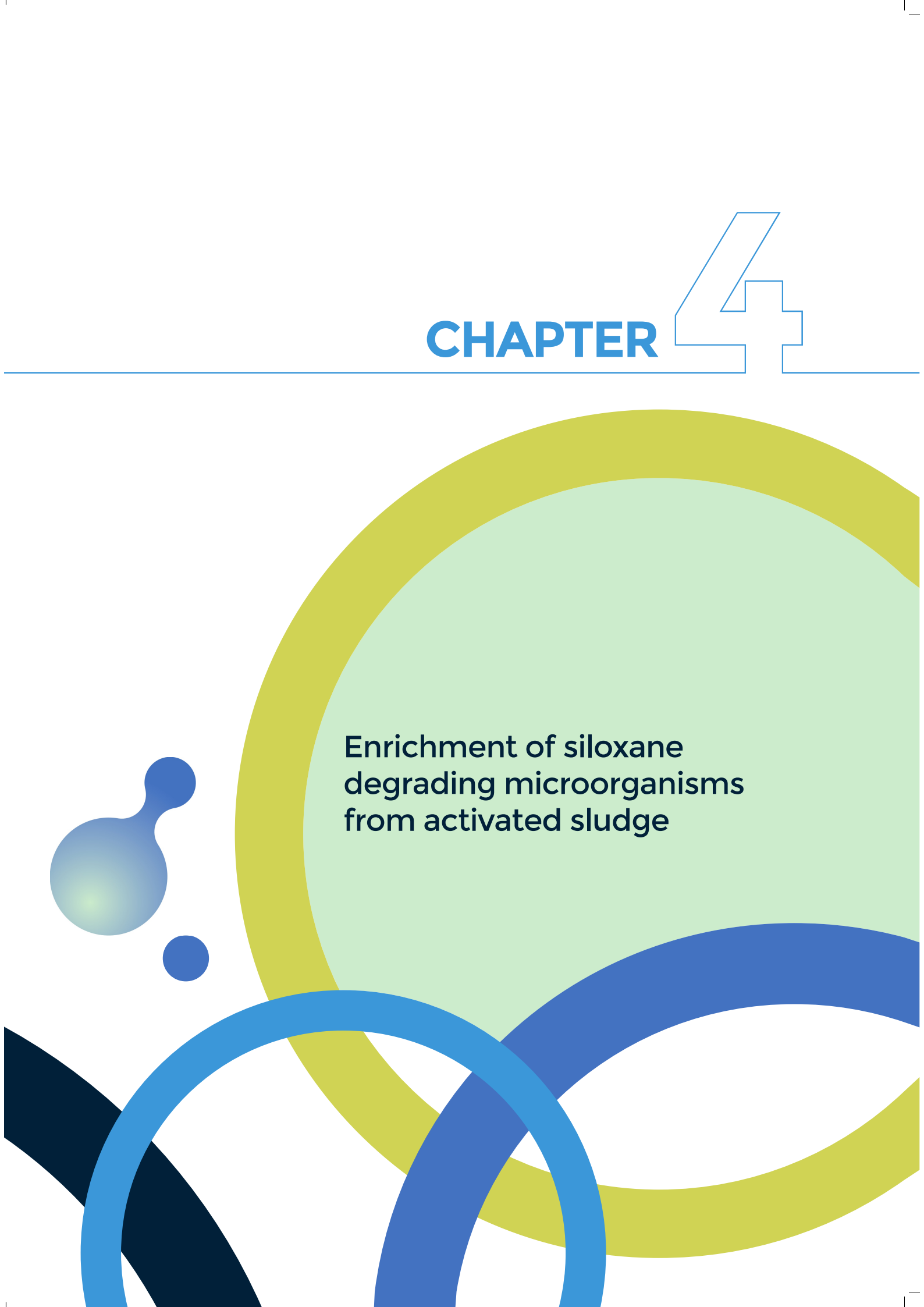
Towards the implementation of
a biotechnology for biogas upgrading:
role of bacteria in siloxane removal

CHAPTER 4

Enrichment of siloxane
degrading microorganisms
from activated sludge

4.1 Background and objectives

Biogas technology is one of the effective ways to ease the energy crisis, due to it is considered a green, environmental and valuable renewable fuel. However, biogas also contains undesirable chemicals such as siloxanes, which could cause damage or corrosion to the energy conversion engines, and prevent a direct use of the biogas from the emission source. Treatment of impurities is complex, induces higher costs and some unwanted side-effects come up.

Commonly used siloxane removal technologies use adsorption onto solid matrices, e.g. activated carbon, zeolite, and others, and have been proven to be effective (Tu et al., 2019). Some studies suggested siloxanes could be removed biotically, although removal efficiencies are limited due to their high hydrophobicity and volatility (Garner & Barton, 2002; Meegoda et al., 2018; C. Wu et al., 2017; H. Wu et al., 2018). These limitations have affected negatively in the development of biologically based methods. In addition to this, microorganisms available so far that exhibit capacity for siloxane transformation are not biotechnologically efficient for distinct reasons. Our main goal in the present chapter was to enrich a steady and specialized bacterial community in the presence of D4 by using two porous packing materials (activated carbon and zeolite) as physical supports for D4 adsorption and cell attachment. Additionally, we characterized and defined a core bacterial community, potentially involved in D4 removal, and tested for the effect of materials in species enrichment and D4 degradation activities.

4.2 Methodology

The microbial community of enrichments (BECs) with activated carbon or zeolite added as packing material and inoculated with activated sludge from WWTP was obtained as described in **Section 3.2.2;** in the presence D4 as the only carbon source. ADN from each enrichment were obtained as described in **Section 3.4.1.2**. The microbial community structure was analysed by molecular methods detailed in **Section 3.4.2** in order to test for the presence of particular degrading bacteria. Microbial diversity and richness were determined and analysed according to **Section 3.4.4**, the Simpson's diversity indices and Richness values were defined by molecular tools as described at the end of this section. Finally, statistical analysis described in **Section 3.6.2.1** supported and validated the defined members of core community. BEC culture samples were observed thought SEM images as described in **Section 3.3**.

4.3 Results and Discussion

4.3.1 Enrichment of bacterial cultures obtained from activated sludge in the presence of D4.

A successful application of enrichment culture techniques for D4-degrading bacteria was obtained using activated sludge of WWTP as inoculum. Two solid substrates were used separately, activated carbon and zeolite. During the bacterial growth period (~60 days), solid samples were recovered and formed biofilms analysed by SEM. High cell densities of different bacterial morphologies were observed and distributed homogenously on the porous adsorbents, thus, demonstrating bacterial adherence and colonization (**Figure 4.1.**). Pili like structures were recognizable for most of the cells and suspected to participate in the adhesion process.
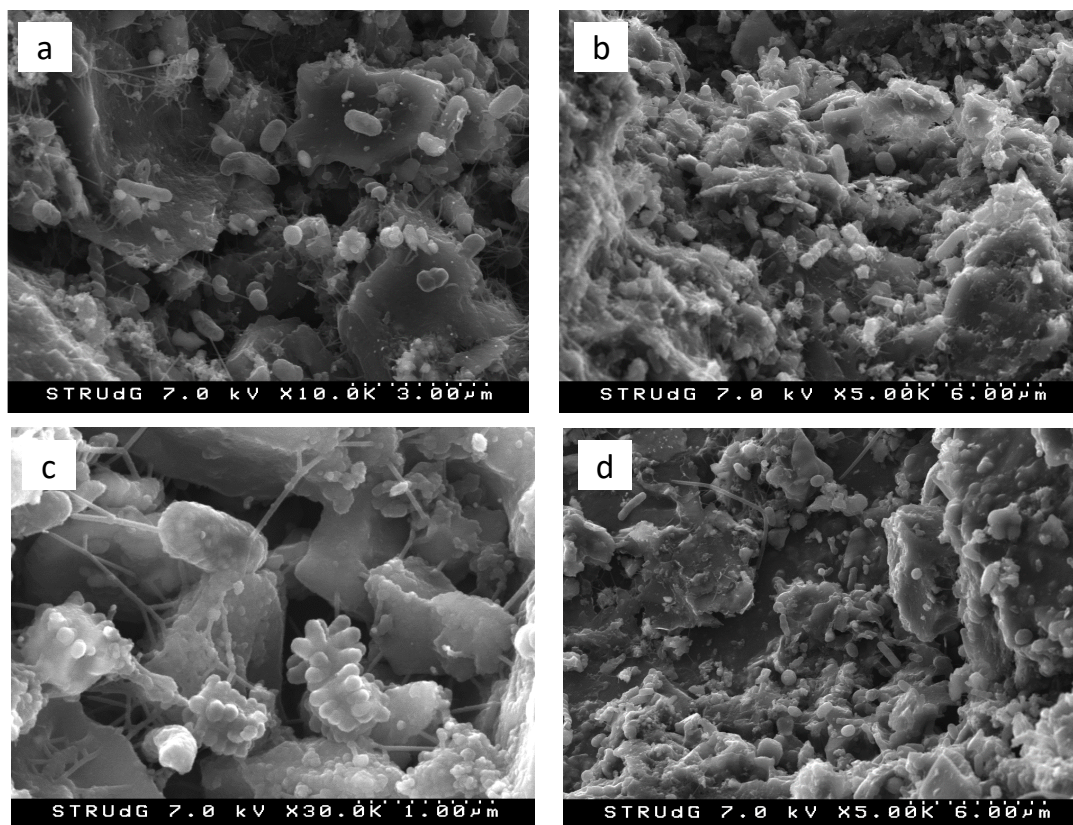
Figure 4.1. Scanning electron photomicrographs of activated carbon Norit: NRT-2-1 (**a.**) and NRT-2-2 (**b.**); Airpel 10: DST-2_1 (**c.**) and DST-2_2 (**e.**) present in anaerobic batch enrichment cultures.

Although not analysed quantitatively, the number of cells attached to packing materials exceeded the number of cells in suspension, suggesting the preference for biofilm growth. Moreover, some studies (Y. Li et al., 2014; Tu et al., 2019) have reported that using packing materials effectively improved

conditions for the growth of microorganisms and the removal performance. Tu et al., (2019) reported the removal efficiencies of more than 92.5% on four biogas compounds (mostly sulphur associated) with a BTF packed with a combination of plastic balls and lava rock compared to a BTF with sole lava rock as packing material. As well as, Li, Zhang, & Xu, (2014) reported studies about siloxanes removal with *Pseudomonas aeruginosa* S240, the outlet D4 concentrations were 12 ± 3 mg m^{-3}, with the REs of $76 \pm 6\%$ in BTF packed with lava rock.

Different physicochemical properties of packing materials may not fulfil ideal surface conditions for the biofilm formation and improved cell growth of microorganisms with specialized metabolism, which lead to differences in colonization (Wang et al., 2019; Tu et al., 2019). Packing materials do not only provide support for bacterial growth but are effective in initial chemical modification of the added siloxanes, affecting degradation capacities too.

Activated carbon is well-known as a suitable material for biofilm formation (Chang & Rittmann, 1987; Nakhla & Suidan, 2002; Z. Wang, Kim, Nakhla, & Zhu, 2016). In addition, the influence of the surface chemistry on the adsorption of biogas impurities on ACs has been also widely studied (Y. Shen et al., 2015; Soreanu et al., 2011b; Yu, Gong, Chen, & Zhang, 2013). However, there is a lack of studies about the influence of the AC surface chemistry on the adsorption of siloxane. Nevertheless, the presence of particular chemical groups over the surface of activated carbon affect some reactions associated to the metabolism of D4 has been reported. A study belong to Cabrera-Codony, (2016), who investigated the polymerization of D4 over the surface of some activated carbons (include NRT-2 and DST-2) during several prolonged contact times, and reported that the of phenolic and carboxylic groups over the surface of ACs, especially the ones chemically activated, catalyse the ring-opening of the D4. This process leads to hydrolysis and promote several condensation reactions that form silanols and even cyclic siloxane of lower molecular weight. However, this process depends compulsorily of the contact time and of quantity of oxygenated functional groups over ACs surface. The present dissertation uses two types of activated, Airpel and Norit, whose chemical capacities are associated to surface chemistry on the adsorption of biogas impurities. For instance, Airpel 10 (DST-2) is an anthracite based carbon activated by a steam process, so that, it has a moderate quantity of oxygen functional groups, which promote the ring-opening polymerization of siloxanes and facilitates degradation according to proposed pathways (Cabrera-Codony et al., 2017; Y. Li et al., 2014). For instance, Airpel 10 (DST-2) is an anthracite based carbon activated by a steam process, so that, it has a moderate quantity of oxygen functional groups, which promote the ring-opening polymerization of

siloxanes and facilitates degradation according to proposed pathways (Cabrera-Codony et al., 2017; Y. Li et al., 2014).

Zeolites were also effective for the adsorption of gaseous pollutants. Compared to activated carbons, the zeolites have some advantages mainly in advanced oxidation process (AOP) (G. Wang et al., (2019). The AOP, are processes based on the generation of very reactive radicals, mainly hydroxyl radicals, which oxidise target organic pollutants. Zeolites can be used as dual functional catalysts. Firstly, siloxanes can be adsorbed (and transformed) in their porous structure and secondly, zeolites can AOP promote advanced oxidation processes, without being vulnerable to oxidation. Thus, zeolites have the potential to be long-lived reusable catalysts.

Likewise, many studies focus on the synthesis of new mesoporous zeolites to fulfil a better adsorption property, which have some unparalleled advantages compared to carbons in D4 removal process. Soreanu et al., (2011) and Ea Sigot et al., (2015) pointed out that siloxane molecules (D4) could interact with hydroxyl groups on the adsorbent surface by H bridges (hydrogen bonds) and Cabrera-Codony, Montes-Morán, Sánchez-Polo, Martín, & Gonzalez-Olmos, (2014) claimed that the surface hydroxyl groups could work as catalytic active centres for cyclosiloxane ring-opening polymerization. To validate this hypothesis, Jiang et al., (2016) dealt with the development and exploration of new zeolite-type materials with mesoporosity for siloxane adsorption. Which were applied in biogas clean up, particularly the adsorption of siloxane D4. In this study, the authors have been attributed the D4 polymerization to the hydroxyl groups on the adsorbent. Besides, zeolites have been widely used as an ion exchanger for the removal of some compounds (e.g. ammonium) due to the effect of the cations (e.g. Na^+, Ca^{2+} and Mg^{2+}) in its crystalline structure. The studies related to D4 adsorption with zeolites are insufficient now. However, as was mentioned; studies of Alba Cabrera-Codony, (2016) verified the efficacy of zeolite as both adsorbent and catalyst for D4 purification, and ascribed the D4 uptake to the catalytic conversion process through which D4 was catalysed to linear product and then entered the inner pores of zeolite. Likewise, Wang et al., (2015) and Montalvo et al., (2012) suggested that, zeolite has favourable characteristics for microorganism adhesion due to is used as support media for the immobilization of microorganisms in different high-rate reactor configurations (e.g. fixed bed, fluidized bed and so forth).

4.3.2 Alpha diversity indicators of enriched microbial communities

The community structures were determined by sequencing the V4 region of 16S rRNA gene by means of Illumina MiSeq sequencing (**Table 4.1.**). As a result, 334,297 raw reads were reported with a length between 243 and 500 nt (average 254.5 nt), 279,984 sequences passed filtering. On average 46,664 sequences were obtained per sample of enrichment, ranging from 31,861 to 56,774 for activated carbon and the zeolites samples had mean number of sequences of 49,066 ± 2,243 sequences. The high coverage estimators (higher than 99%, 0.9997 ± 9.11·10^{-5}) revealed that the microbial community could be well represented by the analysis performed for all samples.

Table 4.1. Detail of microbial communities.

Packing material	Sample code	Number of sequences	Number of OTUs[a]	Good's coverage
Activated carbon Airpel 10	DST-2_1	56,774	101	0.9998
	DST-2_2	31,861	78	0.9998
Activated carbon Norit	NRT-2_1	48,484	217	0.9997
	NRT-2_2	44,732	234	0.9995
Zeolite BEA type	BEA-38_1	51,310	247	0.9996
	BEA-38_2	46,823	141	0.9996

[a] Total number of OTUs: 373 OTUs.

The sufficient coverage of microbial diversity and richness in those samples was confirmed by a rarefaction analysis. The rarefaction methods allow us to estimate that the number of sequences in a given bank is sufficient to yield a correct estimate of the sequence diversity of a sample. The principle is to subsample a set of data and determine whether each new subgroup provides new sequences. The result is a curve that gradually becomes flat, the asymptote representing the maximum possible diversity or richness (Caumette, Lebaron, & Matheron, 2015). Extrapolation of the curves can also be used to estimate maximum microbial richness, i.e., the likely number of sequences in each sample, by using a Chao1 estimate. In this study, rarefaction curves revealed a reasonable coverage of bacterial richness (**Figure 4.2.**) with a representative subsample of 31,800 sequences, corresponding to the minimum number of sequences obtained in a sample (DST_2_2). Subsequent analyses were performed at this sequencing depth.
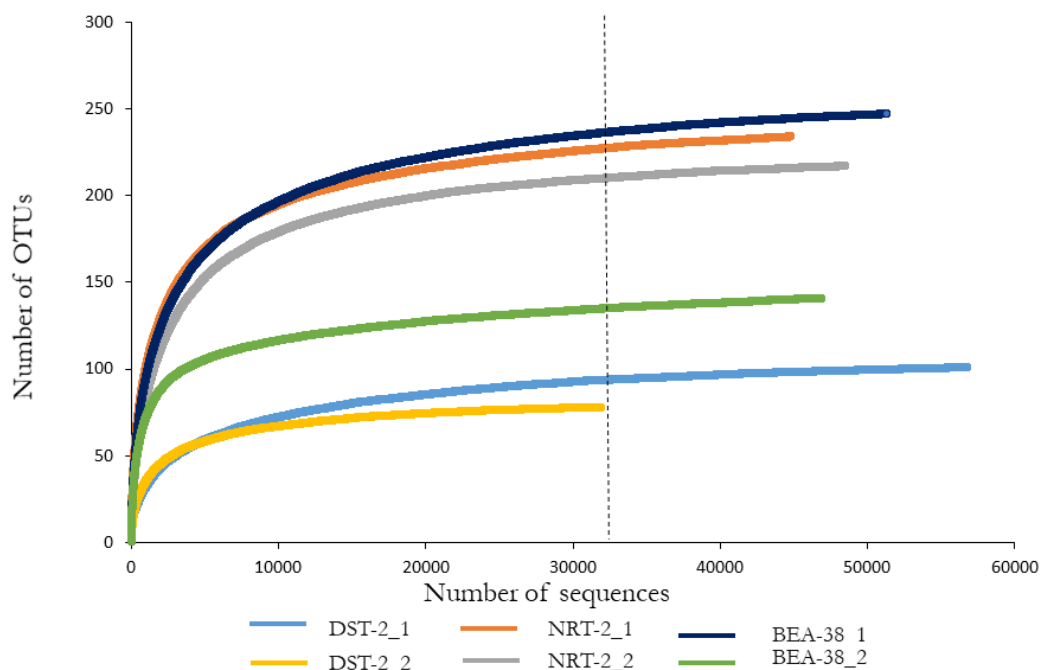
Figure 4.2. Rarefaction curves of samples analysed from activated carbon (DST-2 and NRT-2) and zeolite (BEA-38) enrichments. A subsample of 31,800 sequences was used to analyse alphadiversity (dot line).

Some previous studies reported that several indices that combine measures of richness and abundance has been applied in microbial community analyses (Moris et al., 2014; C. Wu et al., 2017). Foremost among these are the Chao1, Shannon's diversity (H'), and the inverse Simpson's diversity (1-D) indices, which differ in their theoretical foundation and interpretation (**Table 4.2.**).

Table 4.2. Alpha diversity indicators of Richness (S and Chao1) and Diversity (Shannon's and Inverse Simpson's) obtained from an OTU based analyses at 0.03 cut-off.

Packing material	Sample code	*Richness*		*Diversity*	
		Sobs	*Chao1*	*Shannon's (H')*	*Simpson's (1-D)*
Activated carbon Airpel 10	DST-2_1	101	107.8 ± 12.9	2,22 ± 0.010	0.826 ± 0.002
	DST-2_2	78	080.1 ± 6.5	1,93 ± 0.015	0.776 ± 0.002
Activated carbon Norit	NRT-2_1	217	221.6 ± 8.3	3,02 ± 0.014	0.908 ± 0.001
	NRT-2_2	234	244.7 ± 14.4	3,54 ± 0.014	0.935 ± 0.001
Zeolite BEA type	BEA-38_1	247	252.5 ± 8.7	3,19 ± 0.015	0.899 ± 0.001
	BEA-38_2	141	153.0 ± 17.8	2,98 ± 0.014	0.900 ± 0.001

[a] 0.03 are the OTU cut-off in distance units. (Sobs, number of OTUs)

Chao1 is a nonparametric method for estimating the maximum number of species an environment can harbour. The Chao richness estimator was developed by Anne Chao and is based on the concept that rare species infer the most information about the number of missing species (C. Wu et al., 2017; Falk, Chaganti, & Weisener, 2018). As shown in **Table 4.2.**, at the OTU level, the Chao1 richness

indexes for the activated carbon and zeolite were between 80.1 ± 6.5 and 252.4 ± 8.65, belonging to DST-2_2 and BEA-38_1, respectively. Statistical analyses reported not significant differences among evaluated samples (Kruskal-Wallis tests, p value $= .416$; $p > 0.05$). The zeolite BEA-38 enrichments presented higher values of Chao1 indices and their values represent higher richness; consequently, this support material could play an important role during growth related to D4-specialized metabolism with a wide range of species. Tyagi, da Fonseca, & de Carvalho, (2011) suggested the use of carrier materials often provides a physical support for biomass, along with a better access to nutrients, moisture and aeration, which extends the survival rate of the microbes, mainly in presence of particular substrates (such as recalcitrant or with intermediate biodegradability), depending on their chemical structure and physical state. In this case, the hydrophobicity of D4 is an important factor that has a direct influence in the survival rate and "diversity" of the microorganisms with D4-related metabolism.

Simpson's Diversity Index is a measure of diversity, which takes into account the number of species present, as well as the relative abundance of each species (Huang et al., 2019). The higher Simpson's diversity indices were found in NRT-2 enrichments (0.935 and 0.908). However, statistical analysis reported that not significance differences are present among Simpson diversity indices from all enrichments (Kruskal-Wallis tests, p value $= .416$; $p > 0.05$).

Finally, the Shannon diversity index (H) is another index that is commonly used to characterize species diversity in a community. Like Simpson's index, Shannon's index accounts for both abundance and evenness of the species present. The Shannon's index increases as both the richness and the evenness of the community increase (Morris et al., 2014). Typical values of Shannon index are generally between 1.5 and 3.5 in most ecological studies, and the index is rarely greater than 4 (Caumette et al., 2015). In this study, the values were between 1.93 ± 0.0015 (DST-2_2) and 3.54 ± 0.014 (NRT-2_2), suggesting that despite the number of OTUs is high, only a minimum number of them occur at high relative abundances. Statistical analyses reported not significant differences were found among evaluated samples (Kruskal-Wallis tests, p value $= .416$; $p > 0.05$). Based on the average value, samples from NRT-2 had higher Shannon index and greatly higher Simpson index than those from DST-2 and BEA-38 indicating that microbial communities in NRT-2 were evenly distributed than the others. The results suggest that among evaluated enrichments, non-differences are found at alpha diversity level and new analyses are necessary associated to influence of substrate (D4) into bacterial community.

4.3.3. Beta-diversity indicators of enriched microbial communities

Bacteria were clearly dominant over Archaea in all samples. The phylum *Proteobacteria* was dominant in all samples and accounted for more than 50% of all sequences. At order level (**Figure 4.3.**), *Rhizobiales* and *Betaproteobacteriales* were the dominant orders in all samples and accounted a range between 19.82% (BEA-38_2) and 58.14% (DST-2_2) for *Rhizobiales* and 2.72% (DST-2_2) and 55.35% (BEA-38_2) for *Betaproteobacteriales*. These percentage values are interesting because suggest an opposite relationship between this order and the packing material activated carbon (DST-2) and zeolite (BEA-38). *Xanthomonadales* (average of 7.03% in all samples) and *Rhodobacterales* (average of 6.34%) were also abundant, 16 orders had relative abundances between 6 and 2%. Additionally, 67 orders were represented at relative abundances less than 1%.

Rhizobiales, *Xanthomonadales* and *Betaproteobacteriales* were the common in all enrichments. However, some others were present in the AC enrichments with higher relative abundance namely *Rhodobacteriales* (18.48% ± 9) and *Bacillales* (7.72 ± 5) in DST-2 and *Opitutales* (6.78 % ± 1) in NRT-2. These orders harbour bacterial members involved in degrading processes of recalcitrant and hydrophobic compounds, such as: *Rhodobacteriales* which studies of Ganesh Kumar, Nivedha Rajan, Kirubagaran, & Dharani, (2019) reported degradation of hydrocarbons with 1% (v/v) of crude oil and 0.05% (v/v) of Tween 80 (non-ionic surfactant) at 28 ± 2 °C for strains in this group.

Likewise, members of *Bacillales* order were identified in packing materials on a thermophilic biofilter for SO_2 removal according to reports of Zhang, Li, & Liu, (2017). Other authors (G. H. Liu, Ye, Tong, & Zhang, 2013) reported that the predominant bacteria in the Upflow anaerobic sludge blanket (UASB) reactor belong to the orders *Bacillales* and *Rhodobacterales*, which may play an important role in removing hydrophobic and cyclic compounds present bioreactors from activated sludge. As mentioned previously, the packing material have implications in promoting biofilm growth and stability. The AC has a larger surface area and together with the reticular structure of zeolite are both key characteristics that favoured the transfer of substrate (D4) in their structure, which in turn improved the growth of microorganisms on their surface.
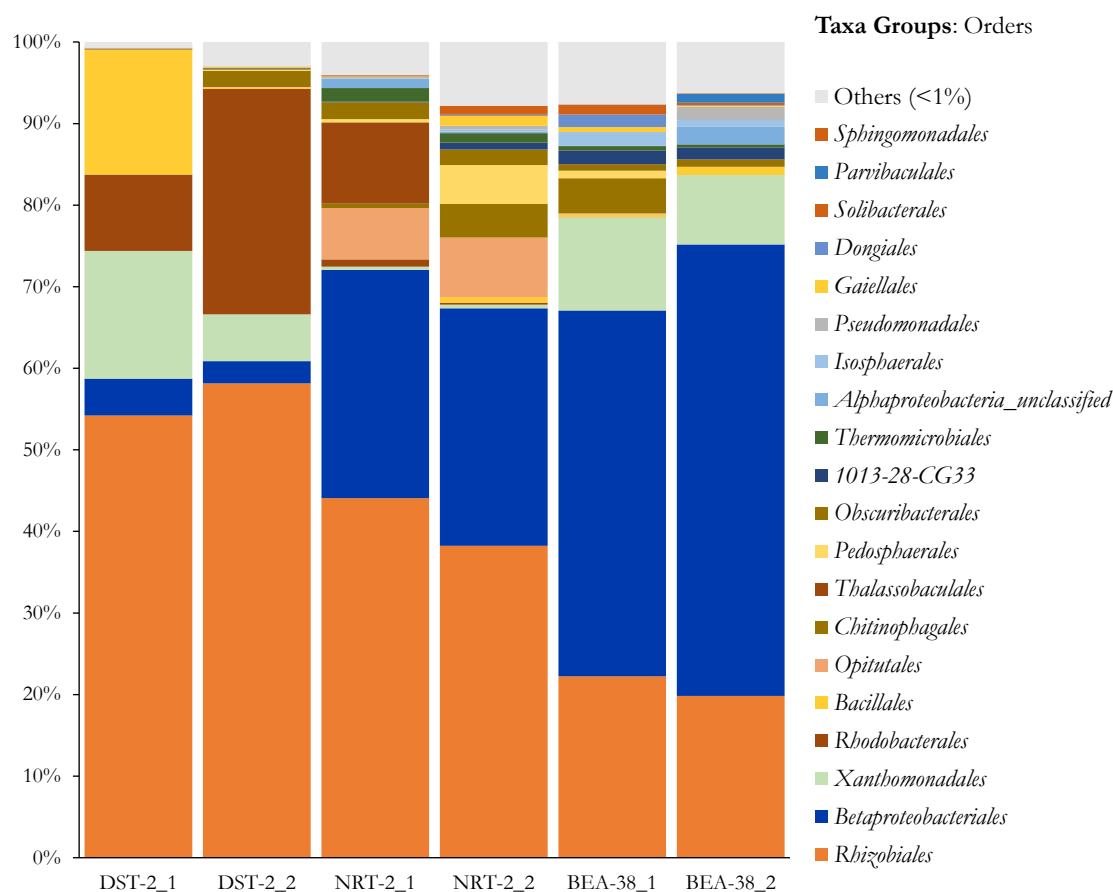
Figure 4.3. Relative abundance of main orders (found >1% of sequences) in each sample in the studied enrichments. "Others" refers to orders that represented <1% of total sequences in all samples.

Complementary analyses of Jaccard's index of similarity and the Yue & Clayton measure of dissimilarity (θ_{YC}) among the structures of every community were performed (**Figure 4.4.**). Jaccard's index of similarity is very straightforward since it is simply the fraction of species shared between the samples. That Jaccard's index only utilizes the richness component of diversity, since it does not entail any information on abundance. However, this index only takes into account the number of species and species proportions of shared species. Generally, all species have equal weight and differences in proportion are not used. To resolve this issue, the Yue & Clayton measure of dissimilarity were calculated. In this study, several interesting observations were being made from this analysis. First, although the dendrograms generated using the Jaccard's and the θ_{YC} coefficients have similar topologies, the terminal branch lengths of the Jaccard coefficient dendrogram are considerably longer between samples DST-2 compare to the others. Despite the variations in diversity, no significant differences were found among enrichments (p values >0.05).

Analysis of Jaccard's index
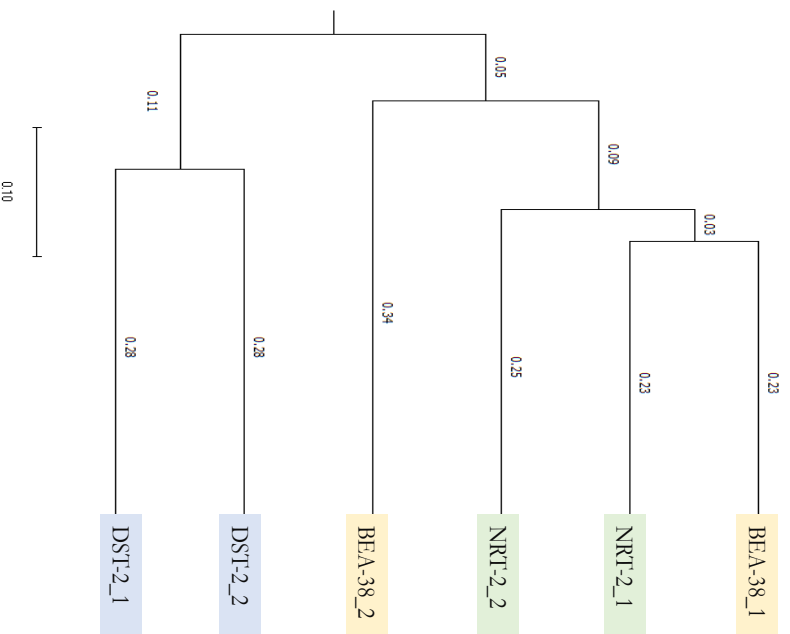
Analysis of Yue & Clayton measure of dissimilarity θ_{YC}
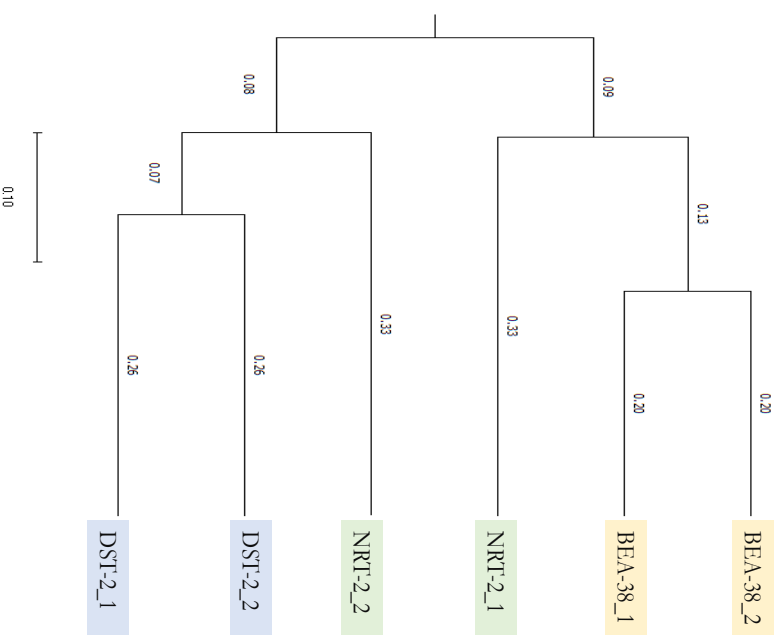
Figure 4.4. Dendrograms of the microbial communities from activated carbon and zeolite enrichments data using matrices computed according analyses of Jaccard's index of similarity and the Yue & Clayton measure of dissimilarity (θ_{YC}). The scale bar (0.10) represents the similarity or dissimilarity index as reference value.

Differences in the structure of microbial communities were analysed with Principal Coordinates Analysis (PCoA), based on the Bray-Curtis similarity matrix. Enrichment samples distribution on the PCoA grouped according to packing material kind (**Figure 4.5.**), did not show significant differences (PERMANOVA, pseudo-F value 3.1035; p value = 0.077).
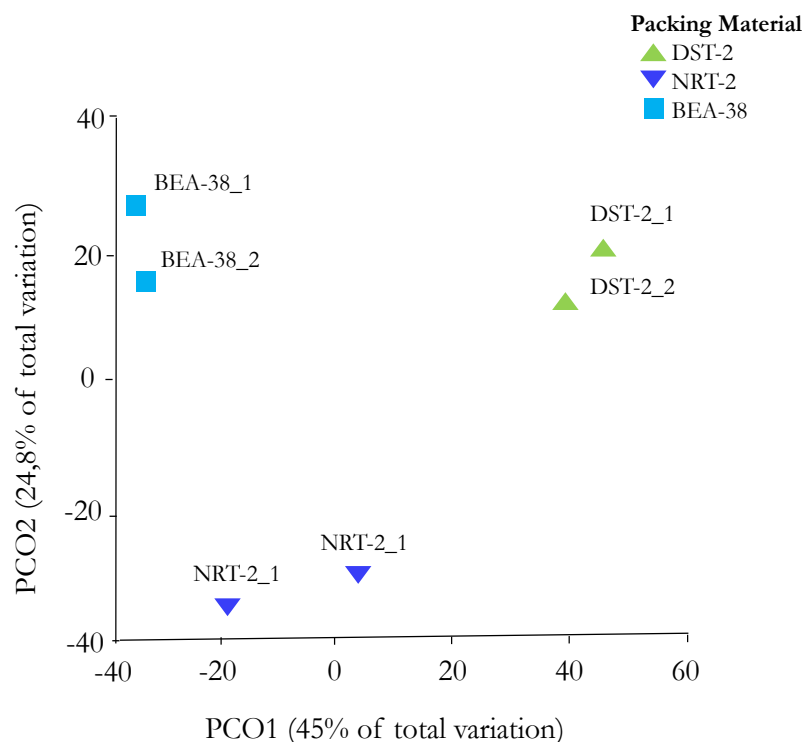
Figure 4.5. PCoA distribution of OTU-based microbial community composition according to packing material: Twp types of activated carbon and zeolite. Resemblance matrix according Bray-Curtis similarity.

4. 3.4 Inference of a "core microbial community" in the enrichments

In order to infer the main taxonomical groups putatively responsible for siloxane-degradation in the enrichments, a core microbial community was defined at the OTU level. Identify a core is the first step in defining a community and predicting community responses to perturbation. Taxonomically defined, the core microbiome is composed of consistent groups (taxa) of bacteria that are present at all tested enrichments and are likely to participate in basic metabolic processes that are taking place in a microbial assemblage (Shade & Handelsman, 2012). In this work, we defined the core microbiome as those OTU appearing at all enrichments tested with a minimum relative abundance of 1%. Sequences belonging to the core community corresponded to an average value of 53.06 % ± 8.64 in all enrichments.

Nineteen (19) OTUs defined the core community (**Figure 4.6.**), the main taxa orders present were: *Rhizobiales* (11 OTUs), *Betaproteobacteriales* (7 OTUs) and *Xanthomonadales* (1 OTU). The most abundant OTU was the OTU001 belonging to genus *Oligotropha,* which was previously reported by Yasuda et al., (2017) as a main member of bacterial community in a full-scale rock-wool biofilter used for treating livestock manure composting emissions (Yasuda et al., 2017). This is the first report about the genus *Oligotropha* associated to siloxane degradation or silicon compounds used as carbon source. The described members of genus *Oligotropha* are able to grow aerobiccaly and facultatively, in autotrophic conditions. For autotrophic growth, the bacteria can utilize carbon monoxide or hydrogen (syngas components) as a source of energy (Komaniecka, Choma, Zamlynska, Sroka-Bartnicka, & Sowinski, 2017).
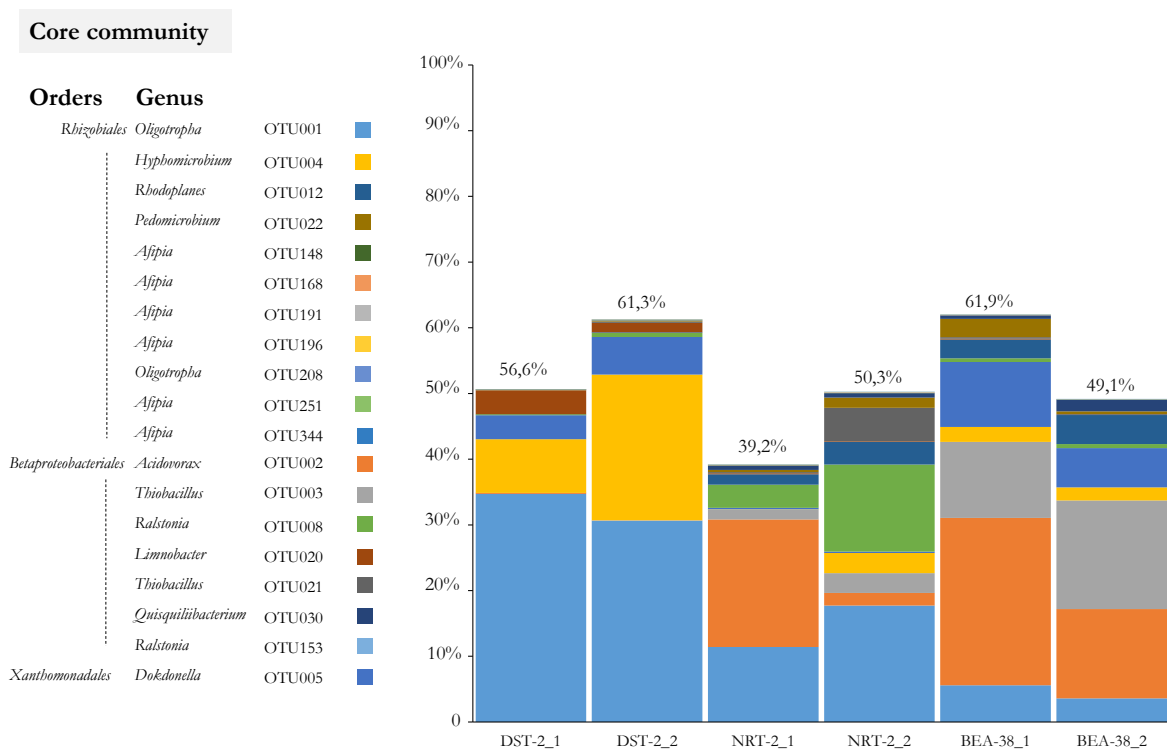
Figure 4.6. Percentage values of OTUs present in the core community in each sample of the studied enrichment. OTUs are grouped by square bracket according to the taxa group: Order and Genera.

The enrichments of activated carbon DST-2 (1 and 2) had the OTU004 (*Hyphomicrobium*) as an abundant OTU. Some authors (Bringel et al., 2017; Salerno et al., 2016) reported several species of *Hyphomicrobium*, which are commonly identified in biofilms obtained from microbial populations of the so-called activated sludge work together in the abatement of pollutants or as member of bacterial community in biofilter, reactors and BTF in cleaning biogas process. For instance *Hyphomicrobium* sp. strain GJ21, was isolated from samples of environments contaminated with halogenated pollutants

and capable of using dichloromethane as its sole carbon and energy source (Bringel et al., 2017). *Hyphomicrobium zavarzinii* appeared as the most prevalent bacteria in samples of a lab-scale plant treating industrial pharmaceutical wastewater.

The enrichments of NRT-2 include two OTUs with percentages greater than 10%: OTU002 (genus *Acidovorax;* 19.4%) and OTU008 (*Ralstonia;* 13.2%). Some acetate utilizers and several carbons sources have been identified as closely related to *Acidovorax* (Yasuda et al., 2017). In a report of Timmis et al., (2019), this genus are able to remove sulphur from organosulfur compounds consisting of benzene rings. Moreover, representative betaproteobacterial are capable of growth on single ring substituted and unsubstituted aromatic hydrocarbons. The most common genera in the *Betaproteobacteria* that are known to degrade hydrocarbons and related substituted molecules include *Acidovorax, Burkholderia, Comamonas, Delftia, Polaromonas,*and *Ralstonia.* At biogas level, analysis of salicylate-, naphthalene-, and phenanthrene-degrading microbial communities in a bioreactor treating contaminated soil detected primarily sequences belonging to the genera *Pseudomonas*, *Ralstonia*, and *Acidovorax* (Rochman, 2016). Mainly, these genera, *Acidovorax*, *Burkholderia*, and *Comamonas* are also capable of degrading multi-ring polycyclic aromatic hydrocarbons (Rochman, 2016). In addition, some studies reported a closely relation between genus *Ralstonia* and the activated carbon as packing material for instance, Mondal, Majumder, & Mohanty, (2008) reported a successful treatment of arsenic contaminated water in a batch reactor by using *Ralstonia eutropha MTCC 2487* and granular activated carbon. Additionally, another study of Miyake-Nakayama et al., (2006), inferred the Biodegradation of VOCs by the polyvinyl alcohol-immobilized methylotrophic bacterium *Ralstonia metallidurans* PD11, the biotic activated carbon granules were packed in a column and subjected for degradation of VOCs including DCM. Thus, the presence of these genera related to VOCs metabolism can be beneficial during D4 removal process, when D4 is mixture with others compounds as toluene, limonene or hexane, which are typical compounds in biogas together to siloxanes.

In the zeolite enrichments the most abundant OTUs are OTU002 (25.5%), OTU003 (*11*.5%) and OTU005 (9.8%), which belonging to the genera *Acidovorax, Thiobacillus* and *Dokdonella* respectively. These genera are commonly found in biofilters and batch reactor (GarcIa & Prats, 2016; Khan, Husain, & Hejazi, 2004). The zeolite are generally used as packing materials in biofilters and can provide more space for microorganisms on the surface for aerobic zone and induce a facultative anaerobic zone into the pores (Garcia & Prats, 2016; Khan et al., 2004).

Table 4.3. Relative abundances of members of the core community among enrichments with Activated carbon (AC) and with zeolite.

Taxonomic OTU information			% Rel. Abund.			
Order	Most probable species identification and sequence identifier	OTU ID	Activated carbon DST-2	Activated Carbon NRT-2	Zeolite BEA-38	*p* value
Rhizobiales	*Oligotropha carboxidovorans*, NR_074142[a]	Otu0001	$3.27 \cdot 10^{1} \pm 2.91 \cdot 10^{0}$	$1.46 \cdot 10^{1} \pm 4.45 \cdot 10^{0}$	$4.60 \cdot 10^{0} \pm 1.41 \cdot 10^{0}$	0.102
	Hyphomicrobium denitrificans, NR_074189[a]	Otu0004	$1.52 \cdot 10^{1} \pm 9.91 \cdot 10^{0}$	$1.55 \cdot 10^{0} \pm 2.12 \cdot 10^{0}$	$2.14 \cdot 10^{0} \pm 1.97 \cdot 10^{-1}$	0.180
	Rhodoplanes roseus, NR_115515[a]	Otu0012	$5.76 \cdot 10^{-2} \pm 7.40 \cdot 10^{-2}$	$2.52 \cdot 10^{0} \pm 1.30 \cdot 10^{0}$	$3.6 \cdot 10^{0} \pm 1.13 \cdot 10^{0}$	0.156
	Pedomicrobium manganicum NR_104841[a]	Otu0022	$8.02 \cdot 10^{-2} \pm 6.86 \cdot 10^{-2}$	$9.35 \cdot 10^{-1} \pm 8.59 \cdot 10^{-1}$	$1.59 \cdot 10^{0} \pm 1.67 \cdot 10^{0}$	0.156
	Afipia felis NR_117715[a]	Otu0148	$3.05 \cdot 10^{-2} \pm 5.73 \cdot 10^{-3}$	$4.91 \cdot 10^{-2} \pm 9.33 \cdot 10^{-3}$	$6.05 \cdot 10^{-3} \pm 2.47 \cdot 10^{-3}$	0.102
	Afipia felis NR_117715[a]	Otu0168	$1.14 \cdot 10^{-2} \pm 6.15 \cdot 10^{-3}$	$4.40 \cdot 10^{-2} \pm 9.90 \cdot 10^{-4}$	$9.95 \cdot 10^{-3} \pm 7.99 \cdot 10^{-3}$	0.156
	Afipia felis NR_117715[a]	Otu0191	$1.62 \cdot 10^{-2} \pm 9.55 \cdot 10^{-3}$	$2.10 \cdot 10^{-2} \pm 2.09 \cdot 10^{-2}$	$1.19 \cdot 10^{-2} \pm 1.07 \cdot 10^{-2}$	0.651
	Afipia felis NR_117715[a]	Otu0196	$1.25 \cdot 10^{-2} \pm 2.12 \cdot 10^{-4}$	$3.11 \cdot 10^{-2} \pm 2.83 \cdot 10^{-4}$	$4.10 \cdot 10^{-3} \pm 2.83 \cdot 10^{-4}$	0.102
	Oligotropha carboxidovorans NR_074142[a]	Otu0208	$1.23 \cdot 10^{-2} \pm 4.88 \cdot 10^{-3}$	$2.49 \cdot 10^{-2} \pm 6.01 \cdot 10^{-3}$	$4.95 \cdot 10^{-3} \pm 4.03 \cdot 10^{-3}$	0.102
	Afipia felis NR_117715[a]	Otu0251	$9.80 \cdot 10^{-3} \pm 3.96 \cdot 10^{-3}$	$1.72 \cdot 10^{-2} \pm 9.90 \cdot 10^{-4}$	$5.05 \cdot 10^{-3} \pm 1.06 \cdot 10^{-3}$	0.102
	Afipia felis NR_117715[a]	Otu0344	$5.60 \cdot 10^{-3} \pm 5.37 \cdot 10^{-3}$	$1.10 \cdot 10^{-2} \pm 9.76 \cdot 10^{-3}$	$4.10 \cdot 10^{-3} \pm 2.83 \cdot 10^{-4}$	0.651
Betaproteobacteriales	*Acidovorax caeni*, NR_042427[a]	Otu0002	$8.63 \cdot 10^{-2} \pm 9.98 \cdot 10^{-2}$	$1.07 \cdot 10^{1} \pm 1.24 \cdot 10^{1}$	$1.95 \cdot 10^{1} \pm 8.41 \cdot 10^{0}$	0.156
	Thiobacillus denitrificans, NR_025358[a]	Otu0003	$4.26 \cdot 10^{-2} \pm 5.52 \cdot 10^{-2}$	$2.26 \cdot 10^{0} \pm 1.10 \cdot 10^{0}$	$1.40 \cdot 10^{1} \pm 3.52 \cdot 10^{0}$	0.102
	Ralstonia pickettii NR_11412⁶[a]	Otu0008	$3.77 \cdot 10^{-1} \pm 3.14 \cdot 10^{-1}$	$8.39 \cdot 10^{0} \pm 6.90 \cdot 10^{0}$	$5.87 \cdot 10^{-1} \pm 3.37 \cdot 10^{-2}$	0.156
	Limnobacter litoralis NR_114291[a]	Otu0020	$2.50 \cdot 10^{0} \pm 1.56 \cdot 10^{0}$	$1.25 \cdot 10^{-1} \pm 4.74 \cdot 10^{-2}$	$6.15 \cdot 10^{-2} \pm 8.39 \cdot 10^{-2}$	0.156
	Thiobacillus thioparus NR_117864[a]	Otu0021	$1.26 \cdot 10^{-1} \pm 1.33 \cdot 10^{-1}$	$2.60 \cdot 10^{0} \pm 3.49 \cdot 10^{0}$	$1.91 \cdot 10^{-1} \pm 8.28 \cdot 10^{-2}$	0.565
	Quisquiliibacterium transsilvanicum NR_159181[a]	Otu0030	$2.31 \cdot 10^{-2} \pm 2.83 \cdot 10^{-3}$	$6.65 \cdot 10^{-1} \pm 1.36 \cdot 10^{-2}$	$1.14 \cdot 10^{0} \pm 8.49 \cdot 10^{-1}$	0.180
	Ralstonia syzygii subsp. NR_134149[a]	Otu0153	$2.79 \cdot 10^{-2} \pm 2.71 \cdot 10^{-2}$	$4.40 \cdot 10^{-3} \pm 3.25 \cdot 10^{-3}$	$4.78 \cdot 10^{-2} \pm 5.93 \cdot 10^{-2}$	0.368
Xanthomonadales	*Dokdonella soli* NR_044554[a]	Otu0005	$4.67 \cdot 10^{0} \pm 1.47 \cdot 10^{0}$	$2.17 \cdot 10^{-1} \pm 2.55 \cdot 10^{-2}$	$7.93 \cdot 10^{0} \pm 2.78 \cdot 10^{0}$	0.102

Core community members were limited to those OTU that were found present in all samples that occurred at a relative abundance (according to number of sequences). Differences were assayed using a Kruskal-Wallis test. Not significant (p values >0.05). [a]: Accession tentatively identified according to BLAST taken of https://blast.ncbi.nlm.nih.gov/Blast.cgi, percentage similarity (PE)> 98%).

Q. Zhang et al., (2019) reported the genus *Dokdonella* (strictly aerobic bacteria) accounted for a smaller proportion of microbial community of hybrid biological reactors, which have zeolite as fibre carrier and X. Zhang et al., (2019), published a study of microbial action for some recalcitrant contaminant removal by zeolite-sand as main carrier. The microbial community defined by (Xiangling Zhang et al., 2019) had species of genera *Dokdonella*, *Acidovorax* and *Thiobacillus*. Our results suggest a possible good relation among these genera with the zeolite as successful carrier during the biofilm formation process. **Table 4.3.** shows the statistical analysis performed whit each member of core community related to the relative abundance with both packing materials. The results confirmed there are not significant differences (Kruskal-Wallis tests*; p>0.05*) between the relative abundances in both materials. However, the data obtained of the higher values of relative abundance in specific packing material can be useful to promote the growth of particular bacterial groups associated with siloxane removal.

4.3.5. Changes in the microbial community structure related to activated carbon presence

The main OTUs shared among both activated carbon DST-2 and NRT-2 and not present in the core community were Otu0013, Otu0016, Otu0024 and Otu0055 all classified as *Rhizobiales,* related OTUs. Otu00013 (identified as a *Labrys* sp.) was previously isolated from a methylamine containing enrichment culture, and representing a novel species of facultative methylotrophic bacteria. Otu00016 (*Hyphomicrobium*) has been cited in biofilms of BTFs. The OTU0024, belongs to the order *Pedosphaerales,* genus *Limisphaera*, is known as a thermophilic, pink-pigmented coccus isolated from subaqueous mud of a geothermal hot spring (Anders et al., 2015). The metabolic versatility of these genera to adapt to particular environment or specific substrates are useful characteristic in siloxane removal adsorbed in activated carbon. Considering the problem in siloxane removal process is mass transfer limitations, this study offers some suitable genera with the ability to form biofilms in activated carbon as carrier material and with the capacity to grow in the presence of hydrophobic compounds, such as D4 as the sole carbon source.

The DST-2 enrichments had 54 OTUS shared, of which 35 OTUs were not present in the core community and interestingly, the OTU016 (6%) and OTU025 (2%) both also belong to the genus *Hyphomicrobium* (order *Rhizobiales*) were the most abundant. The most representative taxa shared between DST-2_1 and DST-2_2 were orders *Rhizobiales* (56.17% ± 2), *Rhodobacteriales* (18.48% ± 9) and *Xanthomonadales* (10.70% ± 5). In contrast, The NRT-2 enrichments had 147 OTUs shared, 127

of which were not present in the core community. The most representative order shared between NRT-2_1 and NRT-2_2 were *Rhizobiales* (41.15% ± 3), *Betaproteobacteriales* (28.55% ± 3) and *Optitutales* (6.78% ± 1). The results suggest, the order *Rhizobiales* can be able to form successfully a biofilm on several packing material. The previous work has been limited to the common and main OTUs as members of a core bacterial community; however, more recent evidence suggests that OTUs with minor abundance but higher specialized characteristics can be able to grow as an isolated pure culture (Xiangling Zhang et al., 2019). For instance, OTU019 belonging to the genus *Ciceribacter,* which include organisms that exhibit extraordinary diversity in the range of substrates metabolized, had a high abundance, with 6.5% of total community in NRT-2 enrichments and 1% in DST-2 enrichments. *Ciceribacter thiooxidans* sp. and *Ciceribacter lividus,* have been identified as potential metabolizers of recalcitrant compounds and detected in coupled aerobic-anoxic reactors (T. Deng et al., 2017). This information is remarkable due to the presence of *Ciceribacter* related OTUs limited to activated carbon. In addition, the study of the effects of carrier material during enrichments cultures provided implications for bioreactor construction when using several activated carbons in biogas upgrading process such as high investment and operating costs (Rasi, 2009).

4.3.6. Changes in the microbial community structure related to zeolite presence

Zeolites work as slow substrate release biofilm carriers and they are used to provide a solid substrate and electron donor for the growth of microorganisms and denitrification for anoxic and/or anaerobic bioreactors whit special substrates (*i.e.*. VOCs, siloxane and sulfur compounds). Zeolites, both natural and synthetic, represent low-cost materials with high adsorption selectivity for cationic contaminants (Medunić et al., 2019). Thus, identify microbial species able to form biofilms in zeolite and whit capacity to growth with D4 as carbon source is relevant for future applications. One hundred and sixteen (116) OTUs were found in zeolite biofilms and 96 were not in the core community. The most representative OTU shared in zeolite BEA-38 were: OTU002 (19.5%); OTU003 (14%); OTU006 (11.9%); OTU005 (7.9%); OTU011 (5.6%); OTU001 (4.6%); OTU012 (3.6%) and OTU023 (2.5%). The genera *Comamonas* (OTU006), *Paradevosia* (OTU011) and *Pseudoflavitalea* (OTU023) were not present in the core community. Thus, these genera could be specific for growing on zeolite as carrier material. In addition, some studies (Xinying Zhan g, Li, Yu, Xu, & Wu, 2016) highlighted the microorganism distribution in biofilm of zeolite is uniform and zeolite showed outstanding superiority on pollutants removal or nitrifying bacteria growing compared to coal absorbents (Xinying Zhang et al., 2016).

4.4 Final remarks
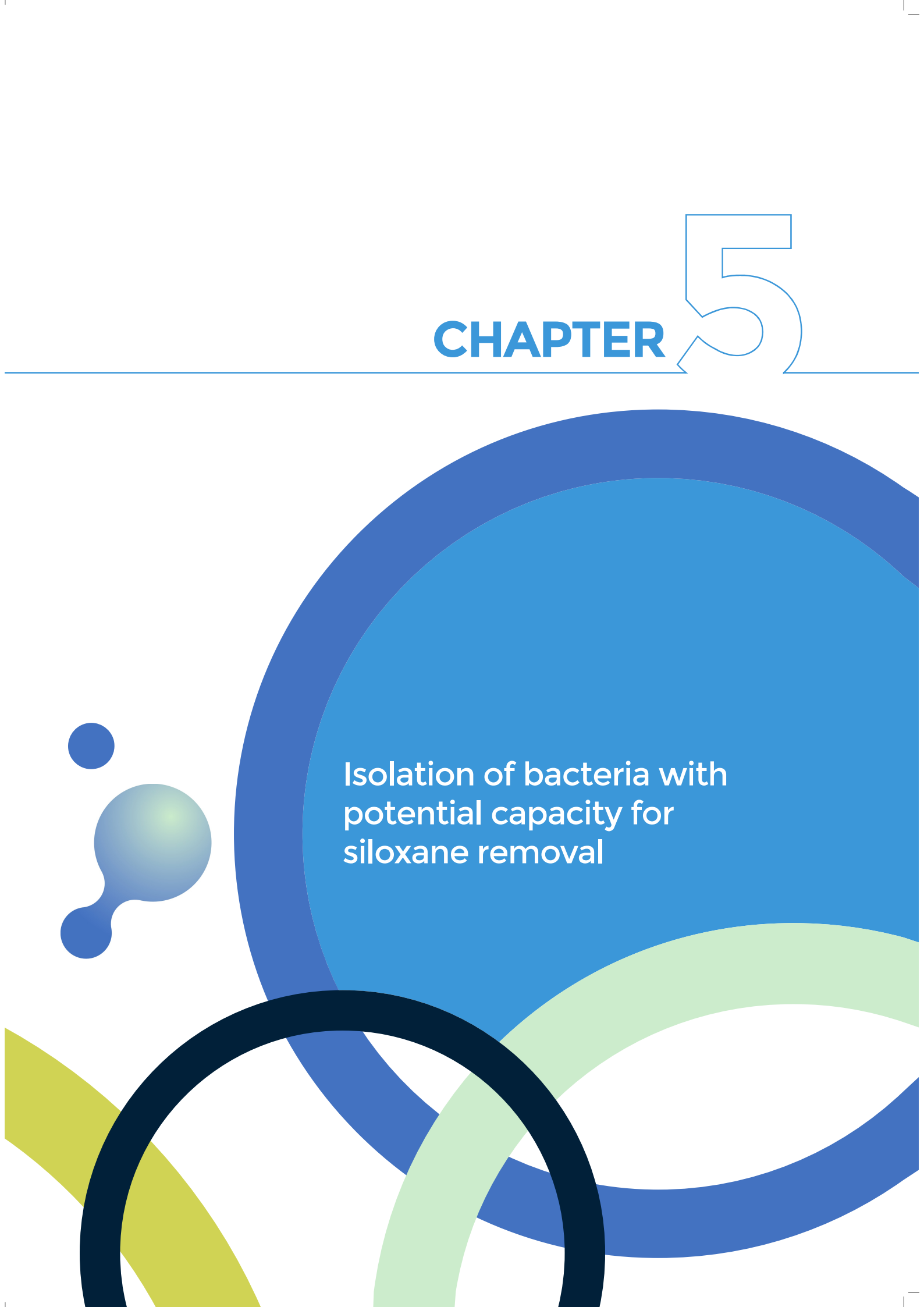
The current knowledge on siloxane bio-removal is limited due to their particular characteristics (e.g. hydrophobicity, volatility, etc.). Hydrophobicity and volatility are the main factors that determine the use of solid matrices as adsorption methods to stabilize the compound for its degradation. Activated carbons, and zeolites have been proposed as being effective for this process (Tu et al., 2019). In this chapter a core bacterial community was identified, which was involved in D4 removal process, and was developed on the surface of activated carbon and zeolite material. Up to 19 phylotypes were defined as the core community regardless of the packing material. Most of them belonged to the *Rhizobiales* and *Betaproteobacteriales*. Furthermore, the results confirmed that packing carriers could enrich biomass, thus improving the richness and diversity of microorganisms in enrichments saturated with D4 as sole carbon source. The larger surface area of AC and the reticular structure of zeolite were suspected to be key determinants that favoured the transfer of substrate (D4), which, in turn, promoted selection of specific microorganisms on their surface. In general, the activated carbon supported higher bacterial growth, but zeolites showed a higher specificity towards certain bacteria.

Towards the implementation of
a biotechnology for biogas upgrading:
role of bacteria in siloxane removal

Isolation of bacteria with potential capacity for siloxane removal
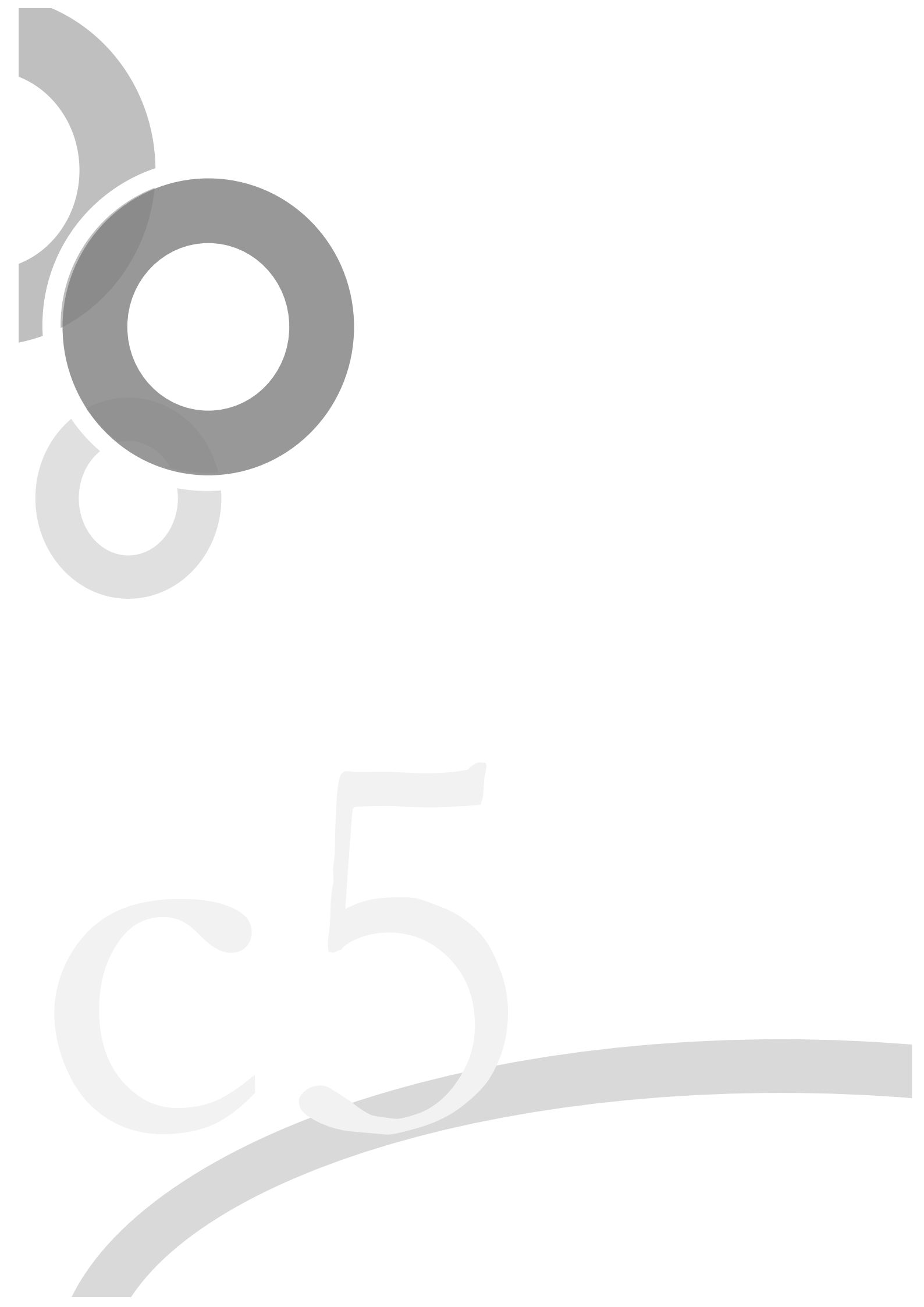
5.1 Background and objectives

Siloxanes are a subgroup of silicone molecules containing Si–O bonds with organic radicals, mostly methyl-groups, widely used in industrial application for cosmetics and personal care products as well as adhesives, lubricants or heat-transfer fluids (Grümping, Michalke, Hirner, & Hensel, 1999; Mojsiewicz-Pieńkowska & Krenczkowska, 2018). Hence, large amounts of siloxanes are disposed in wastewater and urban landfills and volatilize in the biogas generated in such facilities that is used for the generation of heat and power. D5 and D4 are the siloxanes primarily found in biogas (Buser, Bogdal, & Scheringer, 2015; Surita & Tansel, 2015; Tansel & Surita, 2014). During the combustion of biogas, the presence of siloxanes causes serious damage to the gas processing equipment because of their oxidation and deposition as silica particles (M. Shen et al., 2018).

In biological removal, some assays and literature references (Dewil, Appels, & Baeyens, 2006; Hamme & Ward, 2001) have suggested that silicones are still considered as compounds resistant to biodegradation and inert to particular microorganisms. However, it was not until 2008 when the feasibility for the biodegradation of volatile siloxanes for biogas purification in a BTF was studied (Accettola et al., 2008; Popat & Deshusses, 2008). This studies highlighted mass transfer limitations as the main difficulty for the performance of the biotrickling filters.

In this context, the aim of this chapter was isolating and evaluating uncultured facultative anaerobic bacteria able to degrade siloxanes; which will be used for biogas purification in biotrickling filters. Siloxane biodegradation assays with representative isolated species among a previously obtained culture collection were performed to evaluate the removal of D4 alone and in the presence of a multicomponent gas mixture, both using pure cultures and a mixed bacterial community.

5.2 Methodology

In order to isolate D4-degrading strains from both BEC cultures and BTFas' biofilm, samples were treated as described in **Section 3.2.3** at least five times to ensure purity of the isolates. Likewise, several siloxane biodegradation assays were performed. D4 biodegradation was investigated through laboratory experiments with isolates obtained from both the BECs with porous materials (**Chapter 4**) and from initial biofilm of BTFas (**Chapter 6**). The selected isolates were grown as described in **Section 3.2.1**.

Another set of vials were prepared as described in order to investigate the biodegradation of siloxanes in the presence of other organic compounds by supplementing 1 μL of a mix of siloxanes (D4 and D5) and VOCs (hexane, toluene and limonene) according **Section 3.5.1**.

5.3 Results and Discussion

5.3.1 Isolation and identification of D4 degrading bacteria

Activated sludge samples were used for the inoculation of i) anaerobic BECs where D4 was supplied through saturated porous materials, and ii) a lab scale BTFas, where the biofilm was exposed to siloxanes by the gas circulation. D4-degrading bacteria were isolated both from the liquid and the solid phase biomass extraction and grown on agar plates containing D4 as unique carbon and energy source exhibiting distinguishable cell colony morphologies. Colonies from each type were purified on solid agar media until a single colony type could be observed on each plate. Overall, isolation protocols yielded 34 bacterial isolates recovered from the BEC and 17 from the BTF, which are gathered in **Table 5.1.**

Table 5.1. Most probable identification and number of isolates recovered for each enrichment sample. Letter codes refer to activated carbon: DST-2 and NRT-2; a synthetic BEA-type zeolite: BEA-38, and biotrickling filter: BTF.

Source	Most probable Identification of Isolates	Identification code number of Isolates
BEC DST-2_1	*Pseudomonas aeruginosa*	7, 8
	Ciceribacter lividus	9, 10
BEC DST-2_2	*Pseudomonas aeruginosa*	14, 32
BEC NRT-2_1	*Ciceribacter lividus*	1
	Alicycliphilus denitrificans	2
	Pseudomonas aeruginosa	3, 4
BEC NRT-2_2	*Ciceribacter lividus*	5, 15, 18
	Alicycliphilus denitrificans	6, 16, 17
BEC BEA-38_1	*Pseudomonas aeruginosa*	11, 12, 13, 29, 30, 31
BEC BEA-38_2	*Pseudomonas aeruginosa*	19, 20, 27, 33, 34
	Pseudomonas citronellolis	21, 22, 23, 24
	Alicycliphilus denitrificans	25, 26, 28
Biofilm from BTFas	*Dokdonella* sp.	39
	Nocardioides sp.	40, 46
	Rhodococcus sp.	41, 51, 57
	Rhodococcus erythropolis	52, 53
	Rhodococcus qingshengii	54
	Sphingopyxis granuli	43
	Mycolicibacterium conceptionense	44
	Gordonia polyisoprenivorans	45
	Methylibium sp.	49, 58
	Ferrovibrio xuzhouensis	50
	Microbacterium foliorum	55
	Novosphingobium sp.	56

Up to 21 isolates were closely related to previously described hydrocarbon degrading *Pseudomonas aeruginosa* strains XM1 (JX093569) and HX-2 (KX461910) at 98% and 96% of similarity, respectively (Chikere et al., 2014). Likewise, the recovered cultures showed 97% similarity to the *Pseudomonas aeruginosa* strain DN1 (KP119458) isolated from petroleum-contaminated soil samples capable to use crude oil as the sole carbon and energy source (Ma et al., 2016) and able to degrade p-nitrophenol (Singh Ningthoujam & Shovarani, 2008). Phylogenetic analysis of the 16S rRNA gene (**Figure 5.1.**) revealed that the isolates were members of the *Proteobacteria* and *Actinobacteria* phyla showing high similarities with previously cultured bacterial species described for their capacity to metabolize complex hydrocarbon compounds.

Isolates identified as *Alicycliphilus* sp. were closely related and showed high similarity (95%) to *A. denitrificans* strain SD-H (AB908107) isolated using cyclohexanol as sole carbon source and nitrate as electron acceptor from a municipal sewage plant (Mechichi, Stackebrandt, & Fuchs, 2003) or to *Alicycliphilus denitrificans* strain ADC-14 (KM210246), which is known as a anthracene-degrading bacterial species (Ntougias, Melidis, Navrozidou, & Tzegkas, 2015). Likewise, *Ciceribacter* sp. strains showed 99 % similarity to *Ciceribacter lividus* strain MSSRFBL1 (NR135717) and *Ciceribacter azotifigens* strain A.Slu09 (KX510117), which were described as nitrogen-fixing bacteria isolated from activated sludge (Siddiqi, Choi, & Im, 2018). The four identified bacterial species namely *Pseudomonas aeruginosa*, *Aliciyliphilus denitrificans* and *Ciceribacter lividus* were recovered from all used material (activated carbon, zeolite and lava rock) except *Pseudomonas citronellolis*, which was only recovered in BEA zeolite-containing BEC. The main factor that may have had an influence with *P. citronellolis* growth probably was the iron dependency and the depletion on the iron availability caused by activated carbon in comparison to zeolites. Some works (Dinkla, Gabor, & Janssen, 2001; Santos et al., 2008) have performed assays and reported the effect of iron on *Pseudomonads* isolates. Dinkla et al., (2001) reported the iron limitation may be an important factor in the degradation of hydrocarbons that require iron-containing enzymes. During iron-limiting assays, *Pseudomonas aeruginosa* and *P. putida* showed 85% reduction in iron-containing enzyme activity but, they were able to use other mechanisms to increase de hydrocarbon degradation (Dinkla et al., 2001; Santos et al., 2008). However, *P. citronellolis* was clearly affected by iron-limited conditions*,* exhibiting an iron dependence metabolism and showing insufficient or insignificant growth. Activated carbon is a widely used adsorbent to remove iron and other metals from water and wastewater. On the other hand, the use of zeolites for removal of metals has been described for low-silica zeolites (Si/Al < 2), while the BEA used in this work is a high-silica

zeolite. Thus, during the anaerobic batch enrichments activated carbon may retain significantly higher amounts of soluble iron than the BEA zeolite and affecting meaningfully the *P. citronellolis* growth.
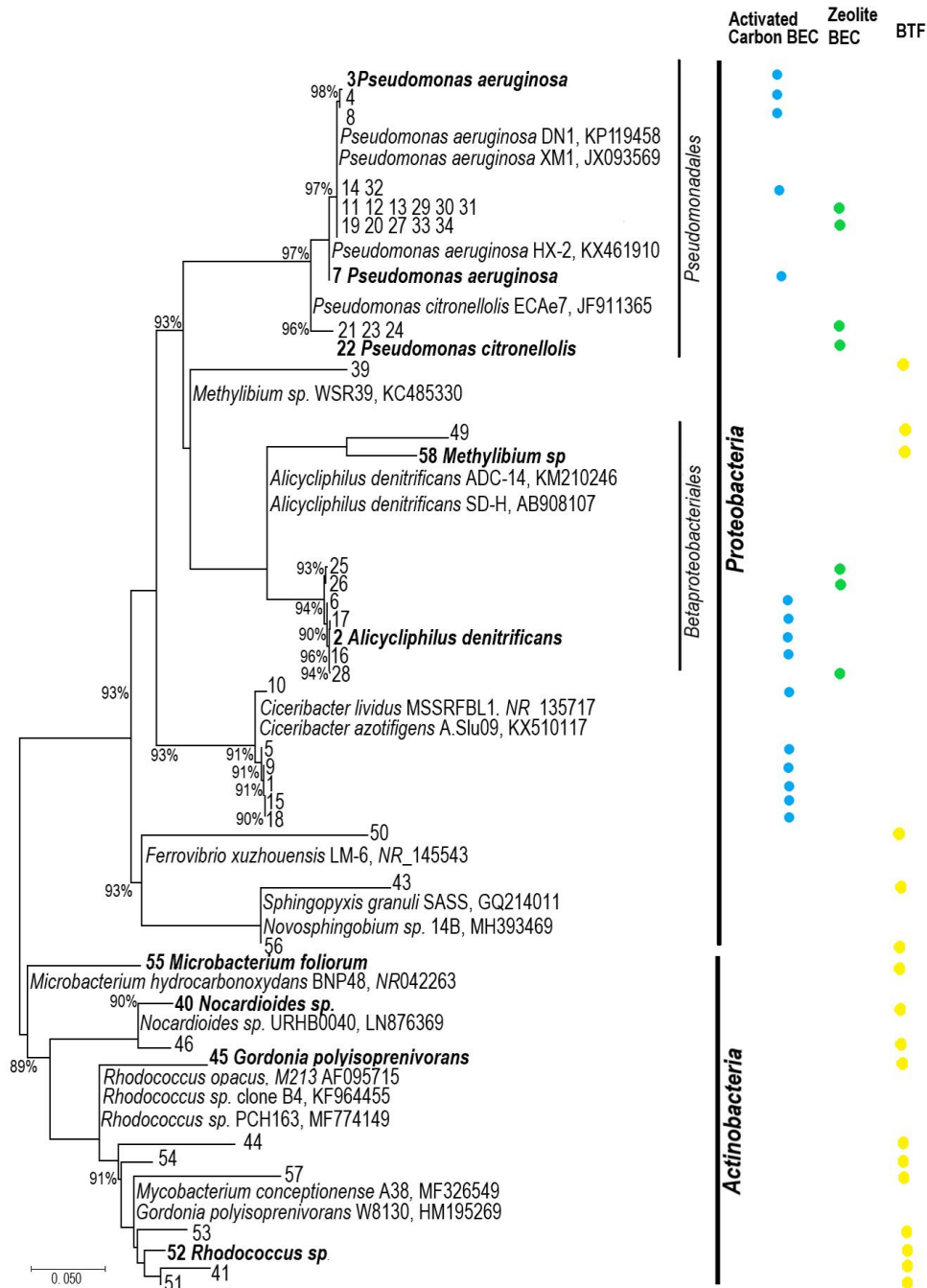
Figure 5.1. Maximum likelihood phylogenetic tree calculated from 16S rRNA gene sequences of bacterial isolates belonging to core communities of the batch enrichment cultures (BEC) using activated carbons and zeolite as support materials for D4 delivery in the growth media and biotrickling filter (BTFas). Members of communities for each sample type are shown as colored circles next to the identification code for the isolates. Percent numbers indicate bootstrap values. Promising bacterial isolates evaluated in siloxane biodegradation assays are shown in bold. Bar, 5 nucleotide substitutions per 100 nucleotides.

The identification of members of *Actinobacteria* recovered from BTFas within the culture collection is relevant since this group is well known by the presence of mycolic acids in the cell wall which confers hydrophobic properties and therefore the capacity to bind nonpolar components as aromatic hydrocarbons and cyclic molecules (Bendinger, Rijnaarts, Altendorf, & Zehnder, 1993). Interestingly, among the identified genera, *Arthrobacter* sp. has been previously described in siloxane biodegradation capacity in soils (Sabourin et al., 1996). Given that, the presence of mycolic acids can favour D4 adsorption on the cell surface since D4 is a low water soluble compound. The 16S rRNA gene sequence analysis showed high similarity of the isolates with previously described bacteria biodegrading hydrocarbons and xenobiotic compounds (**Table 5.2.**), like *Microbacterium hydrocarbonoxydans* strain BNP48 (NR042263) (Schippers, Bosecker, Spröer, & Schumann, 2005), *Rhodococcus* sp. clone B4 (KF964455) and *Rhodococcus opacus* strain M213 (AF095715) which can grow on naphthalene as the sole source of carbon and energy (Grund et al., 1992; Uz, 2000).

Table 5.2. Isolates recovered from BEC and BTF, identified by 16S rRNA gene comparison.

Code	ID	Source	NCBI Accession
2	*Alicycliphilus denitrificans* strain ADC-14	NRT-2_1	KM210246
6,16,17	*Alicycliphilus denitrificans* strain ADC-14	NRT-2_2	KM210246
25,26,28	*Alicycliphilus denitrificans* strain ADC-14	BEA-38_2	KM210246
1	*Ciceribacter lividus* strain MSSRFBL1	NRT-2_1	NR_135717
5	*Ciceribacter lividus* strain MSSRFBL1	NRT-2_2	NR_135717
9, 10	*Ciceribacter lividus* strain MSSRFBL1	DST-2_1	NR_135717
15,18	*Ciceribacter lividus* strain MSSRFBL1	NRT-2_2	NR_135717
39	*Dokdonella sp.*	BTFas	KM016304
50	*Ferrovibrio xuzhouensis*	BTFas	NR_145543
45	*Gordonia polyisoprenivorans*	BTFas	HM195269
49,58	*Methylibium sp.*	BTFas	KC485330
55	*Microbacterium foliorum*	BTFas	NR_042263
44	*Mycolicibacterium conceptionense*	BTFas	MF326549
40, 46	*Nocardioides sp.*	BTFas	LN876369
3,4	*Pseudomonas aeruginosa* strain HX-2	NRT-2_1	KX461910
7,8	*Pseudomonas aeruginosa* strain HX-2	DST-2_1	KX461910
11,12,13,29,30,31	*Pseudomonas aeruginosa* strain HX-2	BEA-38_1	KX461910
14, 32	*Pseudomonas aeruginosa* strain HX-2	DST-2_2	KX461910
19, 20,27,33,34	*Pseudomonas aeruginosa* strain HX-2	BEA-38_2	KX461910
21,22,23,24	*Pseudomonas citronellolis* strain ECAe7	BEA-38_2	JF911365
52,53	*Rhodococcus erythropolis*	BTFas	MF774149
54	*Rhodococcus qingshengii*	BTFas	MF774149
41,51,57	*Rhodococcus sp.*	BTFas	MF774149
43	*Sphingopyxis granuli*	BTFas	GQ214011

5.3.2 Siloxane D4 biodegradation as unique carbon source

In order to determine the capacity of the cultured bacteria to grow in the presence of D4 as unique carbon source, ten representative isolates from both BECs and the BTFas (iso02, iso03, iso05, iso07, iso22, iso40, iso45, iso52, iso55 and iso58) were selected from the culture collection according to their 16S rRNA gene sequence similarity to previously described bacterial species capable to remove harmful contaminants.

The bacterial growth was monitored by optical density, cell counts and protein concentration at the beginning and at the end of the experiment i.e. after 60 days. Growth in mineral medium devoid of D4 did not show significant differences in the absorbance values between 0 and 60 days (*t-test*, $p =$ 0.707, $a = 0.05$). Contrarily, when D4 was supplemented in the growth media all the isolates showed significant differences (ANOVAs, $p < 0.05$, $a = 0.05$) between the absorbance values at 0 and 60 days confirming cell growth. **Figure 5.2.** shows the optical density evolution during the first 20 days of the experiments. Post hoc Tukey test determined the existing subsets, where isolates iso7 and iso58 from BEC and BTFas, respectively, were grouped in a single subset with the highest absorbance values (iso7, $abs_{550nm} = 0.380 \pm 0.020$ and iso58 $abs_{550nm} = 0.405 \pm 0.036$).

Statistical analysis of cell counts and protein quantification revealed similar outcomes, where the p values were more robust and reliable than the rest of the evaluated isolates. Growth in mineral medium devoid of D4 did not show significant differences in the cell counts values between 0 and 60 days (*t-test*, *p value* = 0.281, $a = 0.05$). Whereas, when D4 was supplemented in the growth media all the isolates showed significant differences (ANOVAs, p value = 0.029; $p < 0.05$) between the cell counts values at 0 and 60 days confirming cell growth. For protein quantification, t-test showed a *p value* > 0.05 (*p value* = 0.783), and when D4 was supplemented, the results showed significant differences (ANOVAs, p value = 0.036; $p < 0.05$). Similarly, significant differences for the isolates iso7 and iso58 were determined; consequently, parametrical tests determined the existing of a specific subset with the isolates 7 and 58, which could be considered as potential cultures with higher growth efficiency.
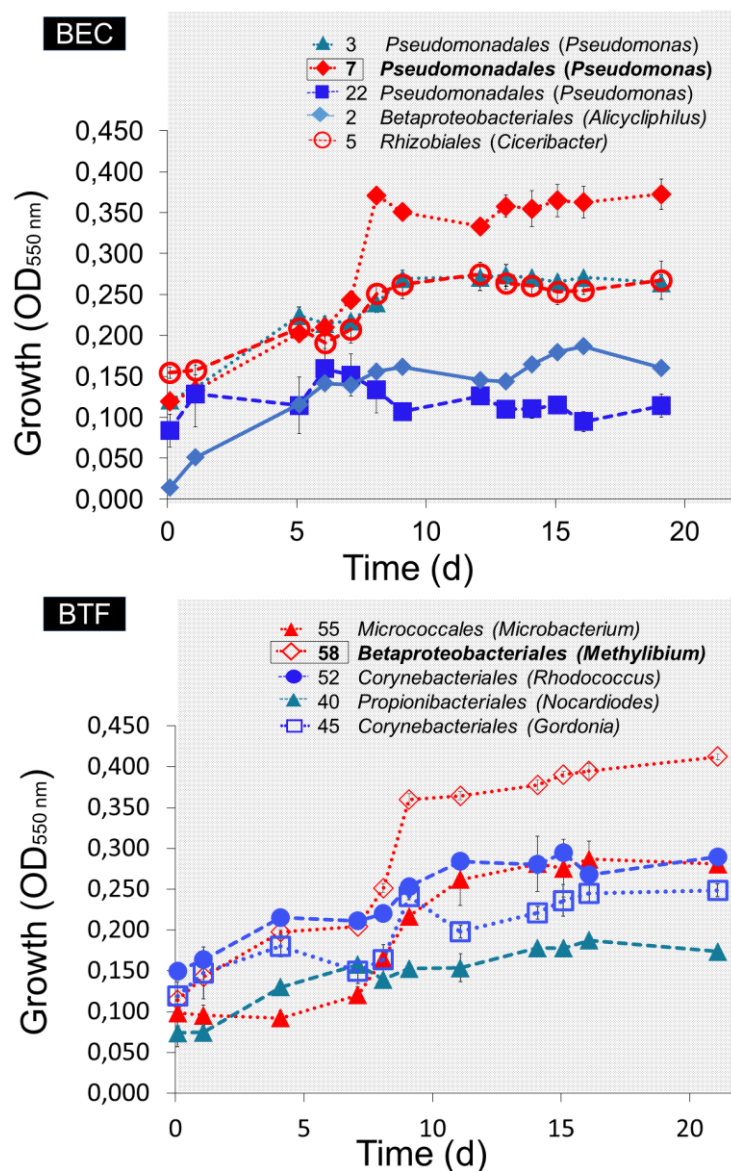
Figure 5.2. Bacterial growth assays measured by optical density at 550 nm in presence of D4 as sole carbon and energy source. Selected growth curves (mean values and SD, n ≥ 3) of representative selected isolates among the culture collection from batch enrichment cultures (BEC) and biotrickling filter (BTFas). Framed codes indicate promising bacterial isolates evaluated in further siloxane biodegradation assays. Error bars indicate SD.

Figure 5.3. shows the D4 consumption after 60 days of the isolates 58 and 7 together with the co-culture composed by all the isolates previously selected in order to elucidate whether the co-culture condition enhances D4 removal.
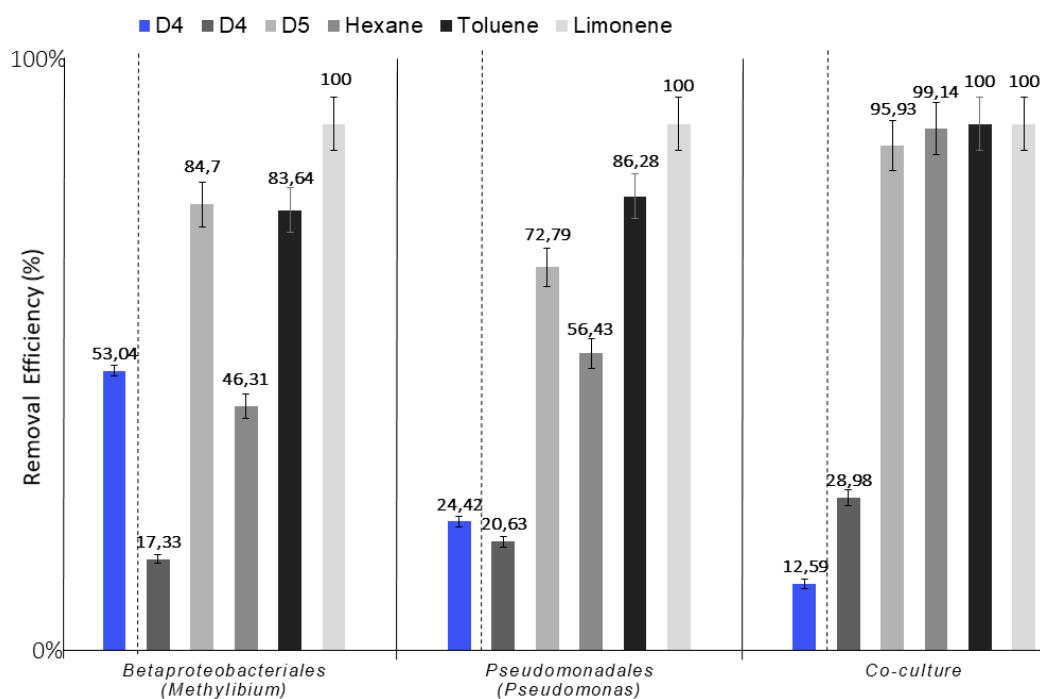
Figure 5.3. D4 consumption during growth assays of selected bacterial isolates 58 and 7 as pure cultures and as co-cultures in the absence and the presence of VOCs (D5, hexane, toluene, limonene). Bar left side of dashed lines represent the percent removal of D4 in the absence of VOCs and bars right side of dashed lines represent percent removal of D4 and each respective VOC compound when are added to the growth media. Numbers (top) indicate mean removal percent values. Error bars indicate SD. Evaluation time was 60 days. ** Isolates present in Co-culture from BEC: 2, 3, 5, 7, 22; from BTFas: 40, 45, 52, 55, 58.

Cultures initiated at early stationary phase displayed a 72 hours lag period followed by degradation along 60 days, ultimately removing 24.42% and 53.04%, for the isolate 7 and 58, respectively (bars left side of dashed lines, **Figure 5.3.**). Interestingly, *Methylibium* sp. (iso58) doubled the D4 removal of *Pseudomonas* sp. (iso7). To our knowledge this is the first study describing the capacity of an isolate of this genus to grow on D4, which moreover showed a removal higher than those of *Pseudomonas*, a bacterial genus commonly considered the paradigm of the bacterial capacity to biodegrade recalcitrant compounds (Dasari, Venkata Subbaiah, Wudayagiri, & Valluru, 2014; Kyaw, Champakalakshmi, Sakharkar, Lim, & Sakharkar, 2012; Varjani & Upasani, 2016; G. L. Zhang, Wu, Qian, & Meng, 2005). In consequence, the isolate 58 represented a potentially promising member of the genus *Methylibium* to be applied for biogas upgrading. Most members of *Methylibium* spp. exhibit methylotrophic activity and are well known for their capacity to grow exclusively on C1 compounds such as methanol or methane (Chaston & Douglas, 2012). For instance, *Methylibium petroleophilum* has been described to degrade methyl-tert-butyl-ether (Nakatsu, 2006). Methyl groups of the D4 molecule are thus suggested here to sustain bacterial growth for *Methylibium* sp.

Regarding *Pseudomonas* several species have been formerly described as good candidates to be applied for siloxane removal in aerobic conditions. Some studies showed that *Pseudomonas* isolates effectively grew on D4. Accettola et al., (2008c) and Y. Li et al., (2014) achieved removal efficiencies from 20 up to 74%, and Grümping et al., (1999) reported D4 degradation after 100 days at least 3% of the spiked D4 was converted into dimethylsilanediol (main degradation product of D4) which are in the range of the efficiencies and evaluation time obtained in the present study. Although the metabolism of siloxane biodegradation is not elucidated yet some authors proposed a biodegradation pathway for D4 by *P. aeruginosa* (Y. Li et al., 2014; J. Wang et al., 2014). In this context, further studies based on transcriptomic analysis are mandatory to identify the genes involved in the biodegradation of D4 for *Pseudomonas* and *Methylibium*, which would stimulate the biodegradation in order to increase the efficiency removal in BTF by means of adding growth factors.

Surprisingly, the removal efficiency of the co-culture decreased to 12.59%, probably due to a metabolic bacterial interaction. Deng & Wang, (2016) suggested that the complexity of the substrate may affect the way bacteria interact in the presence of a recalcitrant substrate. In our experiments, biomass growth was not affected, but D4 consumption was decreased, probably due to the preferential use of other more readily available compounds. Rinkes, Weintraub, DeForest, & Moorhead, (2011) found that C rich labile substrates were preferentially consumed in an organic mixture, and observed a shift in functional groups of microorganisms with different enzymatic capabilities during the biodegradation process. This result suggests that a single bacterial species rather that a microbial community composed by isolates representative of the identified species within the culture collection would be more convenient for D4 removal.

5.3.4 Siloxane biodegradation in multicomponent essays

Biogas composition includes VOCs and siloxanes being D4 content approximately up to 60% of the total siloxanes (M. Shen et al., 2018). Substrate combinations between silicon compounds and other organic compounds influence directly in the biotransformation and biodegradation processes. In order to determine whether the biodegradation of siloxanes D4 and D5 could be stimulated by the presence of other VOCs namely hexane, toluene and limonene, the strains iso7 and iso58 were again tested as pure cultures and as co-culture in the presence of a multicomponent mix (bars right side of dashed lines, **Figure 5.3.**). In this set of biodegradation test, D4 percent removal values for strains 7 and 58 decreased to from 24.42% to 20.63% and from 53.04% to 17.33%, respectively, while showing a similar removal pattern for all VOCs. Contrarily, the co-culture condition doubled the D4 removal

increasing from 12.59% to 28.98%. Moreover, the removal of the other target compounds was almost complete. Thus, siloxane D5 was removed to high extend by both the isolate cultures and up to 95.93% by the co-culture.
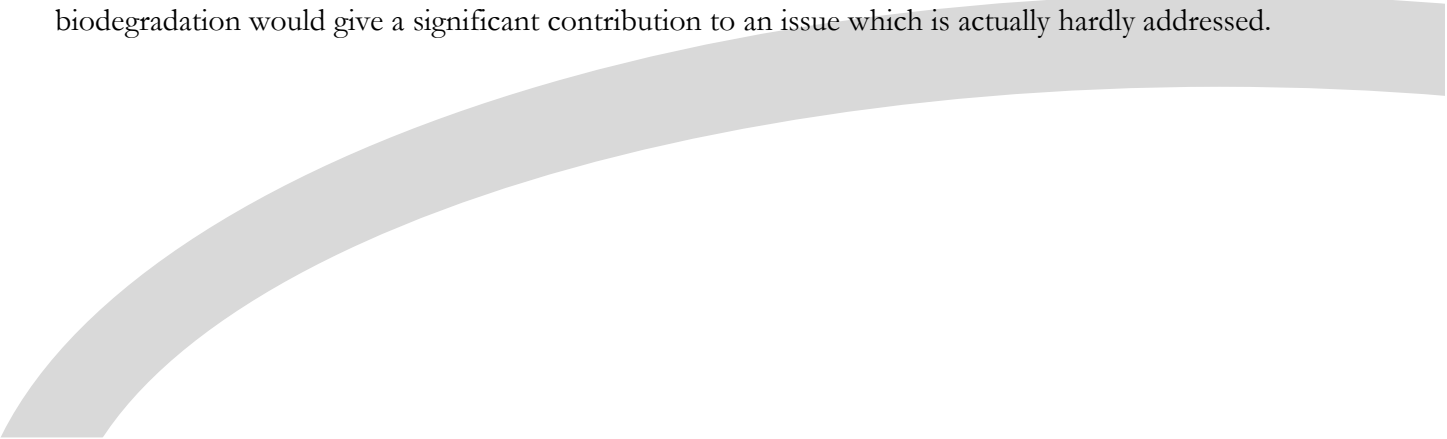
In such conditions, cell counting after 60 days was three times higher than when only D4 was added (results not shown). It is interesting to remark that D4 resulted on the most recalcitrant compound. Therefore, the presence of VOCs in the co-culture condition suggests a positively contribution to bacterial metabolism stimulating both bacterial growth and increasing D4 removal. Likewise, substrate mixtures allow gradual metabolite production and may fuel other metabolic pathways, such as siloxane degradation (Y. Li et al., 2014). Taking into account the increase in D4 removal of the co-culture condition, one would envisage that the removal of D4 could be increased by finely combining different isolates of the culture collection previously characterized, since the combination of bacterial isolates based only on the taxonomic relationship with closely related species or genera rather than on proved biodegradation capabilities can result in different unknown interactions for D4 removal.

5.4 Final remarks

To our knowledge, this is the first report on D4 removal for a member of the genus *Methylibium* (iso58) which was able to degrade D4 in anaerobic conditions as the sole carbon and energy source up to 53.04%, which is double of the removal efficiency of the isolate iso7, a member of the genus *Pseudomonas*. Accordingly, isolate iso58 can be potentially used for biogas upgrading since also partially removes the tested VOCs commonly present in the biogas.

Moreover, a representative selection of the isolates grown in co-culture doubled D4 removal in the presence of D5, hexane, toluene and limonene (VOCs) which were removed close to 100% suggesting that a combination of selected previously characterized isolates would enhance D4 removal in co-culture.

Based on the results obtained in this research we envisage that the present work on siloxane biodegradation would give a significant contribution to an issue which is actually hardly addressed.

Towards the implementation of
a biotechnology for biogas upgrading:
role of bacteria in siloxane removal

CHAPTER 6

Characterization of a core microbial community potentially degrading volatile silicon compounds in an anoxic lab scale biotrickling filter

Submitted paper. Embargo until publication date
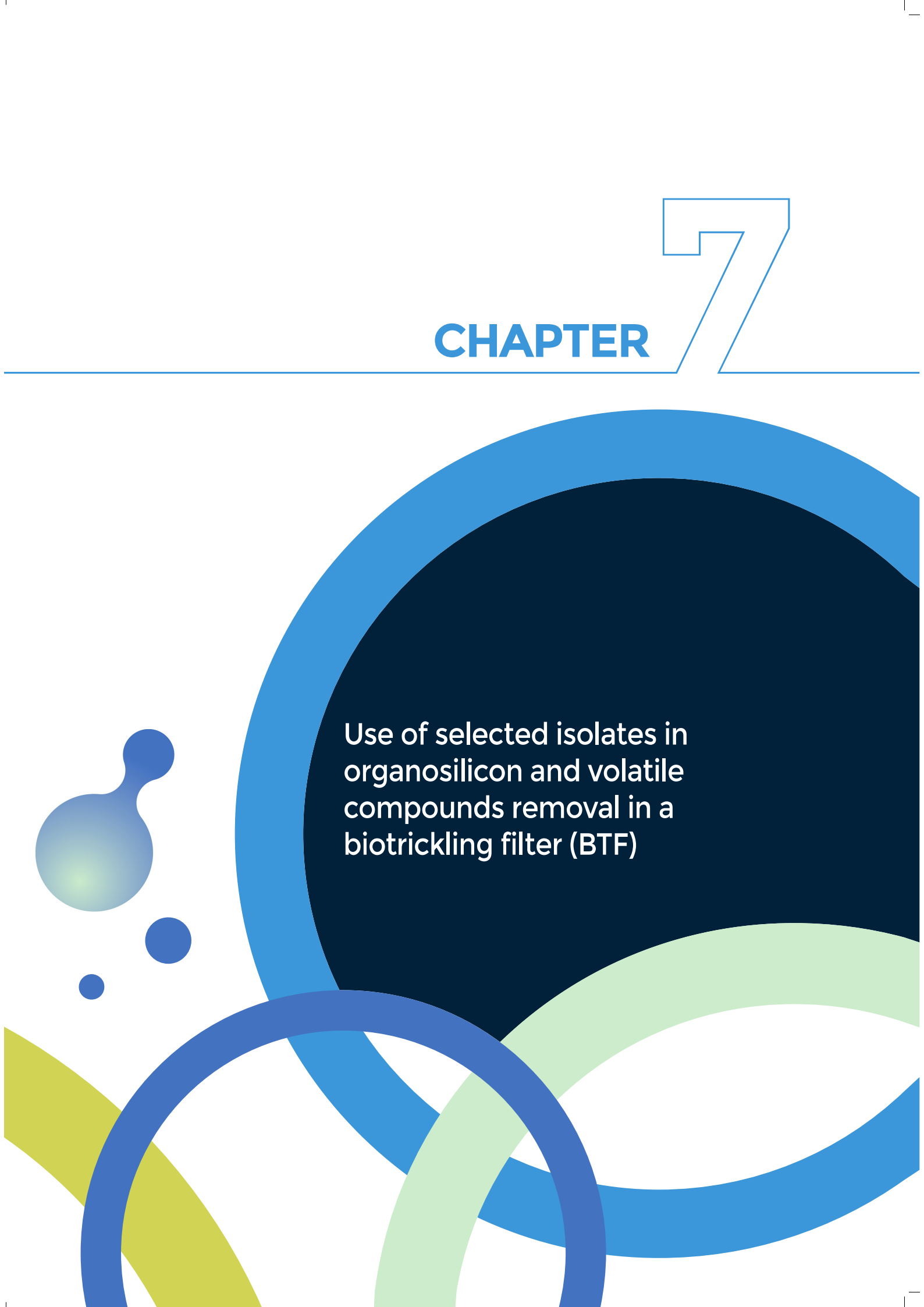
Towards the implementation of
a biotechnology for biogas upgrading:
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CHAPTER 7

Use of selected isolates in organosilicon and volatile compounds removal in a biotrickling filter (BTF)

7.1 Background and objectives

In Chapter 5 *Methylibum* sp. iso58 was identified as the most promising isolate for siloxane biodegradation because it was able to degrade D4 in anaerobic conditions, and use this compound as the sole carbon and energy source. Moreover, it was the first time to report the existence of a *Methylibium* sp. associated to D4 degradation. *Methylibium* sp. is a facultative anaerobic methylotroph (Nakatsu et al., 2006). This is particularly suitable for the removal of methylsiloxanes in biogas treatment, because some amount of oxygen can reach anaerobic digesters biogas.

The aim of this Chapter was to investigate the performance of a lab-scale biotrickling filter (BTF) inoculated with *Methylibium* sp. iso58. More precisely, we aimed at (1) determining the removal efficiency of siloxanes and other VOCs operating at different gas residence time to minimize the required reactor size, and (2) inferring the influence of the final electron acceptor (nitrate and oxygen) disposition in siloxane biodegradation process.

7.2 Methodology

The lab-scale BTF reactor (BTFme) was constructed in Plexiglas, with an internal diameter of 6 cm. The reactor was filled with inert lava rock as packing media and it was operated according to description in **Section 3.5.2.2.** The test gas consisted on a multicomponent mixture of siloxanes and VOCs usually found in biogas with the concentration gathered in **Table 7.1.** The gas flow circulated at counter-current (from the top of the column) respect to the mineral medium solution, and was fixed to 70 $cm^3 \cdot min^{-1}$. The column height of the packing material determined the EBRT and the corresponding load of each compounds on the BTF (**Table 7.1.**).

The system was initially run for 72 h in abiotic conditions in order to rule out the effect of pure physicochemical processes abating siloxanes. For this purpose, concentration of the target compounds were measured at gas outlet and removal efficiencies were calculated according to **Section 3.5.2.** Afterwards, the BTF was inoculated with a pure culture of *Methylibium* sp. iso58, and maintained as described in **Section 3.2.1**. Besides the empty bed residence time (EBRT), experiments in order to test for the effect of electron acceptor were also performed. Tested conditions in BTF operation are detailed in **Table 3.3.**

Table 7.1. Experimental conditions by each operational stage of the BTFme.

Stage	Height [cm]	Gas flow [cm³·min⁻¹]	EBRT [min]	Gas concentration [mg·m⁻³]					Load [mg·m⁻³·h⁻¹]				
				Hexane	Toluene	D4	Limonene	D5	Hexane	Toluene	D4	Limonene	D5
I	36	70	15.5	370.4	23.9	49.4	205.0	99.8	1528	99	204	846	412
II a,b	18	70	7.3	369.7	23.2	45.5	189.3	91.7	3051	192	376	1562	757
III a,b	10	70	4.0	380.2	24.0	46.8	197.5	95.5	5648	356	695	2934	1419

Before the inoculation of BTFme, some kinetic assays were performed in order to determine if *Methylibium* sp. iso58 could be able to grow in the BTF under specific operational conditions. Several operational conditions were evaluated such as: 1) type of substrate, including D4 alone or a mix of hexane, toluene, limonene, D5 and D4 and 2) final electron acceptor, including oxygen or nitrate at different concentrations (**Table 7.2.**). The set of vials were prepared as described in **Section 3.5.1.1.**

Table 7.2. Culture conditions related to final electron acceptor.

e⁻ acceptor	Initial concentration
Nitrate [a] (NO_3) [g·L⁻¹] (mineral medium)	1.0
	2.5
	8.0
Oxygen (O_2) [%] (gas phase)	1.0
	~21.0 [b]

[a] From $NaNO_3$. [b] Air percentage

7.3 Results and Discussion

7.3.1 Bacterial growth

Prior to the reactor operation, bacterial grow assays were performed in order to determine the better culture conditions. These include; physical, chemical, and biological bacterial characteristics, their grow efficiency and future possible operational conditions. **Figure 7.1.** shows the bacterial growth (expressed as OD_{550nm}) at different conditions in order to study the biomass growth using 1) D4 as sole carbon source compared to a mixture of hexane, toluene, limonene D5 and D6, and 2) to compare the growth in oxygen restrict conditions (similar to anaerobic BTF) and in air conditions (similar to aerobic BTF). Tests were performed at different nitrate concentrations in the mineral media because *Methylibium* sp. iso58 is able of mixotrophic and heterotrophic growth and reduce nitrate to nitrite during anoxic o oxygen reduced conditions. Literature the nitrate respiration occupies a central position in facultative metabolism and is a key regulation point for a variety of energy-generating

pathways, and probably includes the siloxane degradation pathways suggests (Hallin, Jones, Schloter, & Philippot, 2009; Johnson, 1967).
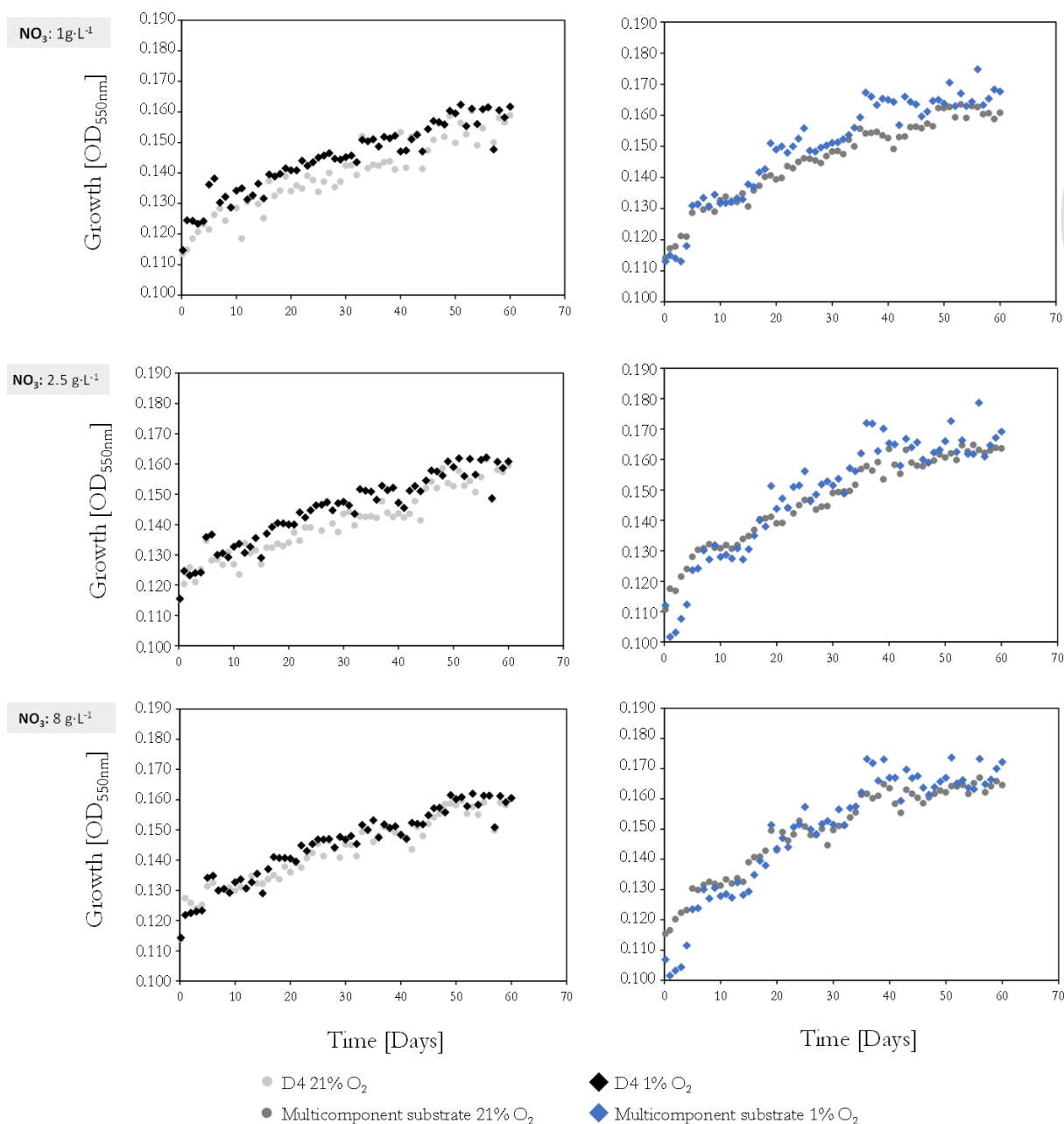
Figure 7.1. Bacterial growth of *Methylibium* sp. iso58 measured by optical density at 550nm in presence of D4 and mixture D4, D5 and three VOCs (hexane, toluene, limonene) as sole carbon and energy source. Oxygen concentrations on culture assays: 1 % and 21% (air conditions). All values are the average of triplicate measurements with deviation below 3%.

During our assays, the biomass growth was measured through optical density and their results were significantly different between oxygen conditions (Kruskal-Wallis tests, *p* value = 0.002; $p < 0.05$).

The biomass growth was low and ranging from 8×10^{-2} and 1.7×10^{-1} OD units (**Figure 7.1.**), and was significantly different between substrates conditions (Kruskal-Wallis tests, p value $= 0.000$; $p < 0.05$), being higher in multicomponent compared to D4. No differences on growth yield could be determined between nitrate concentrations (Kruskal-Wallis tests, p value $= 0.301$; $p > 0.05$).

To determine maximal growth rates (μ, day^{-1}), optical densities of the cultures at exponential period were log-transformed and the slope for each culture used. Although, all growth rates were low ($\mu = 0.521$ day^{-1} +/- 0.081), the literature reports late-log growth for similar assays performed with *Pseudomonas aeruginosa* and D4 as carbon source during 100 days. The main limitations for the relatively slow growth rates of cells in cultures were attributed to the complexity of substrate because the growth rates of the two control cultures, (LB and TSB media as nutrient source) were high, maximum $OD_{550} = 0.280$; $\mu = 0.312$ h^{-1} and maximum $OD_{550} = 0.325$, $\mu = 0.429$ h^{-1}, respectively). The kinetic curves show transient oscillations, and three growth periods are slightly distinguished every 20 days when the substrate is renewed. During the first period the growth rate was $\mu = 0.324$ day^{-1} +/- 0.08, the second period the growth rate increased to 0.496 day^{-1} +/- 0.003, probably because the substrate adaptation phase has been previously achieved. The highest growth rate was during the third period, 0.731 day^{-1} +/- 0.023.

The absorbance values reported by other authors (Accettola et al., 2008) suggest that in presence of D4, the highest optical density reported is close to $OD_{550} = 0.160$. Our assays have similar results with D4 as sole carbon source (**Figure 7.1.**, the culture reaches values close to $OD_{550} = 0.160$ since $50 \pm$ day). Remarkably, in mixed substrate (siloxanes and VOCs), the absorbance values reach 0.160 at 35 ± 3 day, and even reach higher values (0.180). The maximum cell density ranged from $1–3 \times 10^{9}$ cells·mL^{-1} in mixed substrate assays to $6-8 \times 10^{8}$ cells·mL^{-1} in D4.

7.3.2 Lab-scale BTF operation

Stage I

The first operational stage of the lab-scale BTFme was devoted to *Methylibium* sp biomass fixation and acclimation. Thus, long EBRT (15.5 min) was maintained for 95 days. Average test gas concentration of each target compound and the corresponding load in such conditions are gathered in **Table 7.1.** The concentration of each compound at the BTF gas outlet was daily measured from operation day 40 on by means of GC-FID. The elimination capacities were calculated and are shown in **Figure 7.2.**
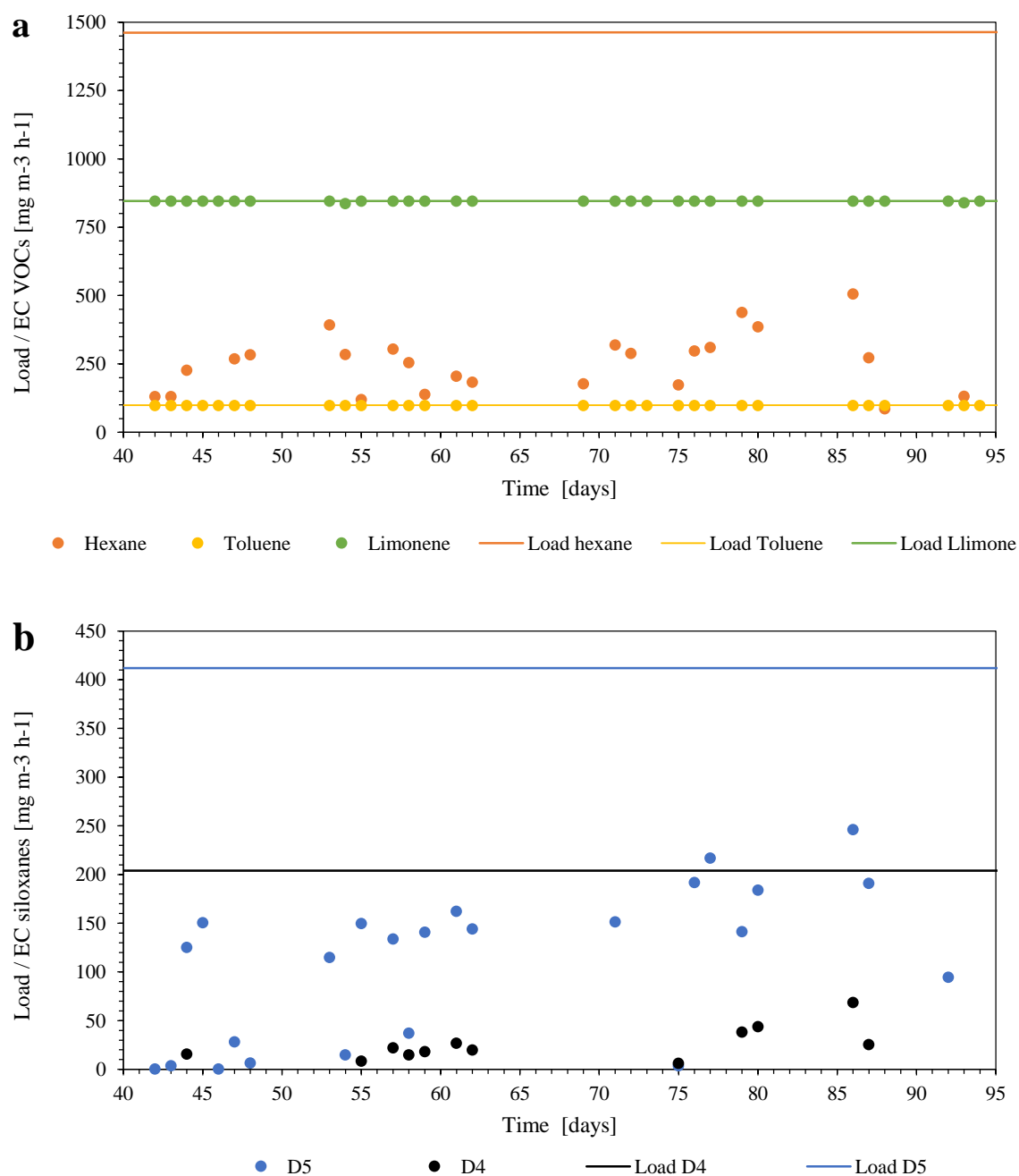
Figure 7.2. Time course of elimination capacity (EC) of **a.** VOCs(toluene, hexane, limonene) and **b.** Siloxanes (D4 and D5) in the biotrickling filter reported during the Stage I Operated at EBRT 15.5 min.

Since the beginning of operation, limonene and toluene were almost completely removed, considering that the concentration of both compounds in the outlet of the reactor was below our detection limit (0.5 mg·m⁻³). Contrarily, hexane was hardly removed biologically, achieving removal efficiencies to 16.9 % ± 6.9, which corresponded to elimination capacities of 248.4 ± 107.3 mg·m⁻³·h⁻¹. The mean removal efficiencies as well as the corresponding standard deviations of each period are presented in

Table 7.3$_{\text{stage I}}$. On the other hand, siloxane D4 was removed in an 11.9 % ± 8.0, while the microbial performance towards the degradation of D5 was higher (28.4 % ± 17.3). This larger cyclic siloxane molecule resulted more biodegradable than the previously studied D4 at the conditions tested.

Stage II

After 95 days of BTF operation the EBRT was reduced up to 7.3 min (**Table 7.1.**) aiming at improving the performance of the bioreactor by reducing the required size. Thus, the column height of the BTF was reduced down to 18 cm and day 95 until day 128 the system was operated in batch NO_3^- loading configuration like in the previous stage (**Chapter 3, Table 3.4.**). During this period, the CO_2 formed was also recorded (**Figure 7.3A.**). The elimination capacities of toluene and limonene increased immediately with the load's rise caused by the reduction of the EBTR up to 181.1 ± 0.02 and 1474.7 ± 8.2 mg m^{-3} h^{-1} respectively, thus maintaining the removal efficiencies at the 100%.

Interestingly, the removal efficiency of hexane experimented a progressive improvement during the first 20 days of operation at higher inlet gas load and stabilized at ca. 40% (35.2 ± 9.9 %), compared to previous operational stage. CO_2 concentration increase in the outlet was observed matching the increase on hexane's removal. Taking into account that hexane's is the most concentrated compound in the test gas (ca. 370 mg m^{-3}), and that its load almost doubles limonene's, its mineralization caused a significant increase on the CO_2 production. Regarding siloxane's biodegradation, an unstable performance was observed for D5 removal (20.6 ±10.1%), while D4 was maintained ca. 15% removal efficiency (**Table 7.3$_{\text{stage IIa}}$.**).

Aiming at improving both the performance and stability of the biodegradation, on day 128 of operation (Stage II-b) an automatic NO_3^- dosage system was implemented in order to maintain a constant nitrate concentration in the trickling solution ca. 2.5 g L^{-1}. This strategy was previously investigated in a lab-scale membrane bioreactor (Santos-Clotas, Cabrera-Codony, Comas, & Martín, 2019) and led to a less oscillating performance regarding siloxane biodegradation.

As observed in **Figure 7.3.A.**, the automatic NO_3^- supply led to an increase on the hexane elimination capacity, which reached complete removal efficiency after 10 days. The performance improvement was also observed on the siloxane removal efficiency, which increased for the first 10 days of operation achieving removal efficiency up to 50% for both compounds. However, after biomass acclimation to

the new operation conditions, from operation day 145 on, hexane removal's efficiency stayed at the 100% while siloxane's drop to values similar to the previous stage II-a.

Figure 7.3. Time course of elimination capacity (EC) of synthetic gas components in the biotrickling filter reported during the Stage II. Horizontal lines represent the load of gas composition. Vertical dashed lines represent operational described stages IIa and IIb. **a.** VOCs: toluene, hexane, limonene. Secondary Y axis: CO_2 formed in the BTF, expressed as ppm v/v. **b.** Siloxanes D4 and D5. EBRT: 7.7 min.

Table 7.3. Removal efficiency (RE) with their corresponding standard deviations for a mixture of two siloxanes and three VOCs calculated by each operation stage of the BTFpi-n inoculated with *Methylibium* sp. iso58.

Substrate	Removal Efficiencies [%]				
	Stage I	Stage IIa	Stage IIb	Stage IIIa	Stage IIIb
D4	11.9 ± 8.0	14.9 ± 4.0	22.5 ± 14.4	13.2 ± 2.8	16.8 ± 19.9
D5	28.4 ± 17.3	20.6 ± 10.1	25.5 ± 15.4	26.7 ± 13.0	20.9 ± 13.5
Hexane	16.9 ± 6.9	35.2 ± 9.9	93.8 ± 13.9	35.8 ± 14.6	85.8 ± 16.4
Toluene	100.0 ± 1.8	99.1 ± 4.9	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Limonene	99.9 ± 0.2	99.1 ± 3.7	99.9 ± 0.6	100.0 ± 0	99.4 ± 2.0

Stage III

The last operational stage was devoted to further reduce the bioreactor size, i.e. achieving high removal efficiency at short EBRT. Thus, the upper layer of packing material of the BTF was removed to get a reactor height of 10 cm corresponding to an EBRT of 4.0 min. This reduction lead to an increase on the load of the target compounds (**Table 7.1.**).

Likewise the previous stage, both limonene and toluene elimination capacities immediately increased up to the complete removal (**Figure 7.4.A.**), i.e. from 181 to 356 mg m^{-3} h^{-1} of toluene and from 1477 to 2935 mg m^{-3} h^{-1} for limonene. On the other hand, hexane biodegradation was slower, showing a progressive increase on the elimination capacity during the first 20 days up to ca. 2000 mg m^{-3} h^{-1} (2038.1 $\pm$8.3). Siloxane elimination capacity was increased up to ca. 100 mg m^{-3} h^{-1} of D4 and mean values of 445.7 $\pm$ 161.4 mg m^{-3} h^{-1} of D5, although the later showed a very unstable performance.

Aiming at improving both the performance and stability of the biodegradation, on day 213 of operation (Stage III-b), besides the automatic NO$_3^-$ dosage system that maintained a stable concentration in the trickling solution ca. 2.5 g L^{-1}, 1% of O$_2$ was added to the feed gas, mimicking the O$_2$ composition in real sewage biogas (Rasi, 2009). Thus, the performance in an oxic BTF was evaluated and compared to the previous anoxic conditions.

Both limonene and toluene elimination capacities were maintained at the complete removal of these compounds was achieved. The biodegradation of hexane increased up to 85.8 $\pm$ 16.4 % during the following days after the addition of O$_2$ (**Figure 7.4.A.**). On the other hand, siloxane's biodegradation increased progressively for the 15 days following the O$_2$ addition, achieving ca. 50% removal efficiency for both D4 and D5 at operation day 193. However, after this initial period, the siloxane biodegradation dropped to 80 to 94.4 mg m^{-3} h^{-1} of D4 and mean values of 370 mg m^{-3} h^{-1} of D5.
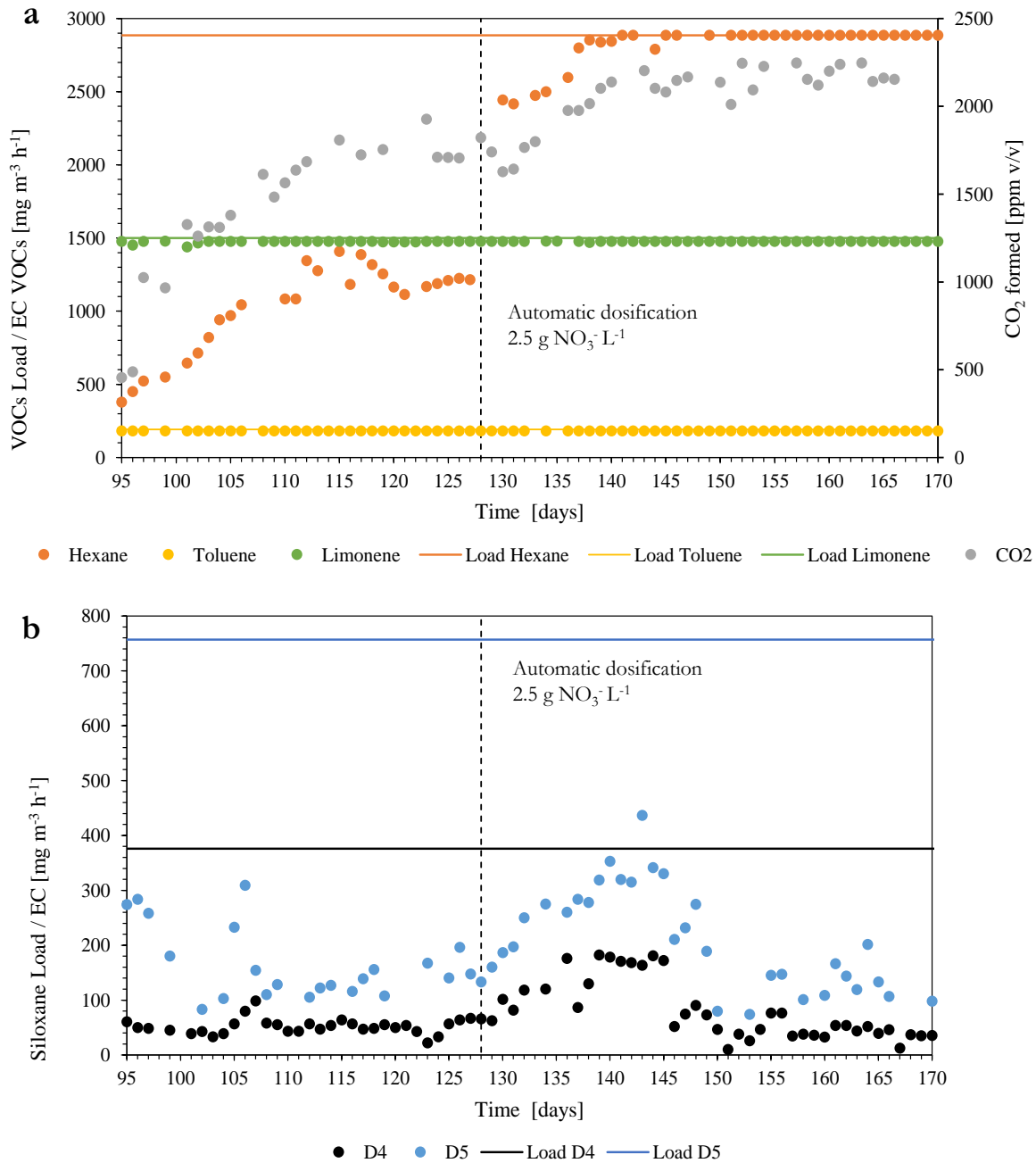
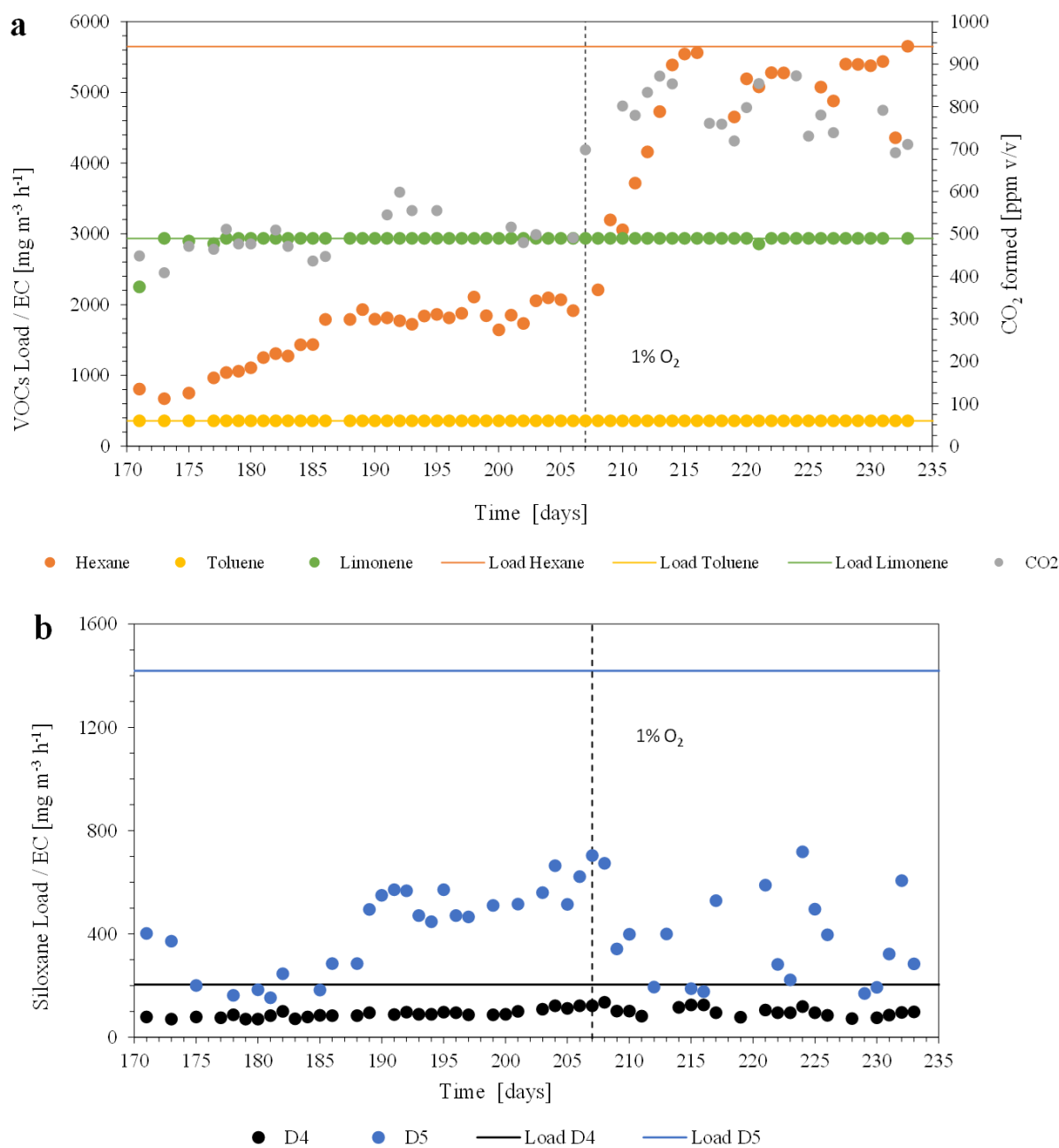**Figure 7.4.** Time course of elimination capacity (EC) of synthetic gas components in the biotrickling filter reported during the Stage III. Horizontal lines represent the load of gas composition. Vertical dashed lines represent operational described stages IIIa and IIIb. **a.** VOCs: toluene, hexane, limonene. Secondary Y axis: CO_2 formed in the BTF, expressed as ppm v/v. **b.** Siloxanes D4 and D5. EBRT: 4.4 min.

7.3.3 Influence of culture conditions in *Methylibium* sp. growth during siloxane removal

Previous studies have demonstrated that the presence of several types of substrates influences bacterial growth. Some authors have reported the metabolic capacity of *Methylibium* in the degradation of organic compounds without carbon-carbon bonds. Such as components of oil sands tailings ponds (e.g. formaldehyde and methylated amines) (Rochman, 2016), fuel oxygenates (e.g. methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE), and tert-amyl methyl ether (TAME)) (Szabó et al., 2015),.

During this work, the metabolic abilities of *Methylibium* sp. were evaluated. The laboratory assays demonstrated the increase in biomass, having as a carbon source a mixture of complex compounds (toluene, limonene, hexane, siloxanes). Moreover, the tests in the BTF reported a decrease in the concentration of substrates. These results confirm the ability of iso58 to grow in siloxane and degrade it in the assayed conditions. The concentration of CO_2 generated during the process and reported in **Figure 7.4.** demonstrates the achievement of basic metabolic processes such as respiration and growth. However, an analysis of the products generated as products of siloxanes degradation such as the concentration of silanediols is necessary in future tests.

The facultative metabolism of the genus *Methylibium* was also evaluated in laboratory tests and BTF tests. The results suggest that the best conditions for the *Methylibium* growth in the two evaluated situations is with low oxygen concentrations, closely to 1%. Couvert and collaborators (Couvert, Divanac'h, Lochardet, Thuault, & Huchet, 2019) proposed a model about the influence of the percentage of oxygen on the growth of facultative bacteria. The Couvert' model reported that there are non-significant differences in the bacterial growth rate associated with the percentage of oxygen in the gas phase.

Biogas digesters have an oxic and anoxic or hybrid operation. For the facultative bacteria, the anoxic or oxic conditions are theoretically irrelevant, because during their metabolism they can have several final electron acceptors depending on each case. During this study, two types of final electron acceptors were evaluated: nitrate (anoxic conditions) and oxygen (oxic conditions). It has been shown that the best condition for the *Methylibium* sp. growth is in conditions closely to anoxic operation, with 1% oxygen both in laboratory tests and in BTF. In addition, three concentrations of nitrate (as

NaNO₃) were used in order to determine if the bacterial growth is associated to nitrate concentration, because nitrate could be a possible final electron acceptor during facultative metabolism of *Methylibium* sp. Laboratory tests showed that there are non-significant differences among the concentrations evaluated, at the level of bacterial growth. However, during the BTF tests, better removal values were observed with higher nitrate concentrations. Therefore, it is suggested that the nitrate concentration could be directly related to the degradation pathway of the evaluated substrates for instance siloxanes.

7.4 Final remarks

A BTF was inoculated with *Methylibium* iso58, which was previously defined with capacity to grow in presence of D4 and other VOCs as the sole carbon and energy source, and was evaluated in different operating conditions.

Complete removal of both toluene and limonene was achieved at all the conditions tested, which corresponds to elimination capacities up to 356 and 2934 mg m^3 h^{-1} respectively at the shortest EBRT. On the other hand, hexane removal experienced a drastic increase with the presence of 1% oxygen in the feed gas. In such conditions, removal efficiencies achieved for both siloxanes were up to 20%. This will confirm is an aerobic bacterium, at least 1% is preferred to no oxygen.

Probably the degradation pathway of VOCs is associated to low concentrations of oxygen, closely to anoxic media, similar to the conditions in typical biogas. This highpoint is crucial because increased the possibility to use the biological methods in removal process in real conditions. Likewise, the presence of nitrate suggested its role during the degradation pathway of the VOCs. Although, the biomass was not affected in the presence of increasing nitrate concentrations, the results suggest that *Methylibium* sp. could be beneficial to general biogas purification, when the bioreactor has an anoxic performance or the biogas possesses high concentrations of supplied nitrate.

The findings of this study confirmed the potential of using *Methylibium* for the inoculation of biotrickling filters for biogas purification. Taking into account that a subsequent step of adsorption would be necessary reduce siloxane concentration, the almost complete biodegradation of VOCs achieved in the BTF drastically increases the lifespan of activated carbon filters.

Towards the implementation of
a biotechnology for biogas upgrading:
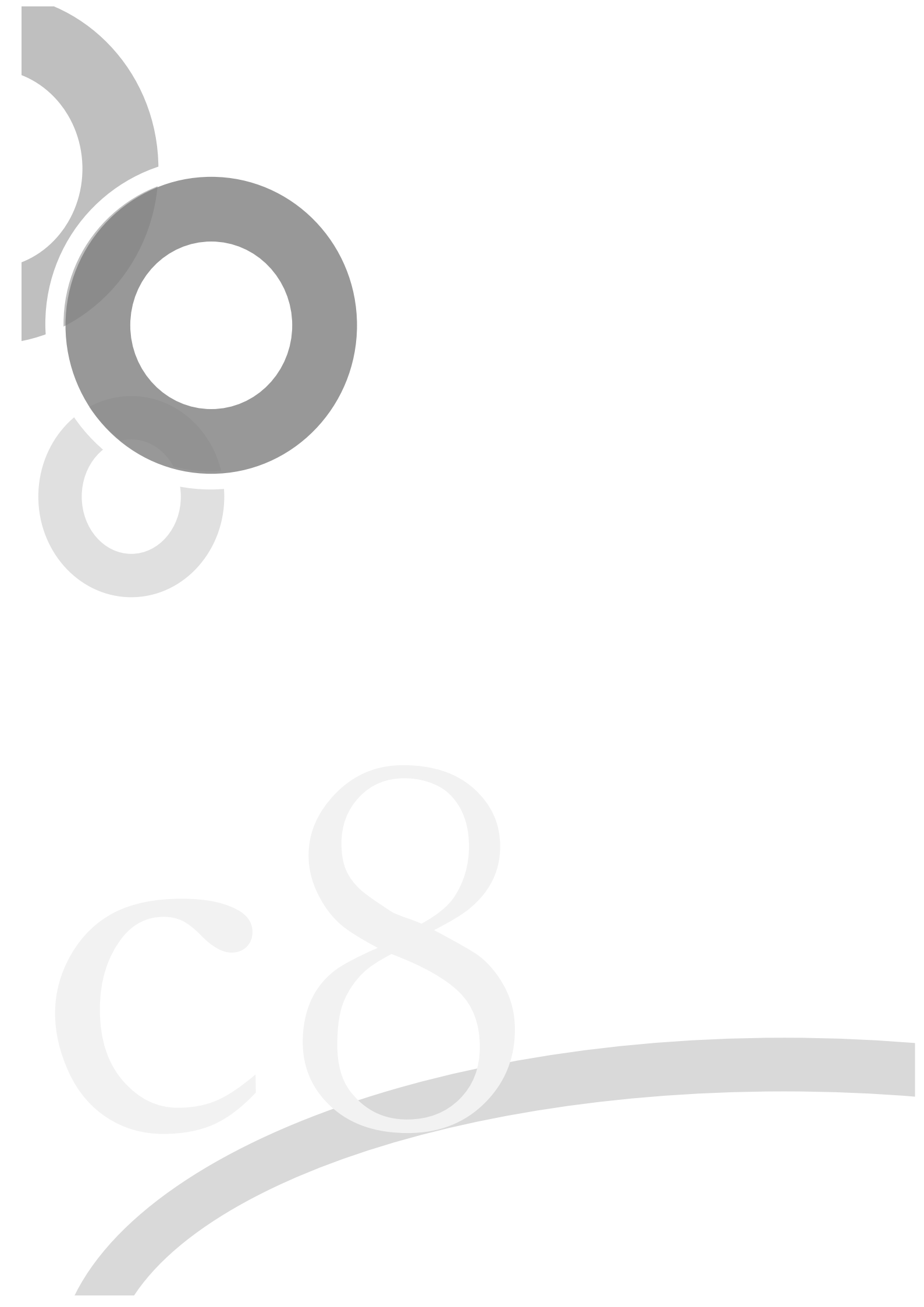*role of bacteria in siloxane removal*

CHAPTER 8

General Discussion

Biogas has emerged as an interesting alternative for more sustainable fuels. Biogas is green energy, environmentally friendly, carbon balanced, and has a high value as a renewable fuel (Adnan, Ong, Nomanbhay, Chew, & Show, 2019; Energiforsk, 2016). However, basic biogas production from anaerobic sludge (one of the main sources) suffer from the accumulation of undesirable chemicals in the final gas stream that may cause serious damages and corrosion to the energy conversion engines, preventing its direct use (Matsui & Imamura, 2010). Among these undesired chemicals, siloxanes, which constituted the main topic of this thesis, have the most adverse effect on the utilization of biogas (Y. H. Liu et al., 2019).

Several technologies have been established to remove siloxanes. The most widely used is the non-regenerative adsorption onto activated carbon because it is easy to operate and it has high removal efficient values (RE 90-99 %) compared to other currently available techniques, such as cryogenic condensation (RE 50 – 80%) and the catalytic process (>80%). However, the after mentioned methods are invasive and environment unfriendly, so there is a demand for the development of less polluting and more sustainable methods for siloxane removal from biogas. Biological siloxane removal methods have been targeted and has become of crucial topic of interest.

The present dissertation explores the abatement of siloxanes from biogas trough biological degradation. The efforts within this work advance towards improving the knowledge about the role of bacteria in siloxane removal and biogas upgrading by biological technologies. We have used a basic "biotechnological approach" that has progressively moved from enrichment-isolation of new bacteria, screening of isolates, and applications in lab-scale bioreactors, such as the biotrickling filters. Approaches to elucidate the microbial community structure of siloxane removing biofilms were also established in chapters 4 and 6. The importance of these chapters reside in the fact that microorganisms are rarely encountered as single species populations and their use as pure cultures for biotechnological applications has some drawbacks in up-scaling. Moreover, the study of the community may help deciphering microbial interactions, which can induce the activation of otherwise silent biosynthetic pathways leading to the degradation of recalcitrant substrates and production of new and more degradable products. One of the main challenges in removal of highly volatile compounds is mass transfer, in this sense the use of packing materials typically used in adsorption methods were used as carrier material in order to increase the availability of those compounds to the biofilm and induce biological treatment. Moreover, solid adsorbents promote biofilm formation and maturity over the surface. In chapter 6 and 7, isolated strains showing potential for D4 biodegradation

were selected to evaluate their capacity to growth with siloxane as sole carbon source or in the presence of a multicomponent mixture of organics in a lab-scale biotrickling filter. Taken all chapters of this thesis together, along with data from the literature, the results found after the analysis of role of bacteria during siloxane removal process and biogas upgrading by biological technologies are discussed below and is schematise in **Figure 8.1.**
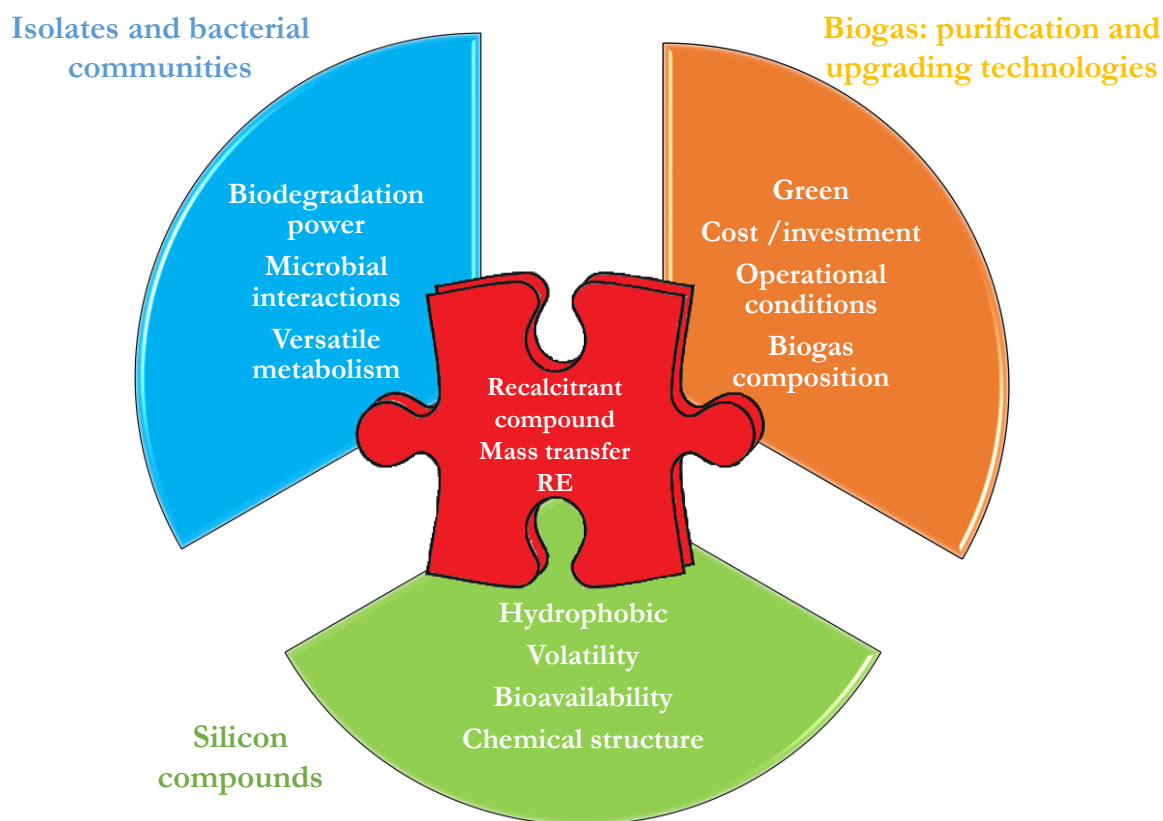
Figure 8.1. Interaction among silicon compounds, isolates and bacterial communities in biogas upgrading technologies.

8.1 Relevance of Biological methods in siloxane removal during biogas treatment

The selection of one method over others depends on several factors, namely economic and operational, but also environmental. Probably the economic factor is the most significant because the industry selects the more efficient and less expensive method in order to obtain cost-effectiveness in a removal process (Matsui & Imamura, 2010). Environmental factors have experienced increased

interest due to the multiple global and state policies have been implemented to counteract the harmful effects of environmental pollution in the world.Several regulations have been applied to control the emission of pollutants generated during biogas production. The main regulations are focused on the control of pollutants, mainly H_2S and siloxanes that may affect the environment and the energy quality of biogas (M. Shen et al., 2018). A large number of investigations aimed at developing strategies for the treatment of sulphur compounds but not yet for siloxanes. The key processes used in siloxane removal are physical or chemical and include high economic and environmental costs due to constant material renewal (Rasi et al., 2011).

The methods used to treat siloxanes were cited in the introduction of this document. The literature suggests that the most commonly used method is the adsorption mediated by activated carbon. Biological technology is, however, less used. The main limitations found in the application of biological methods are: (1) the low biodegradability of some contaminants, (2) the not suitable environment for biological processes (such as bacterial metabolism) and (3) the difficult accessibility of microorganisms to the substrate. This study performs a detailed analysis to overcome these disadvantages. Successful enrichments were obtained using D4 as the sole biodegradable substrate, but a solid matrix to support biomass and stabilize the highly hydrophobic substance in solution was needed. The use of a selective environment was ideal to enhance the growth of bacteria with specialized abilities in the degradation of particular compounds and we provided with an adequate isolation of specialized microorganisms, which could significantly improve the removal efficiency of siloxanes.

The advantage of using biological methods is the lack of formation of secondary pollutants. However, the process is slow (Scarlat et al., 2018). On an economic level, the operating costs associated with the physical/chemical methods depend on the supplies used, the load capacity and the lifetime of the equipment (Ajhar, Travesset, Yüce, & Melin, 2010; Khan et al., 2004). The environmental disadvantages are drastic, due to the environmental management necessary for the waste generated by physical and chemical methods. Instead, one of the objectives of the biological methods is to be economically profitable, sustainable and environment friendly (Ajhar et al., 2010; M. Shen et al., 2018).

8.1.1 Substrate mass transfer and siloxane as volatile compounds

Habitually, the main problems found during the implementation of biological methods are: (1) the formation of biofilm onto surfaces situated in the aqueous phase and (2) metabolize a substrate located

in the gaseous phase (substrates are hydrophobic o poorly soluble) (C. Wu et al., 2017; Yang et al., 2018). In our case, the bacteria are able to growth in presence of a specific carbon source as the siloxane but the substrate is located out of reach of the bacteria: transfer mass problem.

Therefore, the substrate metabolism is difficult and inefficient. In this dissertation, materials with the capacity to keep the substrate in the aqueous phase will be beneficious to the bacteria were determined. Several carrier materials have been tested in order to improve the removal efficiency values associated to their textural properties and usually the removal of D4 or D5 have been the priority. Two support materials (activated carbon and zeolite) were tested for D4 supplementation. As shown in this thesis, cells were able to grow on them in a period of 35 to 60 days. In liquid cultures without support material, bacteria formed aggregates around the available substrate. Theoretically, the availability of the substrate depended on the contact surface with the support material, and is increased in comparison to the liquid phase (Muñoz, Arriaga, Hernández, Guieysse, & Revah, 2006; Ortega & Subrenat, 2009). However, how D4 adsorption into a solid matrix enhances its degradation is not easy to be tested due to the low solubility of this compound in water.

In addition to the problem with siloxane mass transfer, the use of other compounds that may be easier to degrade (i.e. hexane, toluene and limonene) and how their presence contributed to siloxane degradation, was tested. The most biodegradable substrates were toluene and limonene. Hexane showed fluctuating RE values depending on the residence time and packing material. High residence times (greater than 12.5 min) lead to higher hexane removal values (from 20 to almost 50% RE) in the presence of lava rock and activated carbon. The removal efficiency values of the siloxanes increased when two support materials were present in the BTF. Suggesting that pore size, contact surface and type of support influence substrate adsorption capacity. In addition, the availability of hydrophobic substrates increases when two types of support were implemented. Therefore, the establishment of a method that combines various types of support, with good biofilm formation capabilities, would be an excellent strategy for the treatment of hydrophobic volatile compounds.

8.1.2 Packing materials on the siloxane removal and analysis of microbial communities

In Chapter 4, the ability of bacteria to form biofilm on the surfaces of two types of packaging materials was evaluated. The culture media used favoured the growth of microorganisms capable to use D4 as the only carbon source. The used packaging materials improved the ability to contact between the carbon source and the microorganisms. Activated carbon and zeolite proved to be suitable materials for biofilm formation because the concentration of biomass present in the cultures with packing

material was higher than the liquid cultures without support. The problem of mass transfer and difficult accessibility of the hydrophobic substrate in the aqueous phase was minimized thanks to the effective adsorption capacity of the materials.

Activated carbon proved to be an efficient support material in processes of biofilm formation of our uncultured isolates. These results were supported by the literature about the use of activated carbon as a support material for biofilm formation (Lameiras et al., 2008; Xinying Zhang et al., 2016). Its use has also been extended to applications in bioreactors and treatment of recalcitrant compounds, such as siloxanes(Berenjian et al., 2012; Pascual, Cantera, Muñoz, & Lebrero, 2020).

The origin of activated carbon also influenced onto the bacterial growth, and the bacterial communities found in activated carbon of peat origin differs from the community found in activated carbon of anthracite origin. Both communities with growth capacities with D4 as carbon source, but with different representative taxonomic groups whose metabolisms could metabolize siloxanes by different biochemical pathways and obtain different final products.

Zeolite had a great influence on bacterial community compared to activated carbon. Interestingly, underrepresented groups present in the activated carbon enrichment communities were abundant in the community found in zeolite enrichments. These results indicate that the kind of support material influences to the growth of specific taxonomic groups.

8.2. Relevant parameters in Biological technologies to siloxane removal

8.2.1 Residence time and removal efficiency

The efficiency of biological methods depends on the standardization of various operational conditions such as type of microorganism, temperature and residence time. Each condition has been diligently evaluated during this study. Chapters 4 and 6 successfully determined the bacteria and potential taxonomic groups in siloxane degradation processes. During Chapter 5, several assays are established in order to determine the ideal conditions for the cultivation and the maintenance of strains capable to growth with siloxane as the sole source of carbon.

The temperature, the concentration and components of the mineral medium, and substrate concentrations were established for optimum cultivation in the laboratory. Kinetic assays showed that biomass increased during the assay, and the substrate (D4 or others) decreased. Chapter 7, showed interesting results of laboratory tests and in a BTF, where the influence of the electron acceptor in aerobic and anoxic conditions was successfully evaluated.

Residence time was evaluated in all essays and under different operational conditions. The residence times differed between laboratory batch and BTF tests. In batch tests, the residence times evaluated in other studies are ranging 60 to 300 days. The supply of the substrate is divided into periods of 20 to 50 days. Y. Li et al., (2014) worked with residence times of 150 days, and predicted an ideal residence time for laboratory tests of 120 days. The times used in the assays of Chapter 5 were ranging 60 to 120 days. During the first 30 days, the microorganisms had an adaptation phase associated to the specialized substrate, D4 or other compounds (D5, toluene, limonene or hexane). However, according to our results, residence times could be shortened to 70 days, when a stable biomass and VOC consumption was observed.

BTFs were operated with short residence times compared to batch tests. This factor is limiting in the modification from laboratory tests to BTF tests. The evaluated cultures grew in continuous flow reactors with times (EBRT) between 2 and 16 minutes. The chapter gathers the results of a BTF inoculated with activated sludge, and re-inoculated every 50 days with specialized cell culture. RE values showed the substrate consumption in dependent on residence time and the type of support. Toluene and limonene were degraded efficiently during the assay (100% RE). Hexane showed dependence on residence time during its degradation. The longer residence time, the better values of RE. The highest RE reported for hexane was 45% at an EBRT of 12 min with activated carbon and lava rock as a support material. D4 and D5 were degraded at different rates probably due to their physicochemical characteristics. D4 had RE values less than 20% in all the conditions evaluated, although D5 RE increased to 45 and 50% in some conditions. The use of a hybrid support of lava rock and activated carbon (RE 45%), and an EBRT of 12 min was the best conditions reported in terms of RE for D5. The presence of lava rock and an EBRT of 14.5 min reported a RE of 45%. Shorter times (10, 7.3 or 4 min) decrease removal efficiency considerably (RE: 30%, 20% and 15% respectively). In activated carbon the RE values were ranging 20 to 25%, with the shortest residence time (2 min). These results suggested that D5 degradation is the most dependent on residence time. In aerobic tests with RE 43%, and EBRT 19.5 min and REs greater than 43% with an EBRT of 30-

40 min; while anaerobic trials reported 15% RE to EBRT for 15 to 2 min (Chapter 6 and 7). The residence times used in the BTF trials of this study were similar to other previous assays and were even shorter.

8.2.2 Biogas treatment, using Nitrogen as gas stream and perspectives with Methane

BTF has been extensively tested in processes as a useful technology for the purification of H_2S and other biogas contaminants, including siloxanes. BTFs have been operated under anaerobic and anoxic aerobic conditions using mixed, highly enriched, bacterial communities. In fact, due to this heterogeneity of cells that can be found on BTFs, multiple versatile metabolisms occurs, which easies the degradation of several types of substrates. In this thesis, some degradation assays were developed under facultative and aerobic/anaerobic conditions. The used bacteria have mainly facultative anaerobic metabolisms.

Until the moment of this dissertation, no tests had been carried out in anaerobic conditions and using a gas stream closer to the real biogas. Ten strains of bacterial isolates were determined as potential bacteria in the degradation of siloxanes. Among them, *Methylibium* sp. and *Pseudomonas aeruginosa* demonstrated the best growth characteristics in the presence of siloxanes as sole carbon source. *Methylibium* sp. has a facultative metabolism and high ability to degrade methyl groups. This factor is an advantage in the degradation of siloxane.

Moreover, some members of genus *Methylibium* have been described as unable to grow under methane: *Methylibium petroleiphilum* (Kalyuzhnaya & Xing, 2018). Thus, the methane oxidation (microbial metabolic process for energy generation and carbon assimilation from methane) is unfeasible and the degradation of D4 will not be significantly affected by the presence of methane. In addition, herewith the energy capacity of biogas wont altered by methane degradation trough *Methylibium* sp. iso58 during pilot assays with real biogas composition (mostly methane).

Recent studies by (Yang et al., 2018) tested simultaneous purification processes of H_2S and VOCs (including siloxanes) in aerobic BTF, having interesting results in biotic and abiotic removal values. This study, together with this thesis, could serve as antecedent for future tests of purification of siloxanes in aerobic conditions with a tendency to facultative.

8.3 Role of bacteria in siloxane removal and biogas upgrading technologies

Bacterial metabolism is very complex. The bacterial species can metabolize a great diversity of compounds and produce innumerable by-products or secondary metabolites. However, not every bacterium metabolizes the same compounds. Consequently, like in a society, bacteria interact with each other to improve their living conditions. Bacterial interactions are crucial to the success of establishing and maintaining a microbial population. Ten bacterial species were identified as the most representative during siloxane degradation processes. Most isolated species were members of the most representative taxonomic groups in the defined bacterial communities such as the orders: *Betaproteobacteriales* and *Rhizobiales*. In the degradation assays performed with isolated bacteria, some bacterial strains stood out over others. *Methylibium* sp iso58 and *Pseudomonas aeruginosa* iso07 were the strains with the highest reported RE values (53.04%, 24.42% respectively). Interestingly, in assays performed with co-cultures of the ten isolates the RE was lower (12.59%), suggesting negative effects due to bacterial interaction.

The isolated *Methylibium* sp. iso58 was also evaluated in a lab-scale BTF. D4 and other compounds (D5, toluene limonene, and hexane) were used as a carbon source. Initially, greatest RE values were expected (close to 50%). However, the RE was 12% ± 5. This result indicates that the laboratory operational conditions (facultative anaerobic metabolism, temperature, agitation, among others) effectively restricted the degradation of D4. Therefore, greater operational controls are necessary during BTF pilot assays.

Bacterial communities in the BTF were also evaluated with D4 with a single carbon source. The results obtained were similar to BTF inoculated with *Methylibium* sp. iso58. (RE 15% in BTF inoculated with the bacterial community and 12% for BTF inoculated with *Methylibium* sp. iso58). These results confirm a necessity to establish better operational conditions in the BTF. A previous study (López et al., (2015) reported similar limitations in the operation of BTF in anaerobic and aerobic conditions.

8.4 Hybrid technologies and prospects for future research

Traditional technologies for the purification of biogas are based primarily on the use of physical and chemical methods that in addition to generating secondary pollutants, often have a high cost. The

purification of biogas by biological processes using microorganisms is an attractive alternative to overcome such limitations. In addition, the use of complex microbial communities for biodegradation enhances the coexistence of several metabolisms promoting co-degradation of different contaminants at the same time, such as hydrogen sulphide, siloxanes and range of volatile organic compounds.

Two criteria were inferred through this dissertation: (1) the biological technologies for treating silicon compounds are viable alternatives as long as the problem of mass transfer is minimized and (2) using support material may reduce the problem of mass transfer and offer better accessibility to the substrate.

In this context, a cycle with a sustainable trend is consolidated. Since the hydrophobic substrate is absorbed by the packing material, the microorganisms consume the available substrate in the support material, resulting even in a natural recovery of the packing material and in the extension of its useful life.

Therefore, the implementation of hybrid technologies that combine absorption technologies and biological technologies is an economically viable alternative (Santos-Clotas, Cabrera-Codony, Castillo, et al., 2019; G. Wang et al., 2019). Previous studies conducted in our research group (Santos-Clotas, 2019) reported interesting results obtained in scenarios of application of hybrid technologies that support this claim. If the biogas experiences a pre-polishing step in a BTF, a prolonged shelf life of the adsorbent can be obtained, which would result in lower costs as a result of coupling of BTF and adsorption than the treatment of the raw biogas (with VOC) only by adsorption.

Another important factor to analyse in future investigations is the high competitiveness of the VOCs towards the porous sites of packing material. A proposal for sequential debugging could be considered as an alternative to solve this problem, including a first purification step for the removal of the most biodegradable VOCS, and a second step for the most recalcitrant compounds such as siloxanes.

Similarly, one of the objectives for future research is to reveal the mechanism underlying microbial adaptation to the change of packaging materials and the roles of different microbial species in the degradation of target pollutants or in the formation of biofilms. As pointed out in this study, it is likely that the elimination of D4 was mainly limited by the biodegradability of the compounds rather than the mass transfer rate.
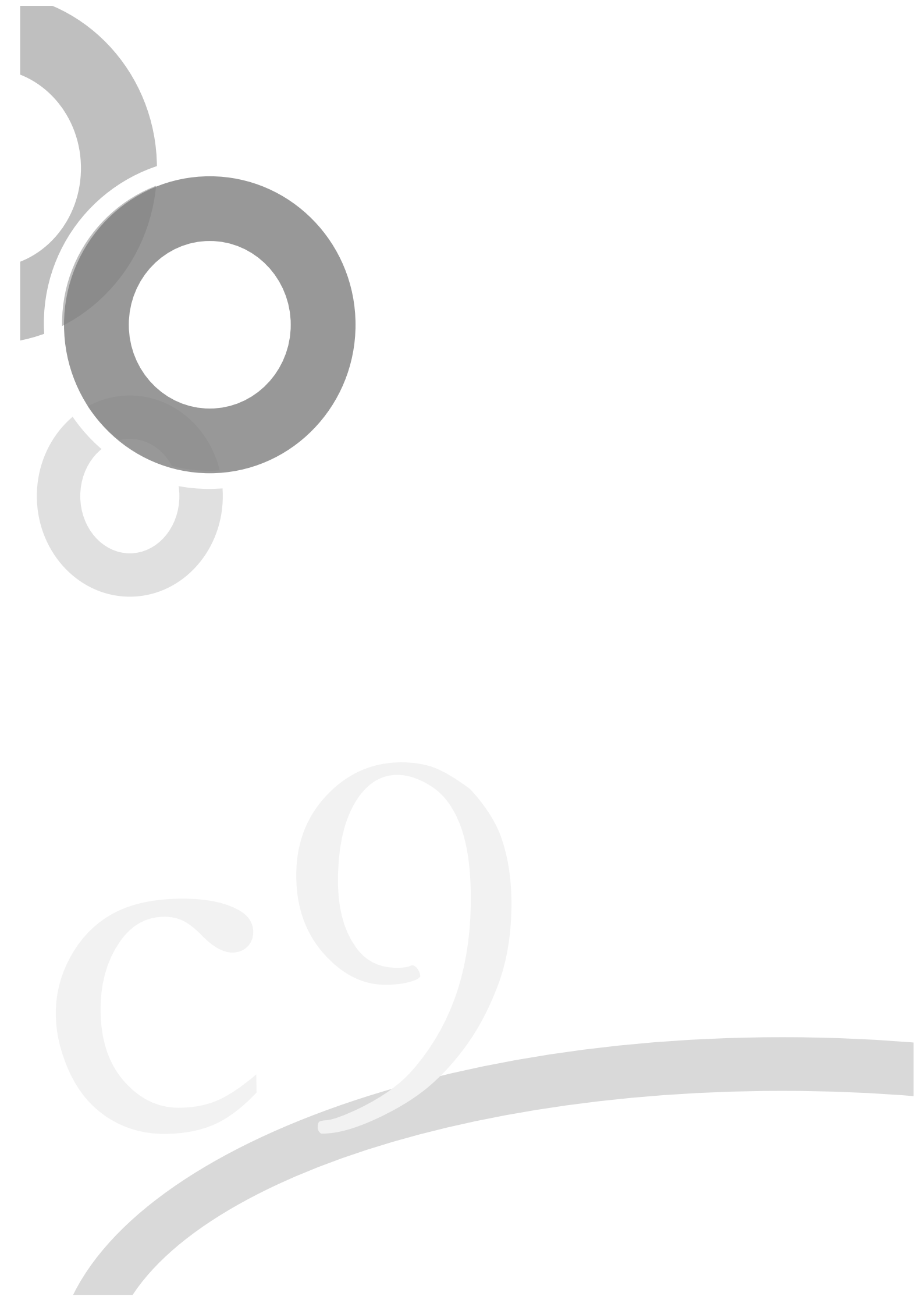
Towards the implementation of
a biotechnology for biogas upgrading:
role of bacteria in siloxane removal

CHAPTER 9

General Conclusions

Several challenges were stated in the body of the present dissertation regarding the problem of siloxane removal from biogas. The biological removal processes are proposed as the "green" solution. This thesis offers an interesting approach to solve the *problem* and to enhance the *"green" solution*. Based on the results obtained in this research we envisage that the present work on siloxane biodegradation would give a significant contribution to an issue which is actually hardly addressed.

A core bacterial community involved in D4 removal process was developed on the surface of activated carbon and zeolite material into liquid enrichments. These materials were key determinants to favour the transfer of substrate (D4), which is hydrophobic *per se,* onto the enrichment aqueous phase. *Rhizobiales* and *Betaproteobacteriales* were the most represent taxonomic groups in the core community, pointing at a high resilience of the bacteria involved in silicon compounds degradation.

Pseudomonas aeruginosa iso07 and *Methylibium* sp. iso58 were the most promising bacteriall strains isolated from the core community. This is the first report on D4 removal for a *Methylibium* sp. iso58, which was able to degrade D4 in anaerobic conditions as the sole carbon and energy source. Removal efficiencies doubled those of iso07. In addition, these species were successful growing in presence of other complex substrates such as toluene, limonene hexane and D5, which are typical components of biogas.

We hypothesize that the complexity of the substrate affects the way bacteria interact with greater cooperation in the presence of recalcitrant substrate. Thus, some members of the bacterial community, included iso58 and iso07, were separately evaluated and studied both independently and in co-culture. The isolates grown in co-culture doubled the D4 removal in the presence of D5, hexane, toluene and limonene (VOCs) suggesting that a combination of selected isolates enhances D4 removal in co-culture.

Transient changes in the microbial community composition and activity due to large operation times of BTF reactors should be further investigated in order to predict and evaluate the occurrence of undesirable activities, such as lower degradation rates in siloxane removal treatment process.

The versatile metabolism of *Methylibium* sp. iso58 contributes interesting highpoints, which could be beneficial to general biogas purification, when the bioreactor has an anoxic performance or higher nitrate concentrations are supplied at the shortest EBRT.

The findings of this study confirmed the potential of using *Methylibium* for the inoculation of biotrickling filters for biogas purification. Taking into account that a subsequent step of adsorption would be necessary to further reduce siloxane concentration, the almost complete biodegradation of VOCs achieved in the BTF drastically increases the lifespan of activated carbon filters.

However, no assays have been performed with real biogas so far. Considering the metabolism of *Methylibium* sp. future assays with real biogas could be successful because the energy capacity of biogas (methane dependent) wont altered by methane degradation while siloxane abatment and VOCs biological removal.
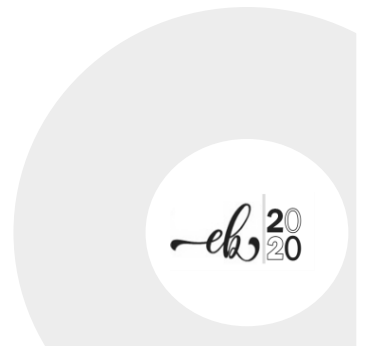
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CHAPTER 10

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