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TESE DE DOUTORADO

Diversidade filogenética da comunidade de procariotos associada à digestão anaeróbica de resíduos orgânicos urbanos e de suínos

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Diversidade filogenética da comunidade de procariotos associada à digestão anaeróbica de resíduos orgânicos urbanos e de suínos

Tese de doutorado apresentado ao curso de Pós-Graduação do Departamento de Biologia Geral da Universidade Federal de Minas Gerais, como requisito à obtenção do título de Doutora em Genética de Micro-organismos.

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ao meu noivo pelo amor incondicional!
Por alegrarem a minha vida! Pela paciência,
dedicação, carinho, amizade e respeito!
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de vocês é especial em minha vida!
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vocês estarem sempre ao meu lado!
Essa vitória é nossa! Amo vocês!
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LISTA DE ABREVIATURAS

acs	Acetil coenzima A sintetase
AD	Anaerobic digestion
AL	Archaeal library
AL	Anaerobic lagoon
ALA	Anaerobic lagoon archaea
ALB	Anaerobic lagoon bacteria
ARDRA	Amplified ribosomal DNA restriction analysis
BL	Bacterial library
BOD	Biochemical oxygen demand
C	Carbono
C	Coverage of library
$CaCO_3$	Carbonato de cálcio
CARD-FISH	Catalyzed reporter deposition - FISH
CO	Monóxido de carbono
°C	Graus Celsius
CH_4	Metano
COD	Chemical oxygen demand
COD/VSS	Food/microorganisms.
CO_2	Dióxido de carbono / Gás carbônico
CoA	Coenzima A
C_q	Quantification cycle
DA	Digestão anaeróbica
DAPI	4′,6′-diaminphenylindol
DA	DGGE archaeal band
DB	DGGE bacterial band
DBO	Demanda bioquímica de oxigênio
DGGE	Denaturing gradient gel electrophoresis
DMSW	Digested MSW

DNA	Ácido desoxirribonucléico
DW	Domestic wastewater
EDTA	Ethylenediaminetetraacetic acid
ETE	Estação de tratamento de esgoto
FISH	Fluorescence in situ hibridization
G	Grams
G+C	Guanina + Citosina
H_2S	Gás sulfídrico
H_2O	Água
$HCOO^-$	Formiato
h	Hour
H_2	Hidrogênio
HRP	Horseradish peroxidase
HRT	Hydraulic retention time
H_4SPT	Enzima tetrahidrometanopterina
Kg	Kilogramas
MSW	Municipal solid waste
m^3	Metros cúbicos
MCR	Metil-coenzima M redutase
N_2	Nitrogênio
NCBI	National Center for Biotechnology Information
NH_3	Ammonia
NH_4^+	Ammonium ion
OF	Organic fraction
O_2	Oxigênio
OD	Oxygen demand
OLR	Organic loading rate
OTUs	Operational taxonomic units
PAST	Paleontological Statistics Software Package

PBS	Phosphate-buffered saline solution
PHA	Polihidroxialcanoato
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
RDP	Ribosomal Database Project
rpm	Rotation per minute
rRNA	RNA ribossômico
RSU	Resíduos sólidos urbanos
S	Supplementary
SAL	Swine-waste-sludge anaerobic lagoon
SD	Sludge sampled from dry season
SM	Supplementary material
SR	Sludge sampled from rainy season
Ton	Tonelada
TSS	Total suspended solids
TVS	Total volatile suspended solids
UASB	Upflow anaerobic sludge bed
VFAs	Volatile fatty acids
δ	Delta
α	Alpha
γ	Gamma
ε	Epsilon
β	Beta
µL	Microlitros
mL	Mililitros
L	Litro

SUMÁRIO

RESUMO

A geração de resíduos sólidos urbanos (RSU) e líquidos é um fenômeno inerente ao cotidiano da sociedade moderna, sendo sua resolução um sério problema: a utilização da digestão anaeróbica (DA) é uma estratégia promissora. Conhecer a diversidade de procariotos, sua dinâmica e sua quantificação é essencial para a otimização deste processo. Neste estudo, usou-se uma combinação de abordagens moleculares para análises filogenéticas e quantitativas das comunidades de procariotos em: reator anaeróbico em escala industrial, em tratamento de RSU (Madri-Espanha), no *start-up* e em condições estáveis; reator em escala piloto, em tratamento de águas residuárias domiciliares (Belo Horizonte), nos períodos de chuva e seca; e lagoa anaeróbica, com resíduo suíno de um frigorífico. Mudanças nas comunidades de procariotos foram observadas como consequência das alterações operacionais e ambientais nestes sistemas. *Bacteroidetes* e *Firmicutes* (*Clostridia)* predominaram, sendo que no reator UASB, na estação de chuva, houve predominância de *Firmicutes*. A classe *Bacilli* foi também identificada no *start-up* do reator em tratamento de RSU. *Proteobacteria*, oxidadores de carboidratos, foram também detectadas no reator UASB, com a dominância de δ-*proteobacteri*a dentre as cinco classes identificadas. Na lagoa anaeróbica, δ- e β- *proteobacteria* foram encontradas. No reator RSU, apenas γ-*proteobacteria* foi identificada no *start-up*. *Chloroflexi*, comum em reatores em tratamento de esgoto sanitário e de RSU, foi identificado apenas na lagoa anaeróbica. Alguns filos foram específicos, ou mesmo comuns a certos sistemas, abrangendo principalmente bactérias hidrolíticas e acidogênicas. Durante a operação do reator em tratamento de RSU mudanças na composição dos procariotos, aumento no número de células e aumento na concentração de ácidos graxos voláteis ocorreu em consequência do carregamento do reator. Em contraste, no reator UASB, na estação de chuva, a diluição do esgoto levou à diminuição do número de células bacterianas. Entretanto, houve aumento da diversidade de espécies deste grupo, o qual foi mais abundante que as arqueias em todos os sistemas anaeróbicos. No reator em tratamento de RSU e na lagoa anaeróbica houve predominância de arqueias hidrogenotróficas, em associação com as bactérias degradadoras de ácidos graxos voláteis, na produção de metano. Em contraste, no reator UASB a produção do metano foi realizada pelas arqueias aceticlásticas e metilotróficas e uma pequena fração pelas hidrogenotróficas. *Crenarchaeota* predominou na lagoa anaeróbica e no reator em tratamento de RSU. Como observado nos três sistemas anaeróbicos de tratamento dos resíduos, aspectos microbiológicos, físico-químicos, operacionais ou sazonais estão diretamente relacionados com a composição e dinâmica das comunidades de procariotos e seu metabolismo na degradação da matéria orgânica e produção do metano.

ABSTRACT

Cities generation of municipal solid waste (MSW) and wastewater is a society inherent phenomenon. Addressing it is essential and anaerobic digestion (AD) is a promising strategy. The knowledge of prokaryotes diversity, dynamics and quantification is essential in order to optimize this process. In this study, we used a combination of molecular approaches to phylogenetically and quantitatively analyze the prokaryotes communities from: a full-scale anaerobic reactor treating MSW (Madrid-Spain) during start-up and in steady condition; a pilot-scale reactor treating domestic wastewater (Belo Horizonte) during the dry and rainy seasons; and an anaerobic pond with pig waste in a refrigerator. Shifts in the prokaryotes communities were observed as a result of operational and environmental changes in these systems. *Bacteroidetes* and *Firmicutes* (mainly *Clostridia*) predominated. In the UASB reactor during the rainy season, *Bacteroidetes* predominated over *Firmicutes, Bacilli* class organisms were observed and also identified during start-up reactor treating MSW. *Proteobacteria*, carbohydrates oxidizers, also prevailed in the UASB reactor, with δ-*proteobacteria* predominating among the five identified classes. In addition, this group was identified in conjunction with β-*proteobacteria* in the anaerobic pond. In the MSW reactor, only γ-*proteobacteria* was identified during start-up. *Chloroflexi*, common in wastewater and MSW reactors, was identified only in the anaerobic pond. Some phyla, mainly consisting of hydrolytic and acidogenic bacteria, were specific or more common to certain systems. During MSW reactor operation substitutions in the prokaryotes composition, increase in cell numbers and in the volatile fatty acids (VFAs) concentration occurred as a result of organic loading of the reactor. In contrast, in the UASB reactor during the rainy season, sewage dilution resulted in a decrease of bacterial cells, but their speciation increased the diversity of this group, which was the more abundant than archaea in all the systems. In the MSW reactor and anaerobic pond, the production of methane was dominated by hydrogenotrophic archaea and its relationship with the VFAs degrading bacteria. In contrast, in the UASB reactor the methane was produced by the aceticlastic and methylotrophic archaea and a minor fraction by hydrogenotrophic archaea. *Crenarchaeota* predominated in the anaerobic pond and was identified in the MSW reactor during steady conditions. As noted in the three waste treatment systems, the physical-chemical, environmental or operational parameters are directly related to the composition and dynamics of the prokaryotes communities and their metabolism in the degradation of organic matter and methane production.

ESTRUTURA DA TESE

Esta tese apresenta a seguinte organização: justificativa do trabalho, uma introdução geral com uma breve revisão bibliográfica, abordando os principais temas propostos no trabalho, e os objetivos gerais e específicos. A seguir, os artigos aceitos e submetidos em revistas nas formas de capítulos, com seu materiais suplementares: I “Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor from start-up to steady-state conditions”, II “Bacterial and archaeal phylogenetic diversity associated with swine sludge from an anaerobic treatment lagoon” e III “Organic loading rate and food-to-microorganism ratio shape prokaryotic diversity in a demo-scale up-flow anaerobic sludge blanket reactor treating domestic wastewater”. Finalmente, discussão geral, conclusão geral, referências bibliográficas e os anexos. Em anexo estão os artigos científicos publicados, projetos aprovados, e um breve histórico das atividades realizadas pela doutoranda durante o período do doutorado.

JUSTIFICATIVA

O conceito de sustentabilidade associado ao tratamento de águas e resíduos, a valorização energética, a prevenção de poluição e a reciclagem destes resíduos, permite constituir uma sociedade mais auto-suficiente (Lettinga, 2001).

Com o crescimento populacional, grandes quantidades de resíduos são produzidas diariamente nas zonas urbanas e rurais, sendo o seu tratamento uma preocupação comum à humanidade. A utilização da digestão anaeróbica para a degradação dos resíduos sólidos urbanos e industriais e das águas residuárias permite além do seu tratamento, a reutilização dos produtos gerados como: o metano, que poderá ser comercializado como crédito de carbono ou queimado para a geração de energia elétrica e motora, pelas próprias plantas de tratamento, e o subproduto final que poderá ser utilizado como adubo orgânico. Isso torna a digestão anaeróbica um tratamento eficiente, auto-sustentável e que previne a acumulação destes resíduos no meio ambiente, evitando a poluição de águas, do solo e do ar. Os principais responsáveis pela digestão anaeróbica são os micro-organismos presentes nestes sistemas e nos resíduos a serem tratados. Bactérias hidrolíticas e acetogênicas são as grandes responsáveis pela degradação da matéria orgânica e as arqueias pela produção do metano. Conhecer a diversidade filogenética destes micro-organismos, a dinâmica de suas comunidades - consequência dos fatores ambientais impostos a estas -, e sua ocorrência nos reatores ou lagoas anaeróbicas, é essencial para entender a eficiência da digestão anaeróbica, como esta pode ser otimizada e como se deve evitar e controlar problemas durante o tratamento. Por isso, neste trabalho várias técnicas moleculares baseadas no gene de rRNA 16S foram associadas a fim de identificar e quantificar as comunidades de procariotos envolvidas na digestão anaeróbica de resíduos sólidos urbanos (RSU), esgoto sanitário e resíduos suíno, com objetivo de entender como a degradação destes resíduos está ocorrendo e comparar as diferentes comunidades envolvidas na degradação de cada tipo de resíduo.

1 – INTRODUÇÃO

1.1 - Resíduos sólidos urbanos

O crescimento populacional, aliado ao intenso desenvolvimento industrial e à crescente urbanização nas últimas décadas, foi acompanhado pela produção de enormes quantidades de resíduos sólidos urbanos (RSU), falta de áreas disponíveis para a sua deposição e pela emissão de grandes quantidades de biogases poluentes. Atualmente, as grandes variações climáticas, o aquecimento global e o tratamento do lixo domiciliar urbano estão entre os principais problemas enfrentados pela sociedade contemporânea.

O processo de geração de RSU é um fenômeno inerente ao cotidiano de qualquer comunidade. A exploração e a transformação dos recursos naturais, a circulação e a aquisição dos bens de consumo modificam a natureza e geram subprodutos ou resíduos. Além da imensa capacidade de crescimento da população humana, a cada dia o homem amplia seus conhecimentos, inventando e descobrindo novos produtos, adicionando novos valores e criando novas necessidades, promovendo o aumento excessivo da exploração e transformação dos recursos naturais e, conseqüentemente, gerando maiores quantidades de RSU (Mucelin e Bellini 2007).

A quantidade de RSU produzida por uma comunidade depende das características sócioeconômicas. Em uma região urbanizada, moderna e economicamente desenvolvida, a quantidade de resíduo sólido produzido será maior que uma região rural. Da mesma forma, o tipo de resíduo produzido poderá variar, como por exemplo, regiões mais modernas produzirão resíduos com proporções maiores de materiais recicláveis como plásticos, papel, e menos matéria orgânica a ser degradada que regiões pobres ou em desenvolvimento (Tabela 1), uma vez que estas consomem tipos de produtos diferentes (OECD, 2007).

No Brasil, o RSU é composto por cerca de 60-65% de matéria orgânica (Tabela 1). Segundo dados do “Panorama dos Resíduos Sólidos Urbanos” (ABRELPE, 2009) são gerados em média150 mil toneladas de RSU por dia, sendo de 0,6 Kg a quantidade média produzida por pessoa. Esta quantidade varia de 0,4 Kg por habitante na região nordeste a 1,1 Kg por habitante na região sudeste. Na Espanha, país modelo no tratamento de RSU, de acordo com o Plano Nacional de Resíduos (2000-2007) são geradas 26,2 milhões de toneladas de RSU por ano, com uma média de 1,7 kg por habitante por dia, sendo que 40-50% constituídos de matéria orgânica (INE, 2009) (Tabela 1).

Tabela 1. Composição dos resíduos sólidos urbanos (RSU) no Brasil e na Espanha.

Compostos (%)	Brasil[a]	Espanha[b]
Matéria orgânica	65	44
Vidro	3	7
Plástico	3	11
Papel	25	17
Metal	4	4
Outros	-	17

Fonte: [a]Jardim e Wells (1995) e [b]INE (2009)

De todo o lixo produzido no Brasil, que varia de acordo com o número de habitantes por região, aproximadamente 20% não é coletado regularmente, e os 80% coletados são dispostos em lixões ou destinados a aterros controlados e aterros sanitários (Figura 1). Os aterros sanitários são classificados como a única forma de destinação adequada para os resíduos no Brasil. Aterros controlados e lixões são considerados como inadequados, pois não garantem a proteção dos aspectos ambientais envolvidos em sua operação (ABRELPE 2009).

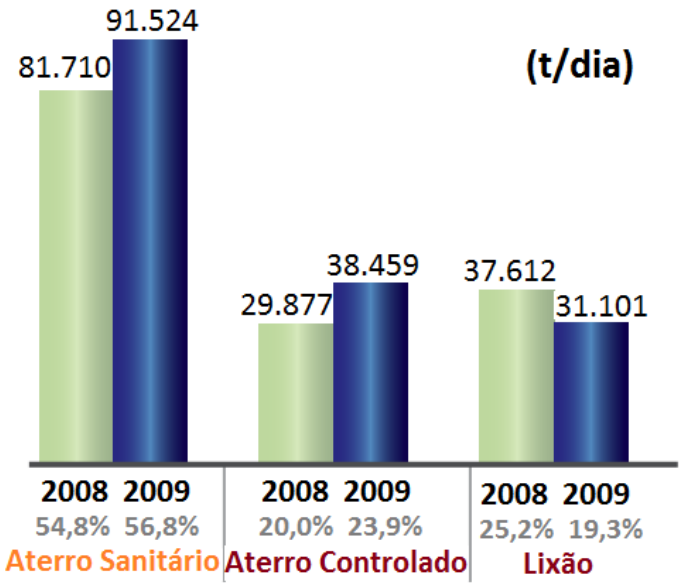

Figura 1. Destinação final dos RSU coletados no Brasil em 2008 e 2009.

Fonte: ABRELPE, 2009.

Do total de RSU depositado em lixões, logradouros públicos, canais, margens de rios ou outro qualquer agente receptor, 65% em média correspondem à matéria orgânica

putrescível, como, por exemplo, cascas de frutas, leguminosas, ossos, restos de comida e gramíneas. Nos lixões essa matéria orgânica sofre por processos aeróbicos e anaeróbicos efetuados pelos micro-organismos encontrados no ambiente e no próprio lixo, gerando chorume (líquido lixiviado), com elevada demanda bioquímica de oxigênio (DBO), ácidos graxos voláteis e, em alguns casos, significativa concentração de metais pesados. Os lixões não dispõem de infraestrutura básica resultando em impactos ao ar, solo e lençóis freáticos, permitindo assim a proliferação de macro e micro vetores de micro-organismos patogênicos, com consequências sociais e na saúde pública.

A utilização dos aterros para a destinação dos resíduos ainda continua sendo a técnica mais praticada no gerenciamento de resíduos sólidos urbanos no mundo. Por ser uma alternativa mais barata de disposição de resíduos é a mais utilizada no Brasil, na América Latina e em diversos países. Porém, nos países menos desenvolvidos, os aterros se apresentam predominantemente na forma de lixões ou de aterros controlados. A maior preocupação na utilização dos aterros sanitários para o destino dos RSUs são os passivos ambientais gerados durante as operações, que ainda perduram por muitos anos após a sua desativação, inviabilizando a utilização de grandes áreas por longos períodos (ABRELPE, 2009). Além disso, apesar de possuírem sistemas de drenagem de gases, muitas vezes, devido à falta de manutenção após o esgotamento destes aterros, gases como o dióxido de carbono e metano, podem ser emanados no ambiente e serem causadores do aquecimento global (IPT/ CEMPRE, 2000).

A incineração dos RSU é outra opção para o seu tratamento. Entretanto, esta técnica também apresenta diversos aspectos limitantes ao seu uso. As variações de umidade e da quantidade dos materiais inorgânicos no RSU são os aspectos principais limitantes para o uso da incineração. Além disso, a resistência existente quanto ao uso dos incineradores para destinar os RSU deve-se ao fato do processo de incineração produzir substâncias altamente tóxicas e de alto potencial cancerígeno para o homem, tais como as dioxinas e os furanos, que deverão ser posteriormente eliminados em sistemas de filtros adequados (TANGRI, 2003).

Alternativas para o tratamento da matéria orgânica do lixo são cada vez mais atraentes e o processo de digestão anaeróbica dos RSU em reatores anaeróbicos, usado com sucesso na Espanha, parece ser uma boa estratégia. Esta é uma tecnologia que além de tratar o RSU, oferece proteção ao meio ambiente e a possibilidade de recuperação e utilização do gás metano como combustível, despertando por isso mais interesse (Chynoweth et al. 2001; Foresti, 1997).

1.2 - Águas residuárias urbanas (esgotos sanitários)

Todas as cidades produzem suas águas residuárias, também denominadas de esgoto urbano, com características similares e alto poder de poluição. Dentre os maiores desafios da gestão de recursos hídricos no Brasil está a redução das cargas poluidoras nos corpos d'água, principalmente em regiões metropolitanas.

No Brasil, são despejados diariamente nos córregos e rios cerca de 10 bilhões de m^3 de esgoto, (http://ambientes.ambientebrasil.com.br/saneamento/tratamento_de_efluentes/tratamento_de_efluente.html), sendo que apenas 29,4% recebem algum tipo de tratamento e disposição final adequada (IBGE 2008). Os efluentes domésticos representam uma das principais fontes poluidoras dos ecossistemas aquáticos do território nacional e a falta de saneamento básico tem acarretado vários problemas de proliferação de doenças de veiculação hídrica à população, gerados através dos esgotos que correm a céu aberto e também pela poluição dos mananciais.

Os esgotos domésticos contêm aproximadamente 99,9% de água, a fração restante inclui matéria orgânica e inorgânica, suspensas e dissolvidas, bem como microrganismos. Portanto, é devido a essa fração de 0,1% que há necessidade de se tratar os esgotos, e os parâmetros físicos, químicos e biológicos é que irão definir a qualidade deste (Von Sperling, 1996). Segundo Von Sperling (1996) para que a água possa ser utilizada para abastecimento doméstico deve se obedecer aos seguintes requisitos de qualidade: ser isenta de substâncias químicas prejudiciais à saúde; adequada para serviços domésticos; esteticamente agradável (baixa turbidez, cor, sabor e odor) e com ausência de microrganismos. A resolução do Conselho Nacional do Meio Ambiente – CONAMA Nº 357 de 2005 apresenta padrões de qualidade dos corpos receptores, como também do lançamento de efluentes a fim de garantir a qualidade dos mananciais para a população. O tratamento de esgotos em estações de tratamento de esgotos (ETE) é fundamental para qualquer programa de despoluição das águas.

1.3 - Aguás residuárias de suínos

O consumo de carne de porco vem crescendo a cada dia no Brasil e no mundo. O Brasil consumiu 2,5 bilhões de toneladas de carne de porco em 2010 e está no quinto lugar entre os outros países (USDA / Abipecs - http://www.abipecs.org.br/pt/estatisticas/mundial/consumo-2.html). Em regiões com alta

concentração de suínos, grande parte dos dejetos (excreta e urina) é lançada no solo e em cursos de água sem tratamento prévio, poluindo o ambiente e contribuindo para a proliferação de insetos ou outros vetores de doenças. A maioria dos suinocultores utiliza sistemas de produção que propiciam elevada produção de dejetos e que necessitam de posterior tratamento. Os porcos abrigam vários patógenos humanos, como por exemplo, *E. coli*, *Salmonella*, *Campylobacter* e *Cryptosporidium* que podem produzir gastrenterites no homem causadas principalmente pelo consumo da carne contaminada (Venglovsky et al. 2006). Entretanto, nos últimos anos devido ao grande aumento da prática de criação de suínos e a falta de tratamento do dejeto produzido, problemas de saúde vêm sendo causados, principalmente, pelo consumo da água e produtos orgânicos contaminados (Guan and Holley 2003; Franz and Van Bruggen 2008).

A quantidade de dejetos produzida na criação de porcos, a concentração de substâncias poluidoras e de nutrientes nos efluentes são os aspectos básicos para se definir a melhor forma de tratamento e reutilização dos mesmos. Adicionados a estes estão também os resíduos produzidos nos frigoríficos onde os animais são abatidos. A quantidade de dejetos produzida é grande e são compostos não apenas das excretas e urina, mas também dos restos de sangue e carne não utilizados para o consumo humano e que também necessitam de posterior tratamento antes de serem descartados.

O sistema de tratamento recomendado para o tratamento do efluente suíno é a combinação de decantadores para separação das frações sólida e líquida e, posteriormente, o tratamento da fração sólida orgânica em lagoas de estabilização anaeróbicas, facultativas e aguapés (UN Division of Sustainable Development 2000; US EPA 2003; Han et al. 2007). A sua principal função é reduzir a carga orgânica do efluente e, em alguns casos, a remoção de nutrientes, especialmente nitrogênio, fósforo e potássio que podem ser reutilizados para a adubação orgânica do solo (EMBRAPA Suínos e Aves 1997). O elevado pH das lagoas também favorece a redução do número de bactérias patogênicas, a precipitação dos fosfatos de cálcio e a volatilização da amônia (Uehara e Vidal, 1989).

1.4 - Aplicações da digestão anaeróbica

Entre os processos de tratamento de RSU e de águas residuárias, a digestão anaeróbica é a mais adaptada para a degradação de resíduos com alta concentração de carbono orgânico (Leitão et al. 2004). A geração de energia, devido à elevada produção do biogás, faz da digestão anaeróbica uma alternativa vantajosa para o tratamento de resíduos

orgânicos,diferentemente do processo aeróbico que consome energia, levando a cada ano ao desenvolvimento de biodigestores mais modernos (Ahring et al. 1992, 1995; Alves e Oliveira, 2004).

A digestão anaeróbica apresenta várias vantagens: proteção ao meio ambiente – uma vez que o processo de digestão anaeróbica previne o acúmulo de material no ambiente e, consequentemente, a disseminação de doenças para a população–; produção de pouco lodo; permite a recuperação e reutilização do gás metano a partir de sua combustão ou para ser comercializado como crédito de carbono entre os países, devido ao seu alto valor agregado – impedindo a sua liberação ao ambiente e o agravamento do aquecimento global (Wuebbles and Hayhoe, 2002; Leitão et al. 2004); e reutilização do material tratado como adubo orgânico na agricultura e na recuperação de áreas degradadas (Taf Drup e Hjort-Gregersen, 1999).

A aplicação da digestão anaeróbica para o tratamento de águas residuáriasdomésticas iniciou-se em 1880 por Béchamp, com reator semelhante a um tanque séptico (Oremle, 1988; McCarty, 2001). Posteriormente, os digestores anaeróbicos foram utilizados para o tratamento de resíduos industriais, sendo implantados inicialmente na Dinamarca, em 1929 (Henze, 1997). A utilização do processo anaeróbico foi então intensificada a partir da década de 60 e 70, estabelecendo-se em larga escala no tratamento de uma variedade de efluentes e resíduos (McCarty, 2001) ocorrendo também no Brasil, principalmente entre os suinocultores. Programas oficiais estimularam a implantação de muitos digestores focados principalmente na geração de energia, na produção de biofertilizantes e diminuição do impacto ambiental, mas, infelizmente, no Brasil, os resultados não foram os esperados e a maioria dos sistemas implantados foi desativada. Na década de 80, ocorreu então a introdução de sistemas de alta carga orgânica, como o sistema de leito de lamas com fluxo ascendente (upflow anaerobic sludge bed, UASB), desenvolvido na Holanda pelo grupo de Gatze Lettinga (Lettinga et al. 1980). Na década de 90, os sistemas de digestão anaeróbica foram reimplantados no Brasil com a finalidade de estabilizar a fração sólida de esgotos sanitários e de resíduos agrícolas (Peres et al. 1991; Kunz et al. 2004;).

Em países como Dinamarca, Bélgica, Alemanha e Espanha a utilização de biodigestores anaeróbicos para o tratamento de RSU é crescente (Alves e Oliveira, 2004). De acordo com a Agência Internacional de Energia (IEA, 2003), existem 400 plantas de tratamento de resíduos industriais (260) e de tratamento anaeróbico de RSU (140) espalhadas pelo mundo. Destas, 130 estão distribuídas pela Europa (93%) e Ásia (7%) (Biogas Works, 2005).

A difusão da utilização de biodigestores anaeróbicos na Espanha já é uma realidade, sendo hoje considerado o país com maior capacidade e potencial de tratamento de RSU da

Europa. Trinta a cinquenta por cento das plantas de tratamento utilizam esta tecnologia, sendo que, destas, 19 plantas de biometanização, em escala industrial, são responsáveis pela digestão anaeróbica de 1.100.000 toneladas de RSU por ano (De BAERE, 2005).

Atualmente, 24 plantas de tratamento de RSU estão em operação na Espanha. Nas proximidades de Madri, sete plantas de biometanização estão em funcionamento, duas recentemente inauguradas no Parque Tecnológico de Valdemingómez, Las Dehesas (Figura 2) e Paloma, sob responsabilidade da prefeitura de Madri. Elas são consideradas as maiores e mais modernas da Europa e de referência internacional no tratamento de resíduos. Estima-se que as duas plantas serão responsáveis pelo tratamento de 370.000 toneladas de RSU e pela produção de 34 milhões de m^3 de biogás por ano, o que diminuirá o uso de energias não renováveis, uma vez que o biogás produzido é equivalente a quase 17.000 toneladas de petróleo (http://www.madridiario.es/2008/Noviembre/medioambiente/gasnatural/118868/plantas-biometanizacion-valdemingomez-empiezan-rodaje.htm). A energia produzida será reutilizada como combustível para uma frota de 250 ônibus de transportes coletivos que circularão em Madri e também como energia elétrica. Além disso, as duas plantas serão responsáveis pela redução da emissão de biogases do efeito estufa para a atmosfera, pois 300.000 toneladas de dióxido de carbono deixarão de ser liberados anualmente, melhorando ainda mais a qualidade do ar na região da capital espanhola (http://www.paginadigital.com.ar/articulos/2007/2007prim/tecnologia2/plantas-biometanizacion-miercoles-070307.asp).

Figura 2. Planta de biometanização Las Dehesas – Madri

Foto: J.Cardinali-Rezende

Em relação ao tratamento do esgoto sanitário no Brasil, nos últimos 20 anos várias cidades adotaram a digestão anaeróbica para o tratamento deste resíduo em digestores anaeróbicos nas Estações de Tratamento de Esgoto (ETEs): Mangueira (Recife - PE) tratando esgoto produzido por 18.000 habitantes; Ribeirão da Onça, 1,5 milhão de hab. (Figura 3), e ETE Arrudas (Belo Horizonte - MG); Piçarrão (Campinas - SP), 200.000 hab.; e Atuba Sul (Curitiba - PR), 370.000 hab. (Jordão et al. 2007).

Figura 3. Estação de tratamento de esgoto Ribeirão da Onça – Belo Horizonte.
Fonte: J.Cardinali-Rezende

A utilização de reatores UASB para o tratamento do esgoto vem aumentando principalmente devido à sua capacidade de receber elevadas taxas de carregamento orgânico, área de implantação necessária reduzida, economia de energia elétrica (Pierre and Doria, 1995). Apesar dos esgotos sanitários brutos serem compostos por apenas 0,1% de sólidos, a produção de lodo nas ETEs é muito grande, principalmente quando o tratamento utilizado é aeróbico, produzindo mais que o tratamento anaeróbico (Campos, 1994). Por isso, o uso dos processos de tratamento de esgotos com menor produção de lodo deve ser estimulado.

Os reatores anaeróbicos UASB são os mais utilizados para o tratamento de esgoto sanitário no Brasil. A concentração de biomassa nesses reatores é elevada, formando a manta de lodo composta de micro-organismos que irão degradar a matéria orgânica do esgoto. Como resultado da atividade anaeróbica, gases serão formados (principalmente metano e gás carbônico) e suas bolhas serão produzidas com uma tendência ascendente no sistema, justificando o nome sistema de leito de lamas com fluxo ascendente (Von Sperling, 1996).

Outro sistema de tratamento utilizando a digestão anaeróbica no Brasil é a Lagoa Anaeróbica de Estabilização, que foi descoberta, acidentalmente, em 1901 após a construção de uma lagoa de armazenamento de esgotos em San Antônio, Texas, EUA (lago Mitchel) verificando-se que os efluentes possuíam melhor qualidade que os afluentes (Silva e Mara, 1979). Em 1924, na cidade de Santa Rosa, Califórnia, para evitar o custo de uma estação de tratamento de esgotos, lagoas anaeróbicas foram implantadas (Kellner e Pires, 1998). A primeira instalação na América do Norte, projetada para o tratamento de esgotos, foi em Maddok, norte de Dakota, em 1948. Nesta mesma época eram realizadas pesquisas sobre a depuração dos esgotos nas lagoas de estabilização na Austrália, conhecidas como “Lagoas Australianas”. No Brasil, a primeira lagoa anaeróbica foi construída em São José dos Campos - SP em 1960. Desde então, muitas lagoas para o tratamento de esgotos sanitários e industriais têm sido construídas no território nacional.

As lagoas anaeróbicas são consideradas como uma das técnicas mais simples de tratamento de esgotos e resíduos líquidos, dependendo unicamente da área disponível, da topografia do terreno e do grau de eficiência desejado (Von Sperling 1996), sendo por isso muito utilizada em fazendas para o tratamento de efluentes de animais (Costa et al. 2000). O tratamento completo é constituído unicamente por processos naturais de degradação da matéria orgânica realizados pelos micro-organismos, através de três fases: anaeróbica, aeróbica e facultativa. O volume de lodo produzido é pequeno, e este se acumula no fundo da lagoa por sedimentação (Figura 4). No descarte do lodo, este se encontra praticamente estabilizado, sendo a eficiência de remoção da demanda bioquímica de oxigênio (DBO) nas lagoas anaeróbias de 50% a 60% (Von Sperling, 1996). As lagoas anaeróbicas são projetadas para receber cargas orgânicas elevadas impedindo a existência de oxigênio no meio líquido. O sucesso do tratamento nessas unidades depende do equilíbrio entre as duas populações: a formadora de ácidos orgânicos (bactérias acidogênicas) e a formadora de metano (arquéias metanogênicas) (König: In Mendonça, 1990).

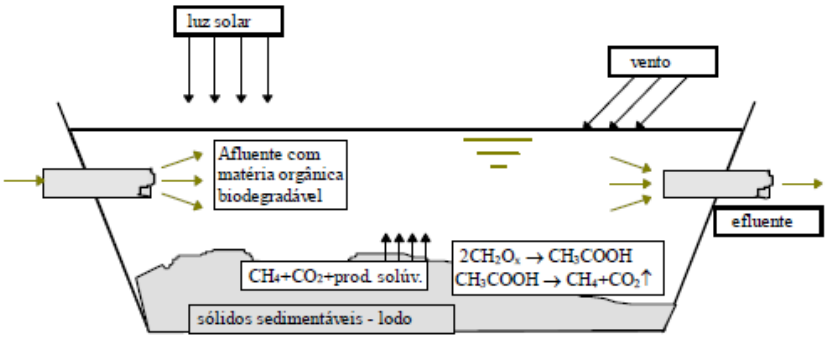

Figura 4. Funcionamento de uma lagoa de estabilização anaeróbica
Fonte: König: In Mendonça, 1990

1.5 – Micro-organismos da digestão anaeróbica

A digestão anaeróbica é um processo complexo e envolve a interação de diversos grupos de micro-organismos que trabalham em conjunto na conversão da matéria orgânica e na oxidação de compostos complexos em metano, gás carbônico, água, gás sulfídrico, amônia e outros produtos (Loreau et al. 2001; Curtis e Sloan, 2004; Bell et al. 2005). É um processo no qual ocorrem trocas recíprocas de substratos entre diferentes grupos bacterianos e de arqueias presentes (Alves e Oliveira, 2004). A Figura 5 ilustra as etapas da degradação da matéria orgânica para a produção de metano.

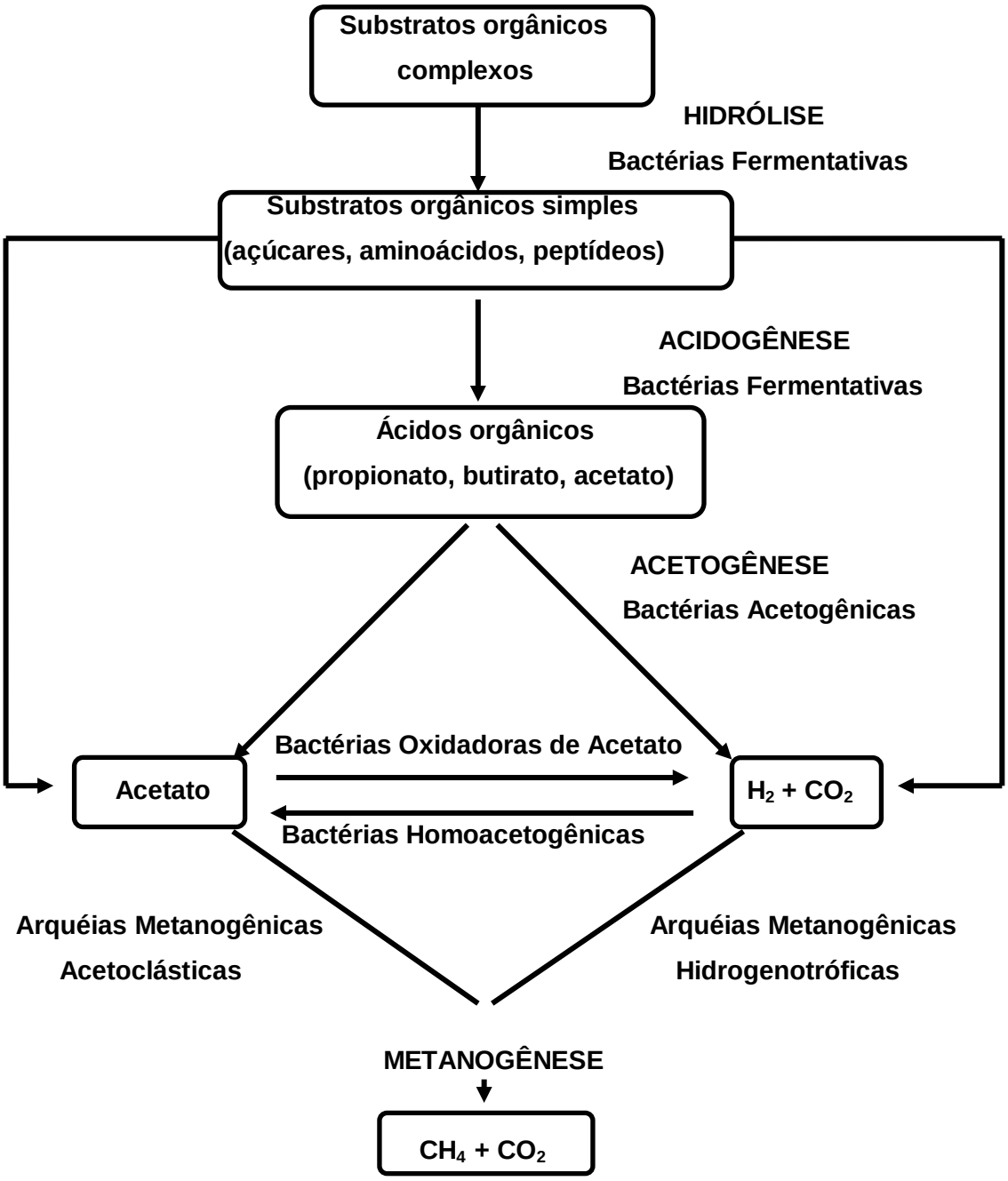

Figura 5. Diagrama do fluxo de carbono da produção do biogás na digestão anaeróbia.
Fonte: Angelidaki et al. 2002, modificado.

Os procariotos que participam do processo de decomposição anaeróbica são distribuídos em três importantes grupos, com capacidades fisiológicas distintas: (1) bactérias fermentativas, que transformam por hidrólise os polímeros em monômeros, e estes, em acetato,

hidrogênio, CO_2, ácidos orgânicos de cadeia curta, aminoácidos e açúcares; (2) bactérias acetogênicas**,** que convertem os produtos gerados pelas bactérias fermentativas em acetato, hidrogênio e CO_2 e (3) arquéias metanogênicas, que utilizam os substratos produzidos pelas bactérias acetogênicas para a produção de CH_4 e CO_2. Durante a digestão anaeróbica, compostos de nitrogênio orgânicos são convertidos em amônia, enxofre em gás sulfídrico, fósforo em ortofosfatos e cálcio, magnésio e sódio em uma variedade de sais (Ferry, 1993; Speece, 1996; Foresti, 1997).

1.6 - A metanogênese e o biogás

A metanogênese, última etapa no processo de degradação da matéria orgânica para a produção de metano, é comum em muitos ambientes anaeróbicos tais como biodigestores (Raskin et al. 1994), ruminantes (Miller et al.1986), arrozais (Joulian et al. 1998), poços de petróleo (Olliver et al. 1997), aterros (Fielding e Archer, 1988) e uma série de ambientes extremos (Garcia et al. 2000).

Estritamente anaeróbicas, as arquéias metanogênicas são responsáveis por toda a produção biológica de metano, estimada em $5x10^8$ ton/ano. Este processo metanogênico é fundamental para o ciclo do carbono (Rogers e Whitman, 1991). O biogás produzido é incolor, insolúvel e de baixa densidade, sendo constituído por 70% de CH_4, 29% de CO_2 e uma pequena fração de H_2S e outros gases (Alves e Oliveira, 2004).

O metano obtido através do processo de tratamento da matéria orgânica dos resíduos domésticos, industriais e da agricultura vem sendo utilizado como fonte de energia alternativa (Wuebbles e Hayhoe, 2002), produzindo energia térmica através da sua combustão ou na geração de energia elétrica. Geralmente, este é utilizado pela própria planta de tratamento, sendo o seu excedente comercializado. A média de produção de biogás é de 100 a 150 m^3 por tonelada de resíduo tratado pela digestão anaeróbica (Chynoweth et al. 2001).

Além de ser uma fonte alternativa de energia, o biogás possui menos poluentes do que os combustíveis fósseis utilizados pelo homem – gasolina, diesel, gás natural e carvão mineral. Entretanto, um dos seus constituintes, o CH_4, é considerado 20 vezes mais prejudicial do que o CO_2, poluindo o meio ambiente e, consequentemente, agravando o efeito estufa (Tilche e Malaspina, 1998). Portanto, o metano pode ser reaproveitado e utilizado como fonte de energia ou comercializado através de “Reduções Certificadas de Emissões” ou “Créditos de Carbono”. A reutilização do metano tem como objetivo diminuir a sua emissão ao meio ambiente (Yadvika

et al. 2004) e impulsionar a criação de tecnologias que diminuam sua geração ou “sequestrem” estes gases em países em desenvolvimento, o que seria de grande interesse a sociedade.

1.7 - As arqueias metanogênicas e a metanogênese

As arquéias metanogênicas (Figura 6) são membros do domínio Archaea, estritamente anaeróbicas, caracterizadas pela produção de CH_4 e energia para o seu crescimento. Pela análise filogenética do gene de rRNA 16S as arqueias metanogênicas são classificadas em cinco ordens: *Methanobacteriales*, *Methanopirales*, *Methanococcales*, *Methanomicrobiales* e *Methanosarcinales* (Boone et al. 1993).

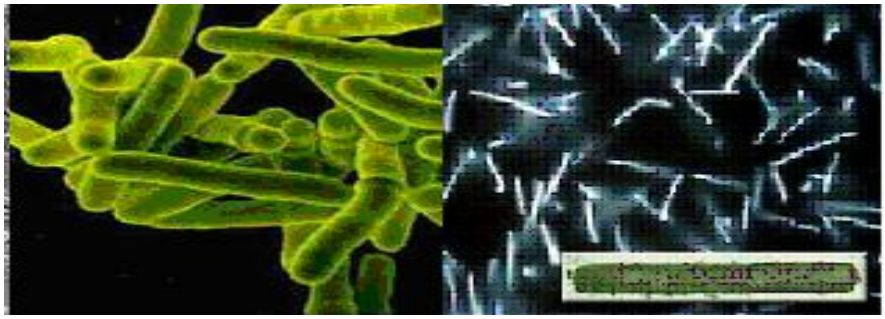

Figura 6. Arquéias metanogênicas.
Fonte: Modificado de Ferry, 1993

O mecanismo bioquímico da metanogênese é bem estudado e o processo depende da participação de uma série de co-enzimas e aceptores de elétrons. Do total de metano produzido pelas arqueias metanogênicas no tratamento de águas residuárias domésticas e industriais, dois terços são derivados da degradação do acetato, enquanto que um terço ocorre a partir da degradação do CO_2, utilizando elétrons derivados da oxidação do H_2 e do formiato (Speece,1983; Welander e Metcalf, 2005). Entretanto, sabe-se hoje que para alguns ambientes, como por exemplo no tratamento de RSU em digestores anaeróbicos, a grande produção de metano ocorre a partir da associação sintrófica entre bactérias que oxidam acetato e as arquéias metanogênicas hidrogenotróficas (Angenent et al. 2002; Cardinali-Rezende et al. 2010).

Estudos bioquímicos extensivos têm revelado quatro vias para a metanogênese (Tabela 2): (1) redução do CO_2, $HCOO^-$ e H_2 (arquéias hidrogenotróficas) – utilizando o hidrogênio como doador de elétrons, (2) redução de compostos metil, metanol e metilaminas em metano (arquéias metilotróficas) – que também utiliza o hidrogênio como doador de

elétrons, porém, reduz o metanol em metano depois de transferir o grupo metil para a metil-coenzima M redutase – enzima presente em todas as arquéias metanogênicas, que cataliza a última etapa para a produção do metano, (3) redução do acetato para metano (arquéias acetoclásticas ou acetotróficas) – o acetato é primeiramente ativado a acetil-CoA e o seu grupo cabonil é então oxidado para CO_2, seguindo a via de redução do CO_2 , e o seu grupo metil é transferido para a enzima tetrahidrometanopterina (H_4SPT) e, subsequentemente, reduzido a CH_4, e (4) a via metilotrófica – envolvendo a redução do metanol e da metilamina em CO_2 e CH_4 (Welander e Metcalf, 2005).

Os substratos mais utilizados pelas arqueás metanogênicas são H_2 + CO_2, formiato e acetato. Porém, alguns compostos com C-1 tais como methanol, trimetilamina e dimetilsufido e alguns alcoóis como isopropanol, isobutanol, ciclopentanol e etanol também são utilizados por grupos específicos de arqueias metanogênicas (Zellner e Winter 1987; Bleicher et al. 1989;).

Durante a maioria das reações metabólicas realizadas pelas arqueias metanogênicas para a produção de metano, a mudança padrão de energia livre (ΔG°´) é muito baixa (Tabela 2). A quantidade de ATP produzida por mol de metano é próximo ou menor que 1 e na maioria dos ambientes onde as concentrações de H_2 e formiato é muito baixa, a mudança na ΔG°´ pode ser muito menor (Thauer et al. 1977; Schink, 1997; Batstone et al. 2002).

Tabela 2. Reações e mudanças nos padrões de energia livre para metanogênese.

Reação	**ΔG°´ (kJ/mol de CH_4)**
4 H_2 + CO_2 → CH_4 + 2 H_2O	–135.6
4 Formiato → CH_4 + 3 CO_2 + 2 H_2O	–130.1
4 2-Propanol + CO_2 → CH_4 + 4 Acetona + 2 H_2O	–36.5
2 Etanol + CO_2 → CH_4 + 2 Acetato	–116.3
Metanol + H_2 → CH_4 + H_2O	–112.5
4 Metanol → 3 CH_4 + CO_2 + 2 H_2O	–104.9
4 Metilamina + 2 H_2O → 3 CH_4 + CO_2 + 4 NH_4^+	–75.0
2 Dimetilamina + 2 H_2O → 3 CH_4 + CO_2 + 2 NH_4^+	–73.2
4 Trimetilamina + 6 H_2O → 9 CH_4 + 3 CO_2 + 4 NH_4^+	–74.3
2 Dimetilsulfido + 2 H_2O → 3 CH_4 + CO_2 + H_2S	–73.8
Acetato → CH_4 + CO_2	–31.0

Fonte: Thauer et al. 1977; Schink, 1997; Batstone et al. 2002

A utilização do acetato como substrato para o crescimento e metanogênese é realizada pelas arqueias metanogênicas afiliadas à ordem *Methanosarcinales* e pertencentes aos gêneros

Methanosarcina e *Methanosaeta*. As espécies pertencentes ao gênero *Methanosarcina* são as mais versáteis, uma vez que utilizam H_2, CO_2, CO, metanol, metilaminas e acetato como substratos para diferentes vias da metanogênese, enquanto que as outras arqueias metanogênicas são capazes de utilizar apenas um destes substratos (Ferry, 1993; Thauer et al. 1993). A capacidade de utilizar mais de uma via para a metanogênese faz das arqueias pertencentes ao gênero *Methanosarcina* bastante atrativas para análise genética com o intuito de estudar os genes e as proteínas envolvidas no processo da metanogênese, como, por exemplo, as metil-coenzima M redutases I e II, que são essenciais para a produção do metano (Guss et al. 2005; Pritchett e Metcalf, 2005; Galand, 2004). Contrastes na função desta atividade catalítica têm resultado em um alto grau de conservação na sequência de aminoácidos das metil-coenzimas M redutases entre linhagens metanogênicas, e até mesmo entre aquelas filogeneticamente distantes. Esta estrutura primária conservada tem sido utilizada para o desenho de iniciadores específicos para a amplificação de fragmentos do gene *mcr* A das arqueias metanogênicas encontradas na natureza, assim como o gene de rRNA 16S, e subseqeente classificação e construção de árvores filogenéticas (Hallam et al. 2003). Todas arqueias metanogênicas conhecidas, inclusive aquelas que já possuem seu genoma completamente sequenciado como, por exemplo, *Methanococcus jannaschii* (Ellermann et al. 1988), *Methanothermobacter thermoautotrophicus* (Ermler et al. 1997), *Methanosarcina acetivorans* (Ferry, 1992), *Methanopyrus kandleri* (Ferry, 1999) *e Methanococcus maripaludis* (http://www.genome.washington.edu/UWGC/) possuem pelo menos um gene *mcr* A – conservado e informativo – que é utilizado em estudos filogenéticos (Ferry, 1992). Atualmente, a presença do gene *mcr* A é considerado um indicador de metanogênese no habitat estudado (Luton et al. 2002; Lueders et al. 2001; Ferry, 1999; Thauer, 1998; Reeve et al. 1997). Entretanto, recentes investigações demonstraram a presença de genes *mcr* A também em arqueias anaeróbicas que oxidam o metano – ANME – (Hallam et al. 2003).

1.8 - Fatores que influenciam a digestão anaeróbica

O sucesso da digestão nos biodigestores anaeróbicos está relacionado a fatores ambientais e operacionais tais como: tipo de resíduo tratado, temperatura, concentração de ácidos graxos voláteis (AGVs), pH, concentração de H_2, nível de amônia, alcalinidade, taxa de carregamento, alimentação do biodigestor, tipo e quantidade de inóculo (fonte adicional de micro-organismos) e tempo de retenção do resíduo e de retenção hidráulico (Angelidaki e Ahring, 1994; Ahring et al. 1995; McMahon et al. 2001).

Mudanças ambientais ocorridas durante o tratamento dos resíduos pode alterar completamente a via de degradação dos substratos e, subsequentemente, produção do metano. O acúmulo de amônia nos biodigestores, por exemplo, levará a uma mudança na via de produção do metano que será dominada não pelas arquéias metanogênicas acetoclásticas (inibidas em altas concentrações de amônia) e sim pela associação sintrófica entre as arquéias hidrogenotróficas e as bactérias acetogênicas, menos sensíveis a estas variações (Schnurer et al. 1994, 1996; Schnurer et al. 1999;Sung and Liu, 2002).

Em relação às arqueias metanogênicas e bactérias acidogênicas, estas diferem amplamente com respeito à velocidade de crescimento e a taxa de utilização de substratos, sendo sensíveis a mudanças ambientais: as arqueias metanogênicas crescem lentamente e são mais sensíveis a variações ambientais quando comparadas às bactérias acidogênicas (Hwang et al. 2009). Durante o processo de digestão anaeróbica o pH ótimo, em torno de 6,8 e 7,2, é exigido pelas arqueias metanogênicas para a produção de metano. Valores de pH abaixo desses níveis levariam à estimulação das bactérias formadoras de ácidos (acidogênicas) e ao acúmulo de AGV no reator, sendo a principal causa disso, o desequilíbrio entre a taxa de produção e o consumo desses ácidos. Por isto, o monitoramento diário da produção de AGV e da alcalinidade no biorreator é essencial, uma vez que o resíduo orgânico a ser tratado necessita ter uma grande capacidade de tamponamento para neutralizar qualquer acúmulo de ácidos (Moosbrugger et al. 1993; Van Haandel e Letinga, 1994; Ahring et al. 1995).

A acidificação devido ao acúmulo de AGV é um problema frequente em biodigestores anaeróbicos e é responsável pelo efeito bioquímico inibitório sobre a acetogênese (Kuninobu et al. 1999). A principal causa é o sobre-carregamento do resíduo a ser tratado e desequilíbrio na manutenção da concentração de hidrogênio no meio e do pH, afetando então as interações microbianas durante a digestão anaeróbica, uma vez que em pH 6,5 o acetato acumulado é protonado (não-ionizado) e se torna tóxico aos micro-organismos. Isso leva ao acúmulo de ácidos graxos voláteis (acetato, propionato e butirato) nos biodigestores anaeróbicos e sua subsequente acidificação, afetando a degradação da matéria orgânica e inibindo a produção de metano (Schink, 2002; Stamatelatou et al. 2003;).

Vários trabalhos relatam que o controle da fase de metanogênese nos biodigestores, como também o monitoramento da diversidade das arqueias metanogênicas, têm sido o fator chave no sucesso operacional da maioria dos processos anaeróbicos (Raskin et al. 1994; Griffin et al. 1998; Angenent et al. 2002; Yu et al. 2006).

1.9 - Ferramentas moleculares para a identificação de procariotos

O advento e a aplicação das ferramentas moleculares têm proporcionado avanços significativos no campo da ecologia microbiana. Estas tecnologias permitiram que micro-organismos não cultiváveis, e anteriormente desconhecidos, pudessem ser estudados. A extração direta do DNA de amostras ambientais e subsequente amplificação do gene de rRNA 16S têm permitido a utilização de estratégias moleculares, independentes de cultivo, para o estudo de comunidades microbianas. A molécula de rRNA está presente em todos os seres vivos e contém regiões variáveis e conservadas que permitem o uso de iniciadores e sondas com diferentes níveis de especificidades, os quais podem ser utilizados em diversas técnicas moleculares (Amann et al. 2006).

Os genes codificadores de rRNA 16S, rRNA 23S e rRNA 5S são tipicamente organizados em um operon (Figura 7), Os genes de rRNA 16S e 23S são separados por uma região espaçadora, denominada ITS (internally transcribed spacer), a qual contém genes de tRNA, que é usada para discriminar espécies intimamente relacionadas, podendo revelar polimorfismos intra-específicos. O gene de rRNA 16S devido a presença de regiões conservadas e variáveis, e a grande disponibilidade de sequências nos bancos de dados, é considerada uma molécula ideal para estudos filogenéticos. O número de operons de rDNAs varia de 1 a 15 por genoma bacteriano sendo esta variação relacionada com a capacidade dos organismos em responder às condições do ambiente (Klappenbach et al. 2000; Lee et al. 2008).

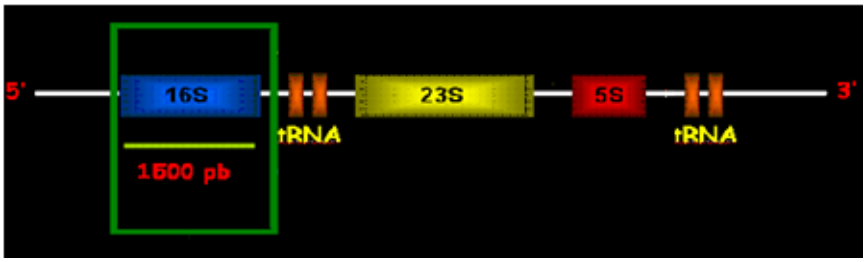

Figura 7. Estrutura do operon de rDNA.

Além dos genes de rRNA, genes funcionais podem também fornecer informações filogenéticas. Por exemplo, os genes metil coenzima M redutase (*mcr*), acetato quinase (*ack*), acetil coenzima A sintetase (*acs*) e polihidroxialcanoato (PHA) sintase são usados para identificar, especificamente, as arquéias metanogênicas, as bactérias oxidadoras de propionato

e acetato e bactérias produtoras de PHA, respectivamente (Luton et al. 2002; Shigematsu et al. 2006; Ciggin et al. 2007).

Análise da comunidade microbiana baseada na amplificação do gene de rRNA 16S, construção de bibliotecas genômicas e sequenciamento vêm sendo utilizada, sendo considerada uma ferramenta importante para caracterizar a diversidade das populações presentes em biorreatores anaeróbicos (Levén et al. 2007; Cardinali-Rezende et al., 2009, 2011;). ARDRA (Amplified Ribosomal DNA Restriction Analysis) é uma técnica amplamente utilizada para uma análise rápida da diversidade de biblioteca de clones de rDNA 16S. A digestão do DNA plasmidiano contendo a sequência inteira ou parcial do rDNA 16S com diferentes enzimas de restrição gera perfis de restrição, visualizados em gel de agarose, permitindo minimizar o número de clones a serem sequenciados na biblioteca, reduzindo os custos, trabalho e tempo (Ziemer et al. 2004).

DGGE (Denaturing gradient gel electrophoresis) é outra técnica amplamente utilizada pela microbiologia ambiental para o estudo da diversidade e mudança nas comunidades microbianas em sistemas complexos, como estrume e reatores anaeróbicos (Liu et al. 2002; Roest et al. 2005; Tan e Ji, 2010). Esta técnica se baseia na eletroforese de fragmentos do gene de rRNA 16S em gel de poliacrilamida, com diferentes gradientes de formamida e uréia, submetido a uma corrente elétrica (Figura 8). Sequências de regiões variáveis do gene de rRNA 16S de bactérias e arqueias, amplificadas com um mesmo par de iniciadores, podem apresentar o mesmo tamanho, mas diferir na sequência de bases, permitindo que os fragmentos sejam separados no gel. O perfil de bandas geradas permite a análise da diversidade microbiana de uma amostra e possíveis mudanças sofridas naquele ambiente (Muyzer et al. 1993; Yu e Morrison 2004).

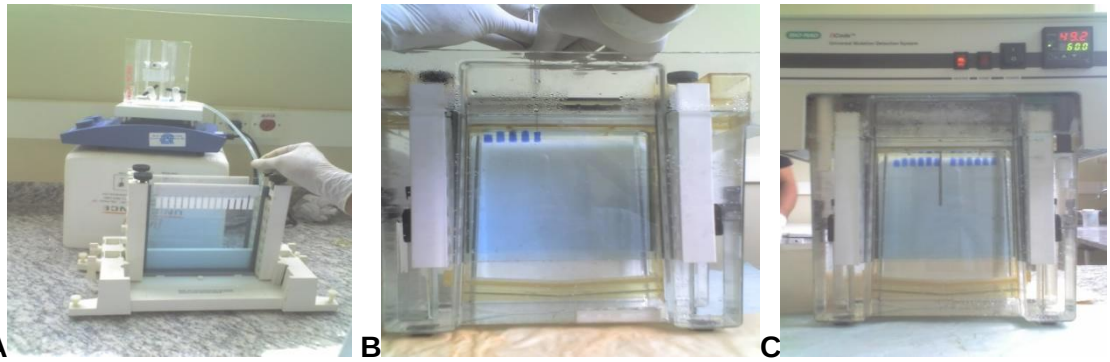

Figura 8. Preparação do gel de DGGE **(A)**, aplicação das amostras**(B)** e aplicação da corrente elétrica **(C). Fonte:** J. Cardinali-Rezende

1.10 - Ferramentas moleculares para a quantificação de procariotos

A quantificação das populações microbianas e sua interação em um biodigestor é de grande interesse econômico e ecológico, pois permite conhecer a contribuição funcional de cada espécie em determinadas fases do tratamento (Zhang e Fang, 2006). Atualmente, três técnicas moleculares muito utilizadas para a quantificação são: qPCR (quantitative real-time PCR), FISH (fluorescence in situ hibridization) e CARD-FISH (catalyzed reporter deposition - FISH).

1.10.1 - qPCR

A técnica do PCR quantitativo em tempo real foi desenvolvida para superar as limitações associadas com os métodos convencionais de quantificação. O uso desta técnica permite o monitoramento contínuo da acumulação do produto de PCR durante a progressão dos ciclos, pela detecção da fluorescência (Hanna et al. 2005). Assumindo que o nível intracelular de rRNA é proporcional à atividade metabólica, a sua quantificação por qPCR pode-se relacionar o conteúdo ribossômico à atividade funcional de uma determinada população microbiana (Hawkins et al. 2006).

O qPCR tem sido usado para o estudo de diversas amostras ambientais e de biodigestores (Zhang e Fang, 2006; Sawayama et al. 2006; Penning e Conrad, 2006;Tatara et al. 2007). Esta permite a quantificação de comunidades de uma série de amostras ao mesmo tempo (Bustin et al. 2009), além de fornecer a oportunidade de quantificar mudanças em populações individuais dentro de um biodigestor nos diferentes estágios da digestão anaeróbica, a partir da quantificação do gene de rRNA 16S, como também a partir da quantificação e expressão de alguns genes funcionais (Yu et al. 2006; Basile e Erijman 2008; Tan e Ji 2010). É uma técnica muito sensível e não exige a manipulação após a amplificação, evitando possíveis problemas de contaminação (Higuchi et al. 1993; Gibson et al. 1996; Heid et al. 1996).

Assim como outras técnicas, o qPCR também apresenta algumas _desvantagens que podem prejudicar o desenvolvimento do experimento e levar a produção de variação nos resultados: má qualidade das amostras (DNA ou RNA), devido a erros na preparação (extração e purificação) ou sua estocagem; escolha inadequada dos iniciadores, que podem formar dímeros ou ser inespecíficos; e escolha inapropriada dos métodos de análise dos resultados (Kubista et al. 2006; Bustin et al. 2009). A sensibilidade do qPCR é dependente do comprimento

do amplicon (que deve ser preferencialmente menor que 250 pb), sendo esperado que uma única cópia de um gene específico pode ser detectado (Livak et al. 1995)

1.10.2 - FISH e CARD-FISH

FISH é outra técnica de quantificação muito conhecida e mais antiga. Esta técnica baseia-se na análise microscópica e contagem de grupos de arqueias ou bactérias por meio de sondas fluorescentes que se ligam por complementariedade às moléculas de rRNA 16S, dentro das células. Esta, também permite o conhecimento da morfologia dos micro-organismos presentes na amostra. A técnica de FISH vem sendo muito utilizada em reatores anaeróbicos para a quantificação de bactérias, arqueias e, principalmente, das arquéias metanogênicas (Amann et al. 2001; Collins et al 2006; Fernández et al. 2008). Assim como outras técnicas, FISH possui algumas limitações: má fixação das amostras pode levar à degradação das células; coloração inespecífica de outros componentes presentes nas amostras como, ácidos húmicos, partículas de colóide e partículas orgânicas e inorgânicas; presença de partículas associadas às bactérias e arqueias, o que impede a sua distribuição homogênea no filtro ou lâmina onde será realizada a contagem; tamanho das células; número de cópias de rRNA celular; acessibilidade ao alvo, devido à permeabilização da parede celular (Wilson et al. 1990; Amann et al. 1995; Crump et al. 1998; Ferrari et al. 2006).

Para tentar resolver alguns problemas relacionados à técnica de FISH como, por exemplo, baixo sinal de fluorescência ou coloração inespecífica, uma opção é o uso do CARD-FISH, técnica capaz de detectar células com baixo número de ribossomos, aumentando drasticamente a sensibilidade do FISH. Esta técnica baseia-se na amplificação enzimática do sinal de fluorescência e foi inicialmente utilizada em ecologia microbiana por Pernthaler et al. (2002). As sondas do gene de rRNA 16S não são diretamente marcadas com fluoróforos, mas covalentemente ligados ao “horseradish peroxidase” (HRP). Durante a etapa da amplificação do sinal esta enzima catalisa a formação de radicais de tiramida marcados com fluoróforos que irão se ligar a regiões ricas em tirosina do ribossomo e em suas proteínas vizinhas. Esta etapa irá aumentar o sinal de fluorescência milhares de vezes, devido à deposição de inúmeras tiramidas marcadas (Figura 9) (Sanz and Kochling 2007).

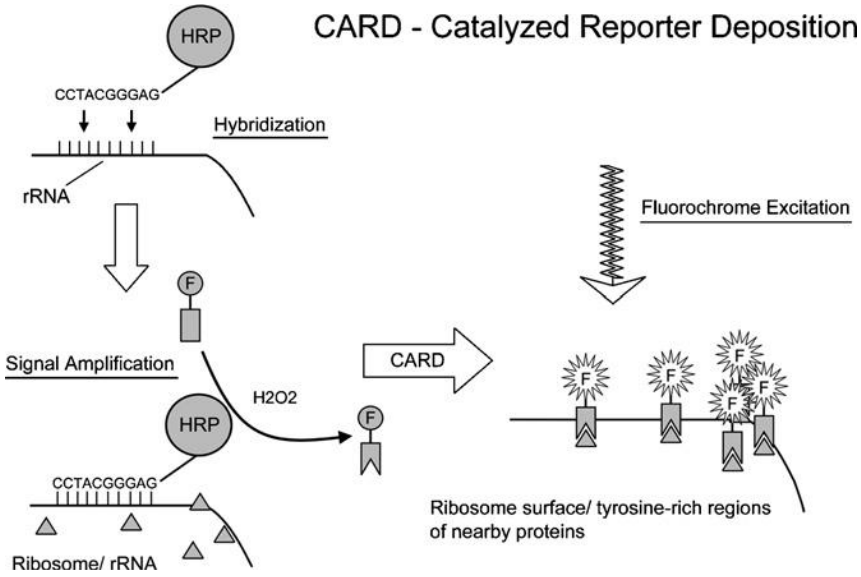

Figura 9. Esquema da técnica CARD-FISH.

Fonte: Sanz and Kochling 2007

A identificação, quantificação dos micro-organismos e de alguns de seus genes expressos em reatores anaeróbicos são importantes para uma melhor compreensão do complexo processo envolvido na degradação da matéria orgânica de resíduos, uma vez que são fundamentais para a otimização da digestão anaeróbica e para o desenvolvimento de biodigestores mais produtivos (Foresti, 1997).

Técnicas de quantificação são frequentemente usadas em combinação com outras ferramentas moleculares, como DGGE ou clonagem e seqüenciamento, para determinação quantitativa da distribuição espacial dos grupos taxonômicos de interesse encontrados nas amostras ambientais. Por possuírem aspectos positivos e limitações, a abordagem conjunta do qPCR e FISH ou CARD-FISH é de grande interesse, a fim de validar ou mesmo comparar seus resultados.

A diversidade da comunidade microbiana presente nos reatores e lagoas anaeróbicas está relacionada à estabilidade do tratamento da matéria orgânica destes resíduos pela digestão anaeróbica e a sua resistência á momentos de desestabilidade nestes sistemas. Mudanças temporais dessa comunidade pode antecipar e até mesmo indicar uma possível falha no tratamento. A associação entre as mudanças na comunidade microbiana e as variações ambientais que podem ocorrer durante a digestão anaeróbica precisa ser bem entendida. Além disso, mudanças nesta comunidade sem aparente alterações no desempenho do reator ou da lagoa anaeróbica podem ocorrer, e estudos nesta área são essenciais para uma melhor

compreensão das funções exercidas pelos micro-organismos que tornam estável o sistema de digestão anaeróbico. O conhecimento de todo o processo da digestão anaeróbica e dos fatores que interferem neste são fundamentais para que se possa aumentar a eficiência da degradação da matéria orgânica e a produção de metano, uma vez que estes são influenciados diretamente pela composição da comunidade microbiana responsável pelas várias etapas da digestão anaeróbica.

2 – OBJETIVOS

2.1 - Objetivo Geral

Analisar e quantificar a diversidade filogenética, por abordagem molecular, das comunidades de procariotos associados à digestão anaeróbica de diferentes resíduos orgânicos: em reator anaeróbico, em escala industrial, com resíduos sólido urbano (RSU) (Madri, Espanha); em reator anaeróbico UASB, em escala piloto, com águas residuárias domiciliares - esgoto sanitário (Belo Horizonte); e em lagoa anaeróbica, com rejeitos suíno de um frigorífico.

2.2 - Objetivos Específicos

2.2.1 - Obter DNA total e analisar sequências do gene de rRNA 16S de bactérias e arqueias, obtidas por construção de bibliotecas de clones, de amostras coletadas: no *startup* e em condições estáveis do reator com RSU; nos períodos de seca e chuva do reator UASB; e de uma lagoa anaeróbica, com resíduo suíno de um frigorífico.

2.2.2 - Analisar e comparar por DGGE a diversidade de bactérias e arqueias presentes no *startup* do reator anaeróbico (RSU) com os resultados obtidos pela análise das bibliotecas de clones.

2.2.3 - Otimizar e quantificar as comunidades de bactéria e arqueias presentes nos reatores anaeróbicos, com RSU e esgoto sanitário, e na lagoa anaeróbica, com rejeito suíno.

2.2.4 - Desenhar iniciadores e padronizar as condições para quantificar por qPCR células de arqueias metanogênicas pertencentes aos grupos de *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcisa sp*., *Methanosaeta sp*. das amostras coletadas no *startup* e em condições estáveis do reator anaeróbico (RSU).

2.2.5 - Quantificar por FISH e CARD-FISH as células de bactérias e arqueias presentes no *startup* e em condições estáveis do reator anaeróbico (RSU) comparando com os valores obtidos por qPCR.

2.2.6 - Tentar relacionar a eficiência dos reatores e da lagoa anaeróbicos nos tratamentos do RSU, do esgoto sanitário e do rejeito suíno com a diversidade e abundância das comunidades de procariotos identificadas nestes sistemas.

3 - CAPÍTULOS

3.1 - CAPÍTULO I

Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor from start-up to steady-state conditions

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Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor from start-up to steady-state conditions

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HIGHLIGHTS

- We identify and quantify microbiota of a full-scale MSW anaerobic reactor.
- Shifts in the diversity and abundance were observed from start-up to steady-state.
- Hydrogenotrophic methanogens dominated the methane production in the MSW reactor.
- *Methanosarcina*, *Methanimicrococcus* and *Methanosaeta* were identified in the reactor.
- Quantification of bacterial and archaeal by FISH and qPCR differ.

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ABSTRACT

The prokaryotic diversity of an anaerobic reactor for the treatment of municipal solid waste was investigated over the course of 2 years with the use of 16S rDNA-targeted molecular approaches. The fermentative *Bacteroidetes* and *Firmicutes* predominated, and *Proteobacteria*, *Actinobacteria*, *Tenericutes* and the candidate division WWE1 were also identified. Methane production was dominated by the hydrogenotrophic *Methanomicrobiales* (*Methanoculleus* sp.) and their syntrophic association with acetate-utilizing and propionate-oxidizing bacteria. qPCR demonstrated the predominance of the hydrogenotrophic over aceticlastic *Methanosarcinaceae* (*Methanosarcina* sp. and *Methanimicrococcus* sp.), and *Methanosaetaceae* (*Methanosaeta* sp.) were measured in low numbers in the reactor. According to the FISH and CARD-FISH analyses, *Bacteria* and *Archaea* accounted for 85% and 15% of the cells, respectively. Different cell counts for these domains were obtained by qPCR versus FISH analyses. The use of several molecular tools increases our knowledge of the prokaryotic community dynamics from start-up to steady-state conditions in a full-scale MSW reactor.

1. Introduction

The environmentally friendly management of municipal solid waste (MSW) has become a global challenge because of limited resources, an ever-increasing population, rapid urbanization and industrialization. An enormous quantity of MSW is produced daily in the European Union (400,000 tons). However, the separate collection of MSW fractions has increased significantly, and the biomethanization (anaerobic digestion, AD) of the organic fraction (OF) of the MSW has become an effective solution (Mata-Alvarez et al., 2000).

Among various biological treatments, the AD of OF-MSW in anaerobic digesters is frequently the most cost-effective. This process results in the reduction of the organic content of the waste, the production of an energy-rich biogas (CH_4, CO_2, and traces of N_2, H_2S and O_2) and a solid residue with a high nutrient content, which can be recycled to recuperate degraded environments. The potential of biogas production throughout Europe could reach in 2020 77.9 billion m^3 of methane, being 10 billion m^3 from biodegradable fraction from municipal solid wastes. (http://www.aebiom.org/IMG/pdf/Brochure_BiogasRoadmap_WEB.pdf).

In 1995, the first biomethanization plant for MSW began operation in Europe. Since then, many plants have opened in countries where the collection of MSW has been established, such as Spain, Germany, France, Denmark, and Belgium (IEA, 2003). Approximately 15% of the OF-MSW is biologically treated in Europe, and AD represents approximately 20% of all biological treatment capacity (http://www.waste-management-world.com). Spain produces 26.2 million tons of MSW

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per year, which amounts to 1.7 kg for person per day (INE, 2009). A total of 24 MSW biomethanization plants are in operation in this country, which includes seven close to Madrid. Recently, two plants commenced operation in Valdemingómez Technology Park (Las Dehesas), with five anaerobic digesters, and in Paloma, both of which are under the responsibility of the municipality of Madrid. These plants are considered the largest and most modern in Europe and are an international model for waste treatment. It is estimated that both plants will be responsible for the processing of 370,000 tons of MSW and the production of 34 million m^3 of biogas per year, which will reduce the use of other energy sources (http://www.madridiario.es/2008/Noviembre/medioambiente/gasnatural/118868/plantsbiometanizacion-Valdemingómez-empiezan-rodaje.html). The concomitant production of electricity, heat and biogas from biomass is an environmentally and economically attractive option.

Previous culture-independent studies of the microbial communities in pilot-scale anaerobic reactors for the treatment of OF-MSW have been performed. Tang et al. (2004) comparing the communities from a thermophilic MSW digester without and with micro-aeration, using complementary molecular techniques, observed that *Firmicutes* dominated and *Methanosarcina* decreased while *Methanoculleus* increased as a result of micro-aerations. Nayak et al. (2009) showed temporal shifts in the archaeal and bacterial community by (DGGE) and the predominance of *Methanosarcinales* and *Methanomicrobiales* by *mcrA* gene libraries. Cardinali-Rezende et al. (2009) studying the microbial community of the MSW before, during and after the AD into a mesophilic reactor, by 16S rRNA and *mcrA* genes clone libraries, observed that *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were represented in all libraries, and only hydrogenotrophic *Methanomicrobiales* (*Methanoculleus* genus) and *Methanobacteriales* (*Methanosphaera* and *Methanobacterium* genera) were identified. The same was observed by Sasaki et al. (2011) in a thermophilic digester, where *Methanoculleus* sp. predominated. Supaphol et al. (2011) observed in the mesophilic anaerobic co-digestion of mixed waste the predominance of *Firmicutes*, *Actinobacteria* and *Proteobacteria* and the shifts of predominance of hydrogenotrophic to aceticlastic methanogens (*Methanosaeta*) from early to end stages of the AD. Bertin et al. (2012) using DGGE analysis in a mesophilic continuous anaerobic reactor fed initially with cattle manure and later with OF-MSW, also observed that *Firmicutes* and *Bacteroidetes* predominated. However, in contrast to the earlier works only two aceticlastic *Methanosarcina* sp. were identified.

Despite the increasing number of full-scale MSW plants in Europe, the prokaryotic composition and dynamics of a full-scale MSW anaerobic digester have been scarcely explored. The performance of AD is linked closely to the structure of the digester's microbial community, and an investigation of its prokaryotic diversity can therefore provide relevant insights. In this study, we sought to illuminate the bacterial and archaeal community dynamics of a full-scale MSW anaerobic digester (Las Dehesas Biomethanization Plant, Madrid) from the start of operations (start-up) to steady-state conditions. To achieve this goal, we applied molecular approaches such as denaturing gradient gel electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA), real-time quantitative polymerase chain reaction (qPCR), fluorescence in situ hybridization (FISH) and catalyzed reporter deposition (CARD)-FISH.

2. Methods

2.1. Reactor conditions

A full-scale, 12-m anaerobic digester for the treatment of OF-MSW commenced operation in February 2009 at the Las Dehesas Biomethanization Plant (Madrid, Spain). The reactor operates at 35 °C, an approximate pH of 7 and a total concentration of 30–40% solids (w/v). The average residence time is 100 days when the reactor is filled. The biogas is extracted from the top of the reactor and injected into the bottom to promote mixing in the reactor tank. Approximately 50–100 tons of fresh OF-MSW is added daily. $CaCO_3$ and water, together with the steam necessary to raise the temperature to approximately 35 °C, are added as needed.

2.2. Sampling and chemical analysis

The digested MSW (DMSW) samples were aseptically extracted from the digester with bottles. The samples were collected on May 21, 2009 (DMSW1), October 22, 2009 (DMSW2), February 18, 2010 (DMSW3), and May 11, 2011 (DMSW4). The first three samples were extracted during the start-up phase of the reactor, which took two years. The first sampling (DMSW1) was performed about one year after the beginning of the operation of the reactor, when 50% of the reactor was filled. The last sample was removed under steady-state conditions while the reactor was completely filled with OF-MSW.

The samples were analyzed for temperature, pH, alkalinity, ammonia content, total suspended solids (TSS) and volatile suspended solids (VSS) according to the Standards Protocols (APHA, 2005). Alkalinity, ammonia content, TSS and VSS were analyzed according to the method numbers 2320B, 2130B, 2540D and 2540E, respectively. The volatile fatty acids (VFAs) were measured by gas chromatography (Varian STAR 3400 CX) using FFAP capillary column (split ratio 1:40, temperature of the column, injector and detector: 250, 140 and 250 °C, respectively).

The general scheme of the experimental design is depicted in Fig. 1. Detailed methodology is described below.

2.3. Pretreatment of the DMSW samples and the total DNA extraction

The samples were pretreated to obtain intact DNA. For this process, 0.91 g of DMSW (wet weight) was resuspended in 10 mL of 0.5 M EDTA (Disodium Ethylenediaminetetraacetate), pH 8.0 and the suspension was stirred at 4 °C for 1 h according to Sánchez-Andrea et al. (2011). Next, the samples were centrifuged (10,000 rpm in an Eppendorf 5430 for 1 h), and the sediments were again resuspended in EDTA by vortexing. The previous steps were repeated twice, and the pellet was resuspended in 10 mL of EDTA. A 1-mL aliquot of the suspension was sonicated with five 30-s cycles (LABSONIC M; Sartorius Stedim Biotech) and allowed to settle for 1 h at 4 °C. The supernatant (900 μL) was centrifuged for 15 min at 12,000 rpm. The supernatant was discarded, and the pellet was resuspended in 1 mL of a 1:1 solution of 0.9% NaCl and 50 mM EDTA, pH 8.0, and stirred overnight at 4 °C according to Sánchez-Andrea et al. (2011). The total DNA of all of the samples was extracted using the FastDNA SPIN kit for soils (Bio 101) according to the manufacturer's instructions. For real-time PCR, the DNA from the DMSW1, DMSW2 and DMSW4 samples was extracted simultaneously.

2.4. PCR amplification of 16S rRNA genes

The 16S rRNA genes of the Bacteria and Archaea domain from the DMSW samples were PCR amplified for cloning (samples DMSW1, 2 and 4) and for DGGE (samples DMSW1, 2 and 3) analyses. The primers and PCR conditions used are listed in Table S1. For the DGGE analysis, the partial bacterial and archaeal 16S rDNA amplicons were re-amplified with the same primer pairs (with a GC-clamp in the 341F and 622F primers).

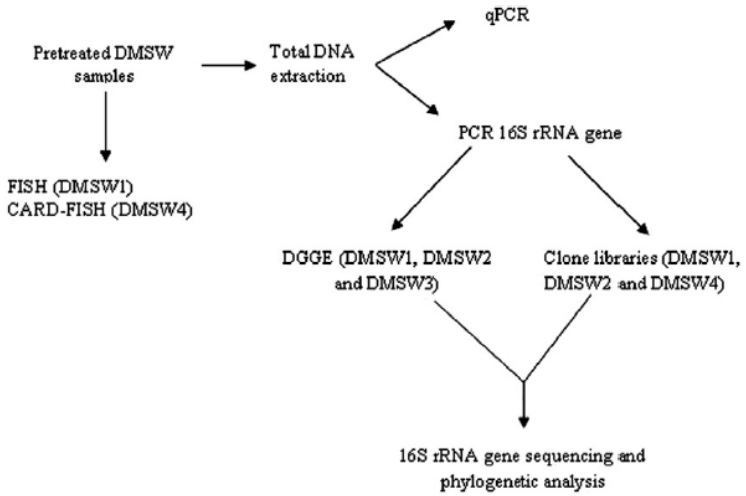

Fig. 1. Flow chart of experimental methods.

2.5. Denaturing gradient gel electrophoresis (DGGE)

PCR products of the bacterial and archaeal 16S rDNA fragments from the DMSW1, DMSW2 and DMSW3 samples were separated by DGGE (DcodeSystem, BioRad, Germany). The 6% polyacrylamide gels (w/v, 37.5:1 acrylamide and bisacrylamide) were prepared with denaturing gradients that ranged from 30% to 60% (in which the 100% denaturant contained 7 M urea and 40% v/v formamide) and were run at 60 °C and 80 V for 15 h. The bands of interest were excised, and their DNA was re-amplified by PCR for sequencing according to the PCR conditions described (Table S1).

2.6. Clustering analysis from the DGGE patterns

For the clustering analysis, the profiles of the bacterial (B1, B2 and B3) and archaeal (A1, A2 and A3) 16S rDNA fragments, which corresponded to the DMSW1, 2 and 3 samples, were converted into a binary matrix in which the digit 1 represented the presence of the band and the digit 0, its absence. The similarity matrix was generated by Euclidean distance, which was used to construct the dendrogram with the UPGMA algorithm (Ryan et al., 1995). The data analysis was performed using PAST (Paleontological Statistics Software Package) (Hammer et al., 2001).

2.7. Libraries and ARDRA analysis

For a further comparison between the DMSW samples, clone libraries were constructed from the DMSW1, DMSW2 and DMSW4 samples and analyzed. The sizes of the amplicons (1465 and 1467 bp for the bacteria and archaea, respectively) were confirmed by 0.8% (w/v) agarose gel electrophoresis. The bands were excised, and the DNA was purified from the gel slices using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited). For cloning, the purified amplicons were cloned into the pGEM®-T Vector (Promega) according to the manufacturer's instructions and transformed into chemically competent *Escherichia coli* DH5α. Plasmids from the clones that contained the 16S rDNA inserts were extracted using a standard alkaline lysis method (Sambrook et al., 1989). The plasmid inserts were screened by ARDRA with the restriction endonuclease *Bfu*Cl (New England BioLabs Inc., New England) according to the manufacturer's instructions. The fragments were separated by 2.5% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining (0.5 μg/mL). The clones were grouped according to their restriction patterns, which defined the different operational taxonomic units (OTUs). Two clones of each OTU were subsequently sequenced.

2.7.1. 16S rRNA gene sequencing and phylogenetic analysis

Sequencing of the bacterial and archaeal 16S rDNA from the clone libraries (BL1, BL2 and BL4 for the bacteria and AL1, AL2 and AL4 for the archaea) and of the bacterial and archaeal DGGE bands was performed in an ABI model 377 sequencer (Applied Biosystems) using standard protocols. All of the sequences were compared with sequences in the Ribosomal Database Project (RDP) using Library Compare and the sequences in GenBank using BLASTN. Prior to these comparisons, the 16S rRNA gene sequences were base-called, checked for quality, aligned, and analyzed with Phred v.0.020425, Phrap v.0.990319 and Consed 12.0. RDP's CHECK-CHIMERA program and VecScreen program (NCBI) were used to detect chimeric DNAs and vectors, respectively. The phylogenetic relationships were inferred by MEGA 4.1 using the neighbor-joining method and Kimura's 2-P model of sequence evolution. The nucleotide sequences generated in this study were deposited in GenBank under the accession numbers JX101959-102021 for the 16S rRNA gene sequences and JX102022-102039 for the DGGE bands.

2.8. Primer design for quantitative real-time PCR (qPCR)

The 16S rRNA gene sequences specific for the methanogenic archaea *Methanosaeta* sp., *Methanosarcina* sp., *Methanobacteriales* and *Methanomicrobiales* were retrieved from the NCBI database and aligned automatically using the CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php) to form a contig. Primers specific for each group (Table S1) were designed using the Universal Probe Library Assay Design Center (Roche Applied Science – https://www.roche-applied-science.com). The primer specificity was tested using the GenBank BLASTN database and qPCR, which consisted of observing the melting and amplification curves.

2.9. Standard DNA for qPCR analysis

A pool of the total DNA that was extracted from the different samples (DMSW1, DMSW2 and DMSW4) was used to establish

absolute quantification standards. The pooled DNA was amplified using conventional PCR in a Mini-cycler™ PTC-100 (MJ Research Inc., Waltham, MA). The specific primers for the bacteria, archaea and methanogenic archaea and the PCR conditions are listed in Table S1. The amplicons were purified using 20% polyethylene glycol (PEG 20%) prepared in 5 M NaCl (Sambrook et al., 1989). The DNA concentration was determined using a Nanodrop ND-1000 spectrophotometer. The 16S rDNA copy number for each group was calculated per amplicon by the URI Genomics & Sequencing Center (http://www.uri.edu/research/gsc/resources/cndna.html). The standards were diluted in nuclease-free water and stored in single-use aliquots at −80 °C. An 8.10-fold serial dilution of the standards (in triplicate) were used for qPCR to generate the standard curve.

2.10. qPCR

Quantitative standard curves were constructed for the following groups: the *Bacteria* and *Archaea* domains and the methanogenic archaea *Methanomicrobiales*, *Methanobacteriales*, *Methanosarcina* sp. and *Methanosaeta* sp. The real-time PCR was conducted in an ABI PRISM 7900HT SDS (Applied Biosystems, Foster City, CA, USA). The reactions, at a final volume of 10 μL, consisted of 5 μL of SYBR Green PCR master mix (QIAGEN, USA), 0.5 μL of each primer (2.5 mM), 1.0 μL of the template (total DNA or dilutions for the standard curve) and sterile H_2O. The quantitative measurement of the samples using real-time PCR was performed in triplicate together with the standards (that were specific for each microbial group) to generate a standard curve. The real-time PCR amplification was performed with 40 cycles of denaturation (20 s at 95 °C), annealing (20 s at 60 °C), and elongation (120 s at 72 °C). The primer set used for measuring the 16S rRNA copy number of the *Bacteria* and *Archaea* domain, *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcina* sp. and *Methanosaeta* sp. is listed in Table S1. The results were analyzed in an ABI PRISM 7900HT SDS (Applied Biosystems, Foster City, CA, USA). The absolute quantitative analysis of the bacterial, archaeal and methanogenic archaeal communities was obtained, and the number of cells per gram of the DMSW samples for each microbial group was calculated considering 4 and 2.5 copies of the 16S rRNA gene per cell bacteria/archaea and methanogenic archaea, respectively (Klamppenbach et al., 2001).

2.11. Fixation of the DMSW samples and standardization of the cell concentrations for FISH

The DMSW1 and DMSW4 samples were collected from the digester and immediately fixed in 4% paraformaldehyde in 1X phosphate-buffered saline solution (PBS) (130 mM NaCl and 10 mM Na_2PO_4/NaH_2PO_4, pH 7.2–7.4) for 4 h at 4 °C for Gram-negative bacteria. The samples were then washed in 1X PBS and centrifuged, and 2.9 g of the sample was re-suspended in 8.5 mL of a 1:1 solution of 1X PBS and ethanol and stored at −20 °C.

2.12. FISH and CARD-FISH analyses

The hybridizations were performed for the DMSW samples following the protocol described for FISH (Amann et al., 1990) and CARD-FISH (Pernthaler et al., 2002). The probes used in this work are listed in Table S1. The oligonucleotide probes were labeled with the cyanin dye Cy3 for the FISH and horseradish peroxidase HRP (Alexa488) for the CARD-FISH analyses. *E. coli* ATCC 12435, *Methanosaeta concilli* DSM2139 and DSM 863 were used as positive controls, and the NON338 *Methanobacterium bryantii* probe was used as a negative control. The total cells present in the samples were enumerated by direct counting of 4′,6′-diaminphenylindol- (DAPI, 1 mg/mL) stained cells when possible. A total of 20 fields for each sample were examined for each probe under a Zeiss Axiovert 200 microscope. The number of microbial cells was calculated and converted to cells per gram of sample.

3. Results and discussion

3.1. Physicochemical characterization of the MSW and their digested samples

The start-up phase of the reactor persisted for almost 2 years. The characteristics of the feed (MSW) and the digested (DMSW) materials are listed in Table 1. Although differences in the TSS and VSS contents of the digested samples can be ascribed to the heterogeneity of the feed material, there was a significant reduction of TSS (50–62%) and VSS (67–71%) during the treatment, which indicates an efficient degradation of organic matter in the anaerobic reactor. The increase in the concentrations of VFA (acetate, propionate and butyrate) with time throughout the study period is likely due to the higher acidogenesis rate versus methanogenesis (the feed material is added daily and includes rapidly degraded carbohydrates). Despite the high VFA amounts detected, the pH values were maintained between 7 and 8 due to $CaCO_3$ addition and ammonium released during the MSW treatment. The average concentration of CH_4 in the biogas ranged from 57% to 60% and is a good indicator of the stability of the process. The production of H_2S was not detected in the reactor, which permits use of the biogas without pre-treatment in engines. Although ammonia was generated in the reactor, its toxicity was overcome via the recirculation of ambient, air-dried biogas back into the reactor. Moreover, the high TSS concentration, which ranged from 19% to 24% (Table 1), diluted the ammonia concentration, which was also observed by Jewell et al. (1999).

3.2. Bacterial and archaeal 16S rRNA gene analysis

To determine the biodiversity of the prokaryotic communities in the MSW digester, the total DNA from the DMSW1, DMSW2 and DMSW4 samples were used to generate six clone libraries. To avoid sequencing clones with identical 16S rRNA genes, the insert-containing plasmids were digested with the restriction enzyme *BfuCl* to generate ARDRA patterns. OTUs were defined as a unique ARDRA pattern. A total of 89 different patterns were generated and sequenced. The chimeras were removed and 64 (48 of bacteria and 16 of archaea) OTUs were phylogenetically analyzed.

The library coverages (82–91% and 94–98% for the bacteria and archaea, respectively) suggest that the number of analyzed OTUs was satisfactory and that most bacteria and archaea present in the samples were detected. The majority of the OTUs displayed relationships with environmental sequences from various uncultured bacterial clones from municipal wastewater, swine waste and solid waste anaerobic digesters (Tables 2–4). The OTU sequences displayed similarity to sequences deposited in the RDPII and NCBI databases, and few sequences were identified at the species level (>97% similarity). These results are in agreement with Martín-González et al. (2011), who also reported that most of bacterial 16S rRNA gene sequences were similar to those of uncultured clones.

In this study, the number of clones selected by ARDRA and analyzed in each clone library was not sufficient to describe the total microbial diversity in detail. However, these results were confirmed by DGGE analysis and combined with qPCR, FISH and CARD-FISH, which accurately reflects the diversity and dynamics of the microbial communities during the operation of this reactor.

Table 1
Physical–chemical analysis of municipal solid waste (MSW) and their digested samples (DMSW) from anaerobic reactor in different periods.

Parameters*	MSW (Average)	Samples			
		DMSW1 05/2009	DMSW2 10/2009	DMSW3 02/2010	DMSW4 05/2011
TSS (%)	48.1	23.6	21.3	18.0	23.7
VSS (%)	28.8	9.0	8.4	9.5	8.7
pH	NA	7.7	7.7	7.9	7.5
Temperature (°C)	NA	35	31	37	35
Ammonium (mg/l)	NA	NA	4139	4760	4460
Conductivity (μS/cm)	NA	NA	49.0	49.3	48.0
VFAs (mg/l)	NA	449	628	961	7184
Acetate (mg/l)	NA	415	555	759	4100
Propionate (mg/l)	NA	34	73	202	2636
Butyrate (mg/l)	NA	NA	NA	NA	448
Alcalinity (mg-$CaCO_3$/l)	NA	14.2	17.1	17.6	17.6
CH_4 (% in biogas)	NA	57	60	60	58

* Municipal solid waste to be treated (MSW); Digested of municipal solid waste treated in different periods of the treatment: start-up (DMSW1; DMSW2; and DMSW3) and stable (DMSW4) conditions, collected inside of the reactor; Total suspended solids (TSS), volatile suspended solids (VSS), Volatile fatty acids (VFAs), NA, not analyzed.

Table 2
Phylogenetic filiations and distribution of bacterial and archaeal clones analyzed from digested municipal solid waste 1 (DMSW1).

Phylogenetic group	OTU	Clon No.	Closest sequence/microorganism	Order/Family	Acession No.	Identity (%)	Habitat of closest relative
Bacteroidetes	BL1-1	1	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	CU919517	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	2	25	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	CU919514	98	Thermophilic biogas reactor fed with renewable biomass
	3	2	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	FN436026	98	Thermophilic biogas reactor fed with renewable biomass
	4	5	Uncultured bacterium clone 01a03	*Porphyromonadaceae*	GQ138680	99	ASBR reactor treating swine waste
	5	1	Uncultured bacterium clone HAW-R60-B-609d-C	*Porphyromonadaceae*	FN436026	95	Thermophilic biogas reactor fed with renewable biomass
	6	1	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	CU919067	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	7	1	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	CU919667	97	Mesophilic anaerobic digester which treats municipal wastewater sludge
Firmicutes-Clostridia	8	3	Uncultured bacterium clone 02f07	*Peptostreptococcaceae*	GQ138525	92	ASBR reactor treating swine waste
	9	1	Uncultured bacterium clone PISD-AIB	*Incertae Sedis*	AM982570	99	Pig saw dust spent bedding
	10	1	Uncultured bacterium clone G35-D8-L-F	*Clostridiales*	EF559144	99	Mesophilic anaerobic digester at 35 degrees Celsius
	11	1	Uncultured *Symbiobacterium* sp.	*Incertae Sedis*	EU639305	99	Thermophilic microbial fuel cell time zero control
	12	1	Uncultured Firmicutes bacterium	*Clostridiales*	CU921622	90	Mesophilic anaerobic digester which treats municipal Wastewater sludge
Firmicutes-Bacilli	13	1	Uncultured bacterium clone		AM982570	99	Pig saw dust spent bedding
Gammaproteobacteria	14	6	*Pseudomonas* sp.	*Pseudomonadaceae*	AY954288	99	Anaerobic digestive reactor of waste water reatment plant
Actinobacteria	15	1	*Actinomyces europaeus*	*Actinomycineae*	NR026363	98	Strain CCUG 32789A
	16	2	*Actinomyces europaeus*	*Actinomycineae*	AM084230	97	Isolate C Strain CUG 32789AT
Euryarchaeota							
Methanosarcinales	AL1-1	4	Uncultured *Methanosarcina barkeri*	*Methanosarcinaceae*	EU857627	97	Nisargruna Biogas Plant
	2	5	*Methanosarcina barkeri*	*Methanosarcinaceae*	AF028692	97	Ricefield soils
Methanomicrobiales	3	38	*Methanoculleus* sp.	*Methanomicrobiaceae*	AJ550158	99	Rumen
	4	1	*Methanoculleus bourgensis*	*Methanomicrobiaceae*	AF095269	99	Strain: MS2
	5	27	*Methanoculleus bourgensis*	*Methanomicrobiaceae*	AB065298	99	Strain:DSM 6216

3.3. Bacterial diversity

A phylogenetic analysis of the bacterial 16S rDNA sequences from the clone libraries revealed that the bacterial composition changed during the study period. The following bacterial phyla were identified in the reactor: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Tenericutes* and the candidate division WWE1 (Fig. 2 and Tables 2–4). According to Nelson et al. (2011), using a meta-analysis approach, the majority of the bacterial communities in anaerobic digesters were classified within four phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Chloroflexi*), and *Actinobacteria* was identified as a 'minor' phylum.

The *Bacteroidetes* and *Firmicutes* phyla contained most of the OTUs (85–95%) identified in all of the samples (Fig. 2). *Bacteroidetes* was represented by the *Porphyromonadaceae* family and accounted for 68% of the sequences in DMSW1, 63% in DMSW2 and 84% in DMSW4. Species from the *Bacteroidetes* phylum are acidogenic, sugar-fermenting, saccharolytic and proteolytic bacteria that produce propionate, acetate and succinate as their primary products. The *Firmicutes* phylum was represented by the *Clostridia* and *Bacilli* classes. The OTUs affiliated with the *Clostridia* class were represented in all of the samples and accounted for 13.5% of the DMSW1 clones, 32% of DMSW2 and 10% of DMSW4 (Fig.2). The sequences from the *Peptostreptococcaceae*, *Clostridiaceae*, and *Ruminococcaceae* families, as well as Incertae sedis, were present, being some of these sequences similar at the genus level (>95%) to *Symbiobacterium* sp., *Clostridium* sp. and *Tissierella* sp. (Tables 2–4). *Ruminococcaceae* and *Clostridiaceae* families are represented by

Table 3
Phylogenetic filiations and distribution of bacterial and archaeal clones analyzed from from digested municipal solid waste 2 (DMSW2).

Phylogenetic group	OTU	Clon No.	Closest sequence/microorganism	Order/Family	Acession No.	Identity (%)	Habitat of closest relative
Bacteroidetes	BL2-1	2	Uncultured *Alkaliflexus* sp.	*Marinilabiaceae*	EU887836	84	Aerobic predigester
	2	6	Uncultured *Bacteroidetes* bacterium	*Porphyromonadaceae*	CU919914	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	3	2	Uncultured bacterium clone PeH15		AJ576333	95	Hindgut homogenate of Pachnoda ephippiata larva
	4	1	Uncultured eubacterium clone LKB108		AJ746506	98	Landfill leachate
	5	26	Uncultured bacterium clone TE-3-E11		JQ337397	99	Composting sample at 12 days
	6	1	Uncultured eubacterium clone LKB106		AJ746505	99	Landfill leachate
	7	2	Uncultured eubacterium clone LKB104		AJ746504	99	Landfill leachate
	8	13	Uncultured *Bacteroidetes* bacterium		CU919517	98	Mesophilic anaerobic digester which treats municipal wastewater sludge
Firmicutes-Clostridia	9	1	Uncultured bacterium clone 03d07	*Incertae Sedis*	GQ134018	98	ASBR reactor treating swine waste
	10	1	Uncultured *Tissierella* sp.	*Incertae Sedis*	GU112188	95	Biogas slurry derived from anaerobic fermentation of pig manure
	11	2	Uncultured bacterium clone LL143-7E10		FJ671370	94	MARC beef cattle feedlot
	12	1	*Clostridium colinum* DSM6011	*Clostridiaceae*	NR026151	91	Strain DSM:6011
	13	1	Uncultured *Clostridium* sp.	*Clostridiaceae*	AB231801	97	Cellulose enrichment culture
	14	2	Uncultured prokaryote clone 08031003-Z7EU_2TH_2_2_A06	*Clostridiales*	HQ156029	97	Biogas Z7 sample
	15	17	Clostridiales oral clone P4PB_12	*Ruminococcaceae*	AF538854	93	Periodontal microflora
	16	2	Uncultured bacterium clone D53	*Ruminococcaceae*	AM500759	93	Composting sample
Firmicutes-Bacilli	17	1	*Bacillus dipsosauri*	*Bacillaceae*	AB101591	99	Strain DSM11125T
	18	1	Uncultured bacterium clone M35-D20-H-B-B		EF586027	98	Solid waste digester fed with methanol
	19	1	*Vagococcus* sp.	*Enterococcaceae*	FJ211190	99	Wastewater treatment factory
Gammaproteobacteria	20	2	*Pseudomonas* sp.	*Pseudomonadaceae*	DQ337603	99	Swine effluent applied soil
Euryarchaeota							
Methanomicrobiales	AL2-1	8	*Methanoculleus bourgensis*	*Methanomicrobiaceae*	AB065298	97	Strain:DSM 6216
Methanobacteriales	2	2	Uncultured *Euryarchaeote* clone 1C		GQ365371	98	Labscale digester Inoculated with anaerobic digester sludge
	3	4	Uncultured archaeote clone T8		EU662689	97	Sludge from a manure pit
Methanosarcinales	4	1	*Methanosarcina* sp.	*Methanosarcinaceae*	EU857627	98	Nisargruna Biogas Plant

cellulolytic and amylolytic bacteria, which have been isolated from several AD reactors (Yu et al., 2010). The *Bacilli* class was a minor component of the reactor community, detected only during the start-up of the reactor, and was represented by the *Bacillaceae* (OTU BL2-17, 99% similarity with *Bacillus dipsosauri*) and *Enterococcaceae* (OTU BL2-19, 99% similarity with *Vagococcus* sp.) families (Table 2). Perhaps the presence of *Bacillus* sp. can be associated with the presence of *Symbiobacterium* sp. According to Ueda and Beppu (2007), ammonium, peptidic substances and amino acids generated by the metabolic activity of *Bacillus* sp. enhance the growth rate of *Symbiobacterium thermophilum* under an atmosphere of mostly CO_2, which is the case in an anaerobic digester.

Pseudomonas sp. was the only OTUs affiliated with *Proteobacteria*. This group decreased with time, eventually disappearing from the reactor once it reached steady-state conditions. The predominance of γ-*Proteobacteria* has been reported in a laboratory-scale anaerobic digester for the treatment of household solid waste (Cardinali-Rezende et al., 2009).

The sequences assigned to *Actinobacteria* were represented by different strains of *Actinomyces europaeus* (Tables 2 and 4). Member of this phylum was previously observed in anaerobic reactors at mesophilic temperatures (Chouari et al., 2005).

Interestingly, the emergence of some groups affiliated with the *Tenericutes* phylum, previously isolated from swine wasters (Table 4) and the candidate division WWE1 (Table 4), also identified in a wastewater reactor (Chouari et al., 2005), were detected in the reactor at steady-state conditions (DMSW4).

The predominance of fermentative acidogenic and hydrolytic bacteria was responsible for the increased VFA content (acetate, propionate and butyrate) in the reactor. The increase of the VFAs and the accumulation of ammonium (Table 1) are typical responses for a reactor during organic overloading. The increase of VFAs was accompanied by an increase of the bacterial and archaeal communities in the reactor (Table 5), particularly in the DMSW4 sample, which was removed when the reactor was completely full and had reached steady-state conditions. VFAs are produced during acidogenesis, and their high concentration could affect the MSW digestion and biogas production. However, the reactor stability was maintained and the digestion occurred normally because a constant pH was maintained (Table 1).

3.4. Archaeal diversity

A phylogenetic analysis of the OTU sequences from the archaeal libraries revealed that most of the OTU sequences (15 of 16) were affiliated with the *Euryarchaeota* phylum (Fig. 3 and Tables 2–4), which was represented by methanogenic archaea from the orders *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*.

The hydrogenotrophic methanogens dominated all of the samples, most of which included the *Methanomicrobiales* order (these OTU sequences were >97% similar to *Methanoculleus bourgensis* and *Methanoculleus* sp.). During the start-up phase and until the steady-state conditions were reached, the production of methane in the reactor was dominated by the syntrophic relationship between the hydrogenotrophic methanogenic archaea, particularly from the *Methanomicrobiales* order (*Methanoculleus bourgensis*) (Fig. 3 and Tables 2–4), and the acetate-reducing and propionate-oxidizing bacteria, which were previously observed by Shigematsu et al. (2006). In an another study, a tracer experiment using 13C-labeled acetate revealed that approximately 80% of the acetate was decomposed via a non-aceticlastic oxidative pathway, whereas the remainder was converted to methane via an aceticlastic path-

Table 4
Phylogenetic filiations and distribution of bacterial and archaeal clones analyzed from from digested municipal solid waste 4 (DMSW4).

Phylogenetic group	OTU	Clon No.	Closest sequence/microorganism	Order/Family	Acession No.	Identity (%)	Habitat of closest relative
Bacteroidetes	BL4-1	22	Uncultured bacterium clone TE-3-E11		JQ337397	97	Composting sample at 12 days
	2	11	Uncultured bacterium clone A35_D28_L_B_A07		EF559196	99	Mesophilic anaerobic solid waste digestor
	3	5	Uncultured *Bacteroidetes* bacterium	*Porphyromonadaceae*		97	Anaerobic digester which treats municipal wastewater sludge
	4	1	Uncultured bacterium clone HAW-R60-B-609d-C	*Porphyromonadaceae*	FN436026	97	Thermophilic biogas reactor fed with renewable biomass
	5	14	Uncultured *Bacteroidetes* bacterium clone De2105		HQ183932	99	Leachate sediment
Firmicutes Clostridia	6	2	Uncultured bacterium partial 16S rRNA gene, clone MS14623-B032	*Ruminococcaceae*	FN994085	99	Biogas completely stirred tank reactor
	7	2	Uncultured bacterium clone A55_D21_L_B_	*Ruminococcaceae*	EF559050	99	Thermophilic anaerobic digester at 55 °C
	8	1	*Clostridiales* oral clone P4PB_122 P3	*Ruminococcaceae*	AF538854	97	Periodontal microflora
	9	1	Uncultured bacterium clone E94		FJ205856	99	Biogas plant
Tenericutes	10	1	Uncultured bacterium clone 04g04	*Acholeplasmataceae*	GQ136883	99	ASBR reactor treating swine waste
Actinobacteria	11	1	*Actinomyces europaeus*	*Actinomycineae*	AM084230	97	Isolate CCUG 32789AT
Candidate Division WWE1	12	1	Uncultured WWE1 bacterium		CU917955	95	Mesophilic anaerobic digester which treats municipal wastewater sludge
	13						
Crenarchaeote	AL4-1	26	Uncultured crenarchaeote TREC89-34	Thermoproteales	AY487102	99	Tomato rhizosphere
Euryarchaeota-Methanosarcinalea	2	18	Methnosarcina siciliae	Methanosarcinaceae	MSU89773	97	Genomic DNA strain C2J
	3	1	Uncultured Methnosarcina sp.	Methanosarcinaceae	EU857628	98	Nisargruna Biogas Plant
	4	1	Uncultured Methanimicrococcus sp.	Methanosarcinaceae	AY487186	95	Food soil of Cubitermes fungifaber
	5	5	Uncultured Methanimicrococcus sp.	Methanosarcinaceae	JN173199	97	Low temperature anaeobic bioreactor
Euryarchaeota-Methanomicrobiales	6	25	Methanoculleus bourgensis	Methanomicrobiaceae	AB065298	99	Strain DSM 6216
Euryarchaeota-Methanobacteriales	7	2	Uncultured Methanobrevibacter sp.	Methanobacteriaceae	FJ919272	95	Rumen

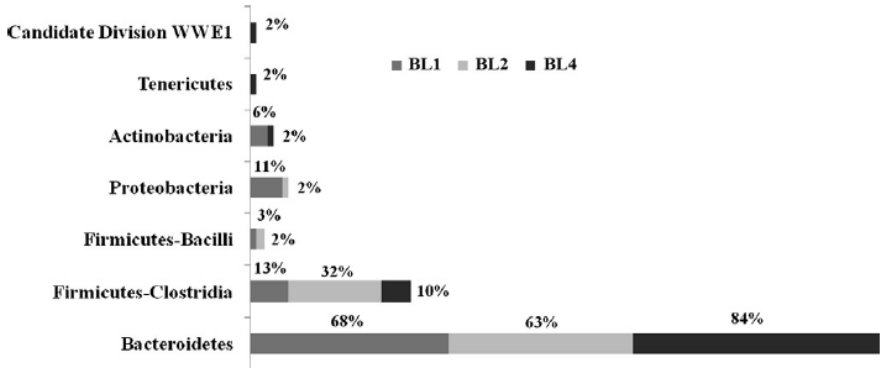

Fig. 2. Distribution of the bacterial clones in the DMSW libraries. BL1, BL2 and BL4 represent the 16S rDNA bacterial clone libraries from DMSW1, DMSW2 and DMSW4, respectively.

way (Sasaki et al., 2011). In the present study, archaeal 16S rRNA analyses demonstrated that the hydrogenotrophic methanogens *Methanoculleus* sp. accounted for >90% of detected methanogens, and the aceticlastic methanogens *Methanosarcina* sp. were the minor constituents. *Methanoculleus bourgensis* also predominated in other anaerobic reactors for the treatment of MSW (Weiss et al., 2008; Cardinali-Rezende et al., 2009; Nayak et al., 2009). *Methanoculleus* sp. requires H_2/CO_2, formate and some secondary alcohols as methanogenic substrates and acetate as a growth factor (Shigematsu et al., 2006).

Over time, some of the CO_2 that was required by the hydrogenotrophic methanogens may have been formed by the acetate-utilizing anaerobic bacteria through the cleavage of acetyl coenzyme A and the oxidation of the methyl and carbonyl groups of acetate, rather than via methanogenic acetate cleavage (Ferry, 1992). This hypothesis may explain the predominance of hydrogenotrophic methanogens in the MSW reactor. A loss of biodiversity in this group from the start-up to the steady-state conditions (88% of the OTUs in DMSW1, 54% in DMSW2 and 32% in DMSW4) was observed (Fig.3). This decrease was accompanied by the emergence of the *Methanobacteriales* order in the DMSW2 and DMSW4 samples (OTU ALA4-7, 95% similarity with *Methanobrevibacter* sp.). The members of this order are bacilli that utilize either H_2/CO_2 or formate as substrates for methanogenesis. The hydrogenotrophic *Methanobrevibacter* sp. (Fig. 3 and Tables 3 and 4) was also identified in a MSW laboratory-scale anaerobic reactor (Cardinali-

Table 5
Number of cell of bacteria and archaea using qPCR, FISH and CARD-FISH techniques.

Sample	Technique	Taxa					
		Bacteria	Archaea	Methanosaeta	Methanobacteriales	Methanomicrobiales	Methanosarcinaceae
DMSW1	qPCR	*2.3×10^{10} (12)	1.3x 10^{8} (21.5)	9×10^{6} (25.7)	(23)	(20)	
	FISH	1.52×10^{9}	3.15x 10^{8}	3.52×10^{7}			
DMSW2	qPCR	1.9×10^{10} (12.3)	4.4×10^{7} (23)	3.6×10^{7} (30)	1.4×10^{9} (21)	(21)	(25)
DMSW4	qPCR	1×10^{11} (10)	2.3×10^{10} (17.7)	1.4x10^{6} (28)	3.5×10^{7} (27)	(19)	(23)
	CARD-FISH	2.27×10^{12}	3.72×10^{11}				

Number in parentheses corresponds to the cycle where the maximum fluorescence crosses the log phase of amplification and the amount of amplicon is detected.
* Cells gr^{-1} of DMSW samples.

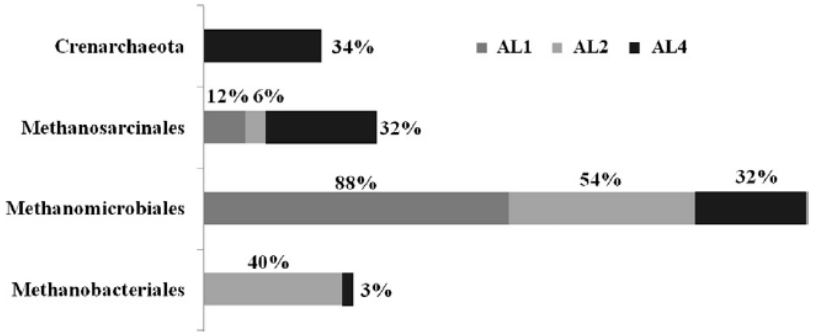

Fig. 3. Distribution of the archaeal clones in the DMSW libraries. AL1, AL2 and AL4 represent the 16S rDNA archaeal clone libraries from DMSW1, DMSW2 and DMSW4, respectively.

Rezende et al., 2009) and in a thermophilic MSW anaerobic reactor (Weiss et al., 2008).

Among the acetoclastic methanogens, the *Methanosarcinales* order was represented in the reactor by sequences from the *Methanosarcinaceae* family. According to Tables 2–4, a shift from *Methanosarcina barkeri* to *Methanosarcina siciliaea* was evident. Additionally, the emergence of OTUs with sequences >95% similar to uncultured *Methanimicrococcus* sp. in the DMSW4 sample was also observed. The genera *Methanosarcina* and *Methanimicrococcus*, both of which were identified in the DMSW samples and previously identified in a thermophilic MSW reactor (Weiss et al., 2008), are methanol consumers and may be competing for this substrate in the reactor. The *Crenarchaeota*-affiliated OTUs were found only in the DMSW4 sample and contained 33.5% of the sequences associated with the *Thermoproteales* order (Fig. 3 and Table 4).

In steady-state conditions and after the methanogenic community had matured and adapted to the MSW reactor, the production of methane was predominantly via the hydrogenotrophic pathway and only marginally through the acetilastic and methanol-degrading pathways.

3.5. Bacterial and archaeal 16S rRNA gene analysis using DGGE

The microbial diversity and the shifts in the bacterial and archaeal communities present in the DMSW1, DMSW2 and DMSW3 samples were observed using DGGE patterns of the partial 16S rRNA gene amplicons. Although the DGGE band patterns shared many of the same bands, some changes in the microbial communities are evident (Fig. 4). A total of 54 bands were excised from the DGGE fingerprints. However, only 17 DGGE bands from bacteria (DB) and four from archaea (DA) were successfully analyzed and phylogenetically identified.

The sequences chosen for analysis were affiliated with the *Firmicutes* and *Bacteroidetes* phyla. Although DGGE is not a quantitative technique, the greater fluorescence intensity of the *Bacteroidetes* bands indicated that this group was more prevalent, an observation that was also confirmed by the clone library analysis. The *Firmicutes* phylum was represented by bands only affiliated with the *Clostridia* class. Some sequence bands exhibited similarities to OTU sequences from the DMSW libraries, such as: DB1-8 (bacterial band 8 from the DMSW1 sample) and DB2-6 (bacterial band 6 from the DMSW2 sample), which displayed 92% identity with an uncultured *Symbiobacterium* sp. (OTU BL1-11, Table 2); and DB2-3 and DB3-9, which displayed 95% identity with the *Clostridiales* oral clone P4PB_12 identified in the OTU BL2-15 (Table 3).

Representatives of other phyla detected in the clone libraries were not identified using the DGGE technique. Some of the sequences from the fragments that migrated to different positions on the DGGE gel (Fig. 4), such as bands DB3-1 to DB3-7, exhibited the same phylogenetic affiliation.

The partial sequence bands of the archaeal communities were affiliated with the order *Methanosarcinales*. The bands DA1-3, DA2-5, and DA3-5 exhibited 99% identity with the uncultured *Methanosarcina barkeri* (Fig. 4), which was also identified in the OTU AL1-1 (Table 2).

A UPGMA cluster analysis of the bacterial and archaeal band sequences from the DMSW 1, 2 and 3 samples was performed to compare the communities in these samples. This analysis demonstrated that the prokaryote communities in DMSW1 were the most dissimilar observed in the dendrograms (Fig. S1).

3.6. Quantitative analysis of the microbial community using qPCR

The abundance of bacteria, archaea and methanogenic archaea in the digester during start-up (DMSW1 and 2) and steady-state conditions (DMSW4) is listed in Table 5. The amplification efficiencies were more than 95% with $r^2 > 0.99$. The bacterial and archaeal communities increased over time, and their cell numbers were one and two orders of magnitude higher, respectively, in DMSW4 than in DMSW1 (Table 5). The increase in these communities accompa-

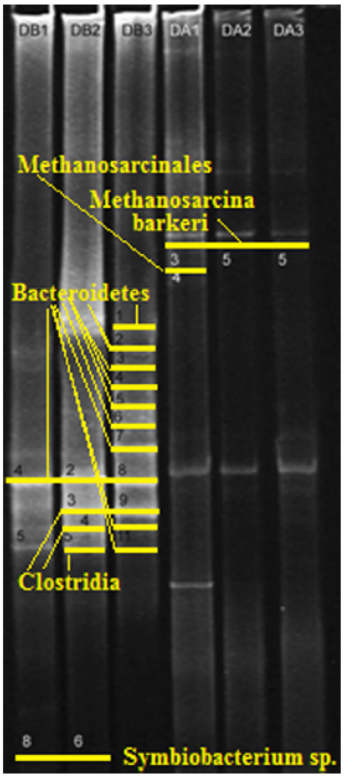

Fig. 4. DGGE temporal analysis of the Bacterial (DB) and Archaeal (DA) communities from the DMSW1, DMSW2 and DMSW3 samples (i.e., DB1 and DA1 are from the DMSW1 sample). The band fragments excised and their phylogenetic identities are represented by the numbers in each lane.

nied the daily increase of the reactor feed material up to complete capacity and the commencement of steady-state conditions in DMSW4.

The changes in the methanogenic archaeal cell counts (Table 5 and Fig. 5) and the substitution of these groups in the community were also monitored (Tables 2–4). The concentrations of hydrogenotrophic *Methanobacteriales*, and particularly *Methanomicrobiales*, were higher than the acetoclastic *Methanosarcina* sp. and *Methanosaeta* sp. The predominance of *Methanomicrobiales* was observed in the community throughout the operation of the anaerobic reactor (Fig. 3 and Tables 2–5). qPCR analysis revealed the increase of the *Methanobacteriales* community in DMSW2 and its decrease in the DMSW4 samples (Fig. 5 and Table 5) and the same phenomenon was observed in the AL libraries of these samples.

The composition of the aceticlastic methanogen community also shifted. The number of *Methanosarcina* sp. cells increased from DMSW2 to DMSW4 and coincided with the emergence of the methanogenic *Methanomicrococcus* sp. in the DMSW4 sample and with the increase of acetate concentration from the start-up phase (415 mg/L) to the steady-state phase (4100 mg/L) (Fig. 3 and Tables 1, 3 and 4). These genera compete when the acetate concentration is <500 mg/L (Jetten et al., 1992). *Methanosaeta* sp. exhibited low concentrations throughout the MSW treatment (Table 5). According to qPCR analysis and the library analysis of archaeal community, the hydrogenotrophic methanogens dominated in the reactor (Fig.3 and Table 5). In contrast, the dominance of *Methanosaetaceae* was previously demonstrated in wastewater sludge reactors (Diaz et al., 2006).

In this work, qPCR analysis of the *Bacteria* and *Archaea* domains uncovered and quantified the diversity of the communities in an environmental sample and incorporated a standard curve generated from a robust control: the PCR products from the pooled DNA of the three DMSW samples. In environmental samples, differences in the G + C content of the 16S rRNA gene in different prokaryotes may lead to the formation of more than one peak in the melting curve (Sharma et al., 2007), which occurred in the samples analyzed here. However, both the standard and the samples present the same melting curve profiles, which confirm the reliability of the results. In this work, shifts occurred in the archaeal community from DMSW1 to DMSW4 (Tables 2–4), being these changes also reflected in the melting curves generated by qPCR analysis. Additionally, the quantification of specific methanogenic archaea, which typically exhibit low biodiversity in anaerobic reactors, resulted in only one peak in the melting curve, as expected. An example was the *Methanobacteriales* order, whose sequences were dominated by *Methanobrevibacter* sp. (Fig. 5).

3.7. Quantitative analysis of the microbial community using FISH and CARD-FISH

FISH was successfully obtained and used to identify the relative abundances of *Bacteria*, *Archaea* and the methanogenic archaean *Methanosaeta* sp. in the DMSW1 sample. *Methanosaeta* sp. was detected only with the use of the more sensitive techniques in this study (qPCR and FISH); particularly in the DMSW1 sample (Table 5).

Several problems occurred during hybridization with the specific probes for other bacterial and archaeal groups in the DMSW1 sample. FISH was also performed with the DMSW2, DMSW3 and DMSW4 samples, as well as the positive and negative controls. However, only the positive controls were successfully hybridized. After several attempts and negative results, the CARD-FISH technique was used to hybridize the DMSW4 sample. The sample and positive control filters were treated with lysozyme and proteinase K to ensure sufficient permeabilization in any individual experiment and to interpret a negative result correctly. The positive controls hybridized with success, which suggests that the permeabilization procedure was sufficient for the bacterial and archaeal groups. However, only the probes specific to *Bacteria* (EUB338) and *Archaea* (Arch915) were successfully hybridized to the cells from the DMSW4 sample. Hybridization was not obtained for other microorganism groups. The negative controls with probe NON338 consistently yielded no fluorescently labeled cells. The MSW samples are characterized by the presence of humic acids, metals, colloids, and organic and inorganic substances, which could have prevented the penetration of the probes into the cells or hybridization with the probes or led to the loss of cell viability in the DMSW samples. These results confirm that pretreatment of the sample is a critical step that may greatly affect the measurement of bacterial and archaeal quantities.

A total of 1.8×10^9 cells g^{-1} was stained with DAPI in the DMSW1 sample. A total of 84% of the cells corresponded to the *Bacteria* (1.52×10^9 cells g^{-1}) and 16% to the *Archaea* domains (3.15×10^8 cells g^{-1}). The *Methanosaeta* sp. was comparatively rare (3.52×10^7 cells g^{-1}), which represented only 11% of the total archaeal cells. Using the CARD-FISH technique, a total of 3×10^{12} cells g^{-1} were stained with DAPI. A total of 86% of the cells that hybridized belonged to the *Bacteria* (2.27×10^{12} cells g^{-1}) and 14% to the *Archaea* (3.72×10^{11} cells g^{-1}). Several cell morphologies, such as rods, long-bowed rods and cocci that occurred singly, in pairs or in chains, were visible in the DMSW1 and DMSW4 samples. According to FISH and CARD-FISH, the total bacterial and archaeal cell numbers increased from DMSW1 to DMSW4, and a similar increase was detected using qPCR (Table 5).

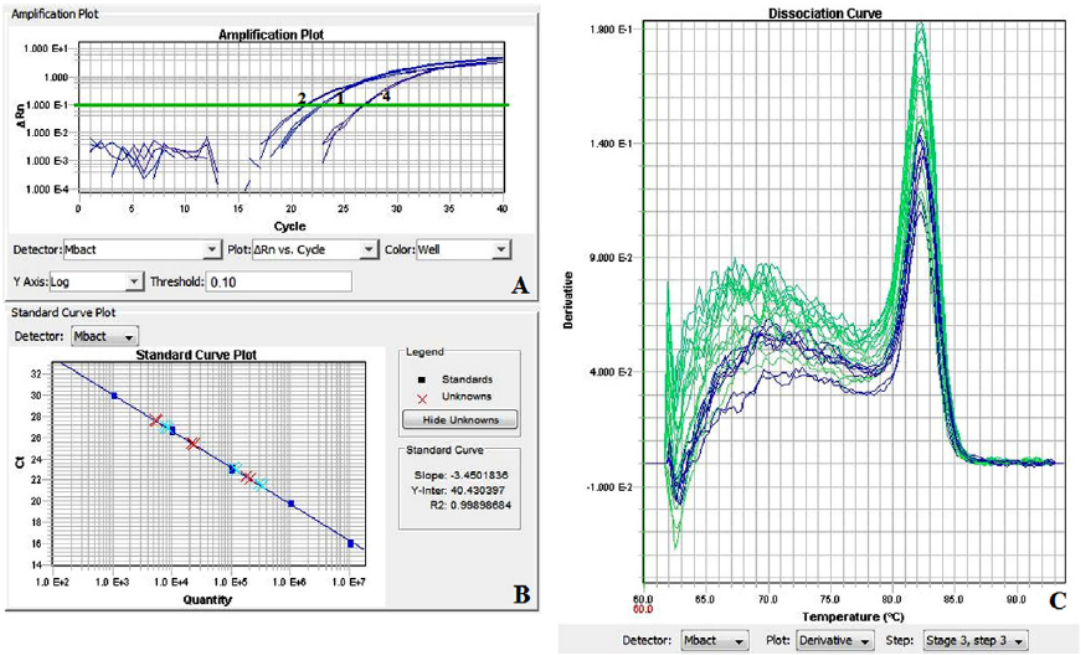

Fig. 5. qPCR analysis of the methanogenic archaeal *Methanobacteriales* order from the DMSW samples. (A) The amplification curves produced by the amplicons from DMSW1 (1), DMSW2 (2) and DMSW4 (4); (B) the standard curve produced from the standards and the sample amplicons; and (C) the melting curves obtained for the Methanobacteriales amplicons and the standards generated during the qPCR analysis of the DMSW samples (1, 2 and 4).

3.8. qPCR versus FISH and CARD-FISH

Variation in the relative abundances of the microbial cells in the digester was evaluated using several quantitative techniques. A comparison of FISH with qPCR (Table 5) revealed that in the DMSW1 sample the number of cells enumerated by FISH from the *Archaea* domain and *Methanosaeta* sp. was 2.4 and 4 times higher, respectively, than by qPCR. In contrast, for the *Bacteria* domain, the number of cells enumerated was one order of magnitude lower by FISH. In the DMSW4 sample, the bacterial and archaeal cell numbers were both one order of magnitude higher using CARD-FISH.

DNA extraction and purification may lead to a significant loss of DNA and considerable change in the quantification data obtained from qPCR. Overall, the hybridization by FISH was proportional to the intracellular level of rRNA, which was also proportional to the metabolic cellular activity in the sample (Wagner et al., 1994). In contrast, this observation was not true for CARD-FISH, which could explain the different results obtained from the use of both techniques.

The analysis of this microbial community revealed temporal shifts in the archaeal and bacterial populations during the operation of a reactor, a phenomenon that was observed earlier in pilot-scale solid waste reactors (Cardinali-Rezende et al., 2009).

4. Conclusions

Shifts in the prokaryotic community took place in a full-scale OF-MSW anaerobic reactor from start-up to steady-state conditions, increasing both bacterial and archaeal cell number over the time. The fermentatives *Bacteroidetes* and *Firmicutes* and the H_2-consumers methanogens *Methanomicrobiales* predominated. Aceticlastic methanogens *Methanosarcina* and *Methanimicrococcus* were identified mainly with the reactor working in steady-state conditions. *Methanosaeta* could be only detected by qPCR and FISH, revealing the sensitivity of these quantitative techniques. The use of several molecular tools to determine the microorganisms that performer the anaerobic digestion is a first effort at understanding and improving performance of anaerobic MSW digesters.

Acknowledgements

We are grateful to the Área de Gobierno de Medio Ambiente of Madrid and the Dirección General Parque Tecnológico Valdemingómez for allowing the collection of the MSW samples from the anaerobic digesters, to J. Antonio Fernández Rodríguez of the Planta de Biometanización Las Dehesas for his assistance, and to Fernando Carrasco of the Centro de Biología Molecular Severo Ochoa de la Universidad Autónoma de Madrid (CBMSO-UAM) for qPCR analysis support. We appreciate the financial support provided by Methanum Environmental and Energy Solutions, the Financiadora de Estudos e Projetos (FINEP), the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Pró-reitoria de Pesquisa da Universidade Federal de Minas Gerais (PRPq-UFMG) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) in the form of a scholarship to Juliana Cardinali Rezende.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.05.136.

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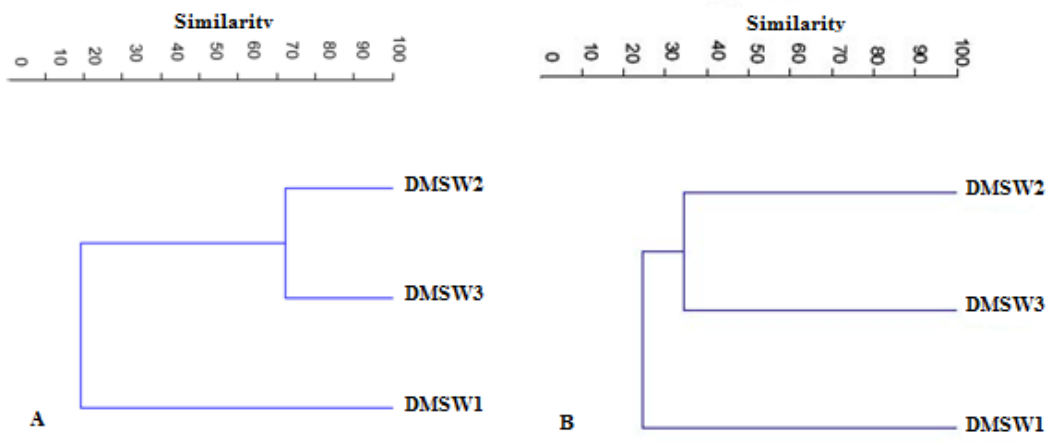
Supplementary Material

Table S1. Specific primers and probes used in several methods in this study.

Taxa/ Methods	Primer/ Probe	Sequence (5'- 3')	Annealing T (°C)*	Lenght of insert (pb)	Reference
Bacteria-Cloning	27F 1492R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	56	1.465	Lane et al. 1991
Archaea-Cloning	25F 1492R	CYGGTTGATCCTGCCRG TACGGYTACCTTGTTACGACTT	52	1.467	Lane et al. 1991
Bacteria- DGGE	341F 907R	CCTACGGGAGGCAGCAG CCGTCAATTCMTTTGAGTTT	52	566	Muyzer et al. 1993 /Lane et al. 1991
Archaea-DGGE	622F 1100R	TGAAATCYYRTAATCCC GGGTTGCGCTCGTTG	46	478	Lane et al. 1991
Clamp-GC- DGGE	Clamp GC	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC			
Bacteria-qPCR	338F 518R	TACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	60	180	Muyzer et al. 1993
Archaea-qPCR	344FM	ACGGGGCGCAGCAGG	60	174	Chan et al. 2002
	518R	ATTACCGCGGCTGCTGG			Muyzer et al. 1993
Methanosarcinaceae qPCR	MsarF MsarR	GGCACGAACCGGATTAGATA GACACCTAGCGAGCATCGT	60	60	This study
Methanosaeta sp. qPCR	McF McR	CAGGCGCGAAAACTTTACA AGATTTGTAACCTGGCACTCG	60	67	This study
Methanobacteriales qPCR	MbactF MbactR	TTAGAATAAGAGCTGGGCAAGAC ACTTGAGCTGCCGGTGTTAC	60	60	This study
Methanomicrobiales qPCR	MmicF MmicR	AGCAAACCGGATTAGATACCC AGCAAACCGGATTAGATACCC	60	61	This study
FISH/CARD-FISH:					
Bacteria	EUB338	GCTGCCTCCCGTAGGAGT			Amann, 1990
Archaea	ARC 915	GTGCTCCCCCGCCAATTCCT			Stahl and Amann 1991
Methanosaeta sp.	MX825	TCGCACCGTGGCCGACACCTAGC			Raskin et al. 1994
Negative control	NON 338	ACTCCTACGGGAGGCAGC			Wallner et al. 1993

*PCR conventional was conducted with initial cycle of 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at °C, extension at 72°C for 3 min (1 min for DGGE use) and post-elongation at 72°C for 10 min.

Figure S1. Dendrogram generated by the DGGE profile cluster analysis indicating the similarity of the Bacterial (A) and Archaeal (B) communities.



3.2 – CAPÍTULO II

Bacterial and archaeal phylogenetic diversity associated with swine sludge from an anaerobic treatment lagoon

World J Microbiol Biotechnol
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ORIGINAL PAPER

Bacterial and archaeal phylogenetic diversity associated with swine sludge from an anaerobic treatment lagoon

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Abstract Over the last decades, the demand for pork products has increased significantly, along with concern about suitable waste management. Anaerobic-lagoon fermentation for swine-sludge stabilization is a good strategy, although little is known about the microbial communities in the lagoons. Here, we employed a cloning- and sequencing-based analysis of the 16S rRNA gene to characterize and quantify the prokaryotic community composition in a swine-waste-sludge anaerobic lagoon (SAL). DNA sequence analysis revealed that the SAL library harbored 15 bacterial phyla: *Bacteroidetes*, *Cloroflexi*, *Proteobacteria*, *Firmicutes*, *Deinococcus-Thermus*, *Synergystetes*, *Gemmatimonadetes*, *Chlorobi*, *Fibrobacteres*, *Verrucomicrobia* and candidates division OP5, OP8, WWE1, KSB1, WS6. The SAL library was generally dominated by carbohydrate-oxidizing bacteria. The archaeal sequences were related to the *Crenarchaeota* and *Euryarchaeota* phyla. *Crenarchaeota* predominated in the library, demonstrating that it is not restricted to high-temperature environments, being also responsible for ammonium oxidation in the anaerobic lagoon. *Euryarchaeota* sequences were associated with the hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*). Quantitative PCR analysis revealed that the number of bacterial cells was at least three orders of magnitude higher than the number of archaeal cells in the SAL. The identified prokaryotic diversity was ecologically significant, particularly the archaeal community of hydrogenotrophic methanogens, which was responsible for methane production in the anaerobic lagoon. This study provided insight into the archaeal involvement in the overall oxidation of organic matter and the production of methane. Therefore, the treatment of swine waste in the sludge anaerobic lagoon could represent a potential inoculum for the start-up of municipal solid-waste digesters.

Keywords Anaerobic lagoon · Swine waste · Anaerobic digestion · 16S rRNA gene · Clone library · qPCR

Introduction

The size of swine-farming operations has increased significantly during the last few decades because of the high demand for pork products (Aranda and Cleary 2002). Accordingly, a large amount of animal waste is produced, and various microbiological and chemical contaminants can be released, leading to human exposure and the contamination of multiple environments. Swine waste is a significant source of fecal pollution of water, groundwater and soil and presents a risk to human health because this waste can harbor a variety of human pathogens (Guan and Holley 2003). Currently, a typical method for swine-sludge stabilization and to treat any animal waste is the anaerobic lagoon (AL), which is widely used in very countries as

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United States, Canada, China, Thailand, Vietnam, Denmark, Germany, India, Sweden, Zaire and many regions of Brazil mainly south region (UN Division of Sustainable Development 2000; US EPA 2003; Bunton et al. 2007). During storage, the effluent becomes anaerobic and supports the biomethanization (anaerobic digestion) of complex organic fractions by microorganisms. The AL offers a low-cost and effective way to treat wastewater that has a high organic content because the AL is mechanically simple, easy to operate by nonprofessionals, and requires less space than facultative and aerobic lagoons.

In AL, most of the bacteria in the liquor are free living, in contrast with the anaerobic reactor, in which the microbial community is associated in flocs; the internal conditions within the floc can be very different from the conditions in the liquor. The microbial dynamics in AL systems are less understood than the microbial dynamics in biological reactors (Grady et al. 1999; Arcand et al. 1994).

Sludge is typically stored in AL on a farm and is applied to agricultural lands as a valuable resource for crop fertilization and soil conditioning (Bunton et al. 2007). Previous work by Cardinali-Rezende et al. (2011) suggests that the microorganisms from the sludge of an anaerobic lagoon for treating swine waste are good candidates for the start-up inoculum in municipal solid waste (MSW) anaerobic reactors. The choice of inoculum is critical to the success of waste treatment, which is the result of the activities and interactions of highly diverse microbial communities—aerobic, facultative anaerobic and anaerobic—from the inoculum and organic material (Bell et al. 2005).

Over the past 25 years, diversity studies have primarily been based on analyses of 16S rRNA clone libraries. The frequent use of the 16S rRNA turned this sequence into a bacterial molecular signature. This was possible because no lateral gene transfer seems to occur between 16S rRNA genes, their structure contains both highly conserved and variable regions and sufficient genetic information to differentiate closely related microorganisms (Woese 1987), although sometimes not informative for species delination. Its application has become obligatory in taxonomy and it has been successfully used to elucidate phylogenetic relationships. Moreover, thousands of 16S rRNA gene sequences have been added to the databases and are available in widely accessible databases.

The knowledge of the microbial communities' composition is important for understanding their function in waste degradation and should aid in developing better strategies for the management and use of stored sludge (Bunton et al. 2007). For this purpose, the phylogenetic makeup of a 16S rDNA clone library and microbial real-time quantification were used to investigate the prokaryotic community of swine sludge from an anaerobic treatment lagoon.

Materials and methods

Anaerobic lagoon and sampling

Swine sludge samples at different sites from an anaerobic lagoon (SAL), at the R & M slaughterhouse, in Minas Gerais State, Brazil, were collected in triplicate and subsequently pooled into a single swine sludge sample. The anaerobic lagoon has a capacity of 2,041 m^3, and receives a daily effluent of 1,280 m^3. The samples were collected in sterilized glass bottles and stored on ice for up to 6 h before subsequent analysis in the laboratory. The sample was stored at −20 °C until DNA extraction.

Swine waste treatment and chemical analysis of SAL

At the R & M slaughterhouse, the swine waste was divided into green line (waste from the stomachs, preparation of bellies, corrals and pens) and red line (from skinning, a division of carcasses, beheading and cutting, deboning and sausage). The swine waste was preliminary treated, involving removal of solids by size, followed by the primary treatment that involved flotation and homogenization of the waste. After the treatments, the waste was mixed and pumped by air injection into the anaerobic lagoon (secondary treatment) for stabilization of the organic matter and reduction of the biochemical (BOD), chemical (COD) and oxygen (OD) demands of this sludge. The physical and chemical analyses of SAL were performed before and after treatment in the anaerobic lagoon for pH, temperature, COD, BOD, acetate, ammonium, propionate, butyrate, isobutyrate, isovalerate, valerate and nitrite. All analyses were conducted in triplicate according to Standard Methods (Apha 2005). The average data from triplicate assays and standard deviations were determined with Excel software. The variables (BOD, COD and pH) were compared using an unpaired *t* test, performed by GraphPad Software. The level of significance was considered at $p \leq 0.05$.

DNA extraction

The sludge sample was concentrated by centrifugation at 14,000×*g* and 4 °C for three rounds of 10 min. DNA was extracted from approximately 5 g (wet weight) of sludge using the MegaPrep Power Max™ Soil Kit (Mo Bio Laboratories) according to the manufacturer's instructions. Genomic DNA from pure cultures of *Escherichia coli* ATCC 25922 and *Halococcus morrhuaea* ATCC 17082 was extracted using phenol–chloroform according to the methods of Sambrook et al. (1989). Total DNA from the sludge and the genomic DNA concentrations were quantified by absorbance at 260 nm using a NanoDrop Spectrophotometer (NanoDrop Technologies). DNA purity was

assessed using the A260/A280 ratio. The DNAs were stored at −20 °C until further processing.

PCR amplification and clone library construction

Bacterial and archaeal 16S rRNA gene fragments were amplified by touchdown PCR using the conditions previously described by Cardinali-Rezende et al. (2009). The reactions were performed using the bacterial-targeted primer set 8F (5′-AGAGTTT GATYMTGGCTCAG-3′) and 907R (5′-CCGTCAATTCMTTTRAGT-3′; Lane 1991); and the archaeal-targeted primer set 21F (5′-CCGGTTGATCCYG CCGGA-3′) and 958R (5′-YCCGGCGTTGAMTCCAA TT-3′; De Long 1992). Amplicons were visualized on a 1 % agarose gel that was stained with ethidium bromide and then were purified directly with a QIAquick Gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cloning, the purified amplicons were cloned into the pJET1.2 vector from the CloneJET^{TM} PCR Cloning Kit (Fermentas, EUA) and were then transformed into electro-competent *E. coli* DH5α.

16S rRNA gene sequencing and phylogenetic analysis

Sequencing was performed in a MegaBace 1.000 capillary sequencer (Amersham Biosciences) using standard protocols and a DYEnamic ET dye terminator kit (Amersham Biosciences). The sequences were compared against sequences held in the Ribosomal Database Project II (RDPII) using Classifier and Seqmatch and against sequences held in GenBank using BLASTn. The 16S rRNA gene sequences were base-called, checked for quality, aligned, and analyzed with Phred v.0.020425, Phrap v.0.990319 and Consed 12.0 software. The Bellerophon program (Huber et al. 2004) was used to detect and omit chimeric DNAs. Phylogenetic relationships were inferred by the MEGA 4.1 (Tamura et al. 2007) molecular evolutionary genetics analysis program, using the neighbor-joining method (Saitou and Nei 1987). The DOTUR program (Schloss and Handelsman 2005) was used to assign sequences to operational taxonomic units (OTUs). Sequences were grouped into an OTU at distance values of 0.03 (≥97 % sequence similarity), which correspond to the species level. The DOTUR program was also applied to calculate rarefaction curves, richness, and diversity indices. The coverage of each clone library was calculated according to the method of Good (1953).

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences obtained in this study have been deposited in GenBank and assigned accession numbers JQ906944-JQ906965 (bacteria) and JQ906938-JQ906943 (archaea).

Quantitation by real-time PCR (qPCR)

The total abundance of bacterial and archaeal cells was estimated by a qPCR analysis of 16S rRNA genes. A limitation of this approach is the variation of the 16S-rRNA gene copy number per genome, which in bacteria, ranges from 1 to 15 depending on the ecological strategy (Lee et al. 2009). The number of copies of 16S rRNA in a cell generally increases with the growth rate, so the abundance of rRNA in an environmental sample is a function of both the growth rate and the population size of the microorganisms in the sample (Ward et al. 1992). With respect to the Archaea domain *Crenarchaeota* only one ribosomal operon copy is present (Lee et al. 2009). This suggests that the crenarchaeal 16S-rRNA gene copy numbers that were measured by qPCR may be truly representative of the cell numbers in the studied sample. However, among *Euryarchaeota*, affiliated with methanogenic archaeal, may possess up to four copies of 16S rRNA genes per genome (Lee et al. 2009). Hence, for the absolute quantification of bacterial and archaeal/methanogenic archaeal communities was considered to be 4 and 2.5 copies of 16S rRNA per cell, respectively, as suggested by Klamppenbach et al. (2001).

An ABI PRISM 7900HT SDS (Applied Biosystems, Foster City, CA, USA) sequence detection system was used for the absolute quantification of bacterial and archaeal cells in the SAL. The choice of primers can introduce a stronger bias than does the template, and thus the primers that were utilized in this study were degenerate primers that had been used with success in previous works. The bacterial primers 338F (5′-TACGGGAGGCAGCAG-3′) and 518R (5′-ATTA CCGCGGCTGCTGG-3′) (Muyzer et al. 1993) and the archaeal primers 344F (5′-ACGGGGCGCAGCAGG-3′) (modified from Chan et al. 2002) and 518R targeted the amplification of a 180-bp and 170-bp (approximate size) region of the 16S rRNA gene, respectively. Real-time PCR was performed using the SYBR Green PCR master mix (Applied Biosystems). The results were analyzed in an ABI PRISM 7900HT SDS sequence detection system (Applied Biosystems, Foster City, CA, USA).

Standard curves for quantitative PCR analysis

The efficiency of nucleic acid extraction and the reproducibility of standard curve reconstruction also can affect the outcome of qPCR. Therefore, the kit for total DNA extraction was the same as previously used and had a precipitation step that was necessary to remove the inhibitors that could affect the amplification by qPCR.

For absolute quantification of the bacterial and archaeal communities, genomic DNA from *E. coli* ATCC 25922 and *Halococcus morrhuaea* ATCC 17082 was used to amplify the partial 16S rDNA. Conventional PCR was performed using the same primers as previously described and was conducted using a MinicyclerTM PTC-100 (MJ Research Inc., Waltham, MA). The 16S rDNA partial amplicons were purified using 20 % polyethylene glycol (PEG 20 %/ NaCl 5 M, Sambrook et al. 1989). The DNA concentrations of the bacterial and archaeal amplicons were determined using a Nanodrop ND-1000 spectrophotometer. The number of copies of 16S rDNA was calculated using the URI Genomics & Sequencing Center's web site (http://www.uri.edu/research/gsc/resources/cndna.html). For all runs, the amplification of a serial dilution of PCR products (in triplicate) from 10^2 to 10^8 copies was used to generate standard curves.

Results

Physical and chemical characteristics of SAL

The physical and chemical characteristics of the swine waste before and after treatment in the anaerobic lagoon are shown in Table 1. The treatment of the swine waste in the anaerobic lagoon achieved a high COD and BOD removal efficiency of 55 % (Table 1). The nearly neutral pH of the medium favored waste degradation.

Table 1 Physical and chemical characteristics of swine waste before and after treatment in the anaerobic lagoon

Measurements	Before treatment	After treatment
BOD (mg/L)[a]	1314 (±143.5)[c]	546 (±63)[c]
COD (mg/L)[b]	2467 (±197.9)[d]	1110 (±117.6)[d]
pH	7.1 (±0.1)[e]	7.0 (±0.1)[e]
Temperature (°C)	35	35
Ammonium (mg/L)	NT	34.8 (±0.9)
Acetate (mg/L)	NT	<2.0
Propionate (mg/L)	NT	<2.0
Butyrate (mg/L)	NT	969.1 (±119.3)
Isobutyrate (mg/L)	NT	44.9 (±1.9)
Isovalerate (mg/L)	NT	70.3 (±2.7)
Valerate (mg/L)	NT	66.9 (±2.5)

[a] Biochemical oxygen demand

[b] Chemical oxygen demand; *NT* not tested. Values are the means of three different experiments and standard deviations

[c] Values significantly different ($p = 0.0011$)

[d] Values significantly different ($p = 0.0005$)

[e] No significant difference ($p = 0.2879$)

General features of the clone libraries

To determine the diversity of the SAL prokaryotic community, two clone libraries were constructed: anaerobic lagoon bacteria (ALB) and anaerobic lagoon archaea (ALA). A total of 103 clones were sequenced, yielding 28 OTUs after quality control and the removal of chimeric sequences. The rarefaction curves did not reach plateau with current sequencing efforts, indicating that further sequencing of more clone libraries would have revealed additional diversity. Nevertheless, the Good's coverage ≥87 % in both libraries suggested that the clone libraries captured the most frequent microorganisms (Fig. 1a, b and Table 2). Furthermore, the calculated diversity indices revealed that the bacterial clone library exhibited higher species richness than did the archaeal clone library.

Bacterial phylogenetic analysis

The bacterial 16S-rDNA clone sequences were distributed into 22 OTUs spanning 15 bacterial phyla. The relative abundance of the various phyla and the phylogenetic tree are shown Figs. 2 and 3, respectively. Most of the sequences presented a high identity to uncultured organisms obtained from several different environments (supplementary Table S1). *Bacteroidetes*, *Cloroflexi* and *Proteobacteria* accounted for the majority, whereas 36 % of the OTUs were affiliated with candidate divisions OP5, OP8, WWE1, KSB1, WS6, *Firmicutes*, *Deinococcus-Thermus*, *Synergystetes*, *Gemmatimonadetes*, *Chlorobi*, *Fibrobacteres* and *Verrucomicrobia*. The sequences belonging to the *Proteobacteria* phylum fell into two of the five classes: β- and δ–proteobacteria. The candidate division WWE1 was represented by one OTU with 99 % identity to *Candidatus Cloacamonas* spp. *Fribrobacteres* was represented by sequences related to *Fibrobacter* spp., and phylum *Chloroflexi* was represented by sequences affiliated with the *Anaerolineaceae* family.

Archaeal phylogenetic analysis

The archaeal library contained six OTUs that were assigned to two phyla *Crenarchaeota* and *Euryarchaeota* (Figs. 2 and 4). Most of the clones were affiliated with *Crenarchaeota*, and 71 % of the sequences were related to uncultured archaea with 91 % identity to *Desulfurococcales* order (Fig. 4 and supplementary Table S2). The *Euryarchaeota* phylum was represented by one OTU affiliated with the new lineage Arc I and by the hydrogenotrophic methanogens associated with *Methanomicrobiales* and *Methanobacteriales* orders. *Methanomicrobiales* was represented by sequences of uncultured archaeal clones, whereas one OTU was closely related to uncultured *Methanolinea* spp. The *Methanobacteriales* order

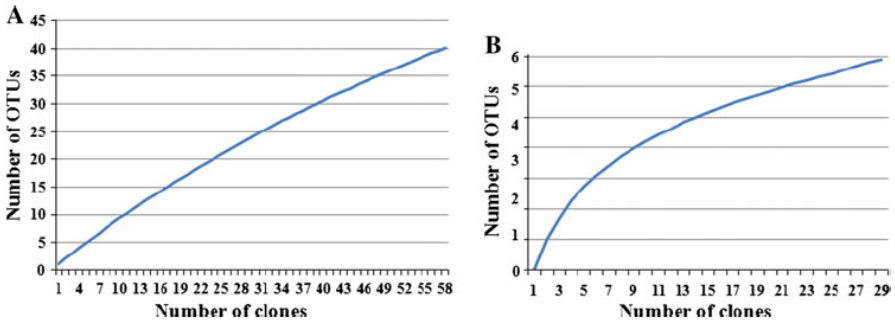

Fig. 1 Rarefaction analysis of the 16S rRNA genes from the bacterial (**a**) and archaeal (**b**) clone libraries that were generated from the SAL sample

Table 2 Sequence diversity and library coverage estimates

Parameters	Bacteria	Archaea
Clones	73	30
Chimera	15	2
OTUs	22	6
Shannon index diversity	3.36	1.19
Simpson index diversity	0.017	0.25
Good's coverage (%)	87	93

was represented by sequences belonging to OTUs related to *Methanobrevibacter* spp. and *Methanobacterium beijingense*.

Quantitative PCR

The total abundance of *Bacteria* and *Archaea* in the SAL was determined by qPCR amplification of the 16S rRNA genes using group-specific primers. Amplification efficiencies for all the reactions were high, with an R^2 value of 0.98 for the standard curve regression (supplementary S3, S4, S5 and S6). The abundance of *Bacteria* was greatest (6.5×10^9 16S-rRNA gene copies/g sludge or 1.56×10^9 cells of bacteria/g sludge [wet weight]) than *Archaea* (1.8×10^7 16S-rRNA gene copies/g sludge or 7.5×10^5 cells of archaea/g sludge [wet weight]). There was a difference of at least three orders of magnitude in the abundance of cells between the *Bacteria* and *Archaea* domains in SAL (supplementary S7 and S8).

Discussion

An important step in understanding any microbial ecosystem resides in our ability to first identify the microorganisms that inhabit the ecosystem and then to assess their metabolic potential, namely, the interactions between these microorganisms and their biotopes. Comprehensive investigations of the bacterial fermentation and methanogenic pathways in anaerobic treatment lagoons are scarce, whereas the wastes generated by swine are increasing. In a previous study (Cardinali-Rezende et al. 2011) based on a

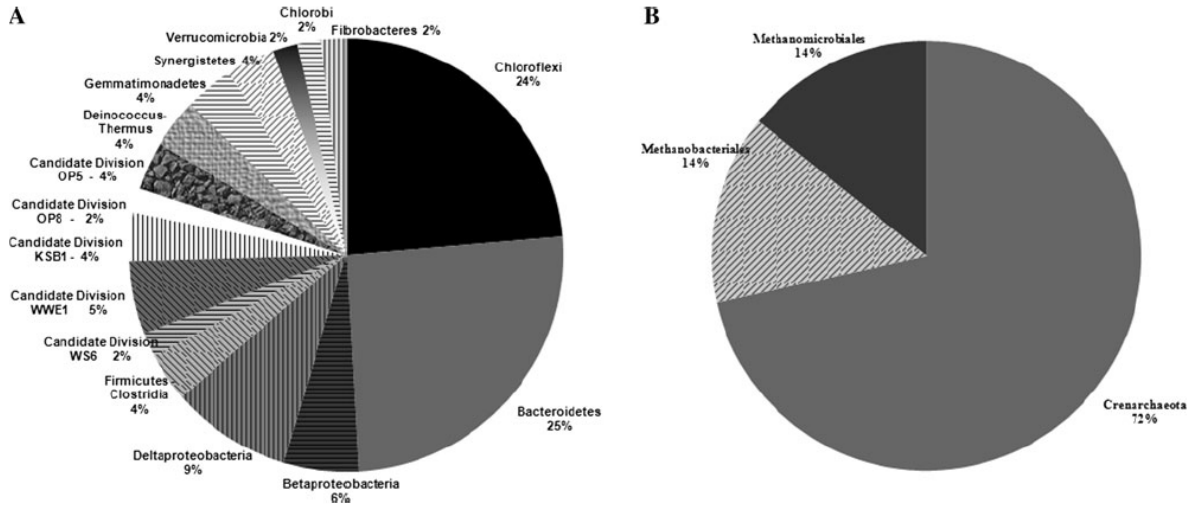

Fig. 2 Phylogenetic distribution of the bacterial and archaeal 16S rDNA sequences that were generated from the (**a**) ALB and (**b**) ALA libraries

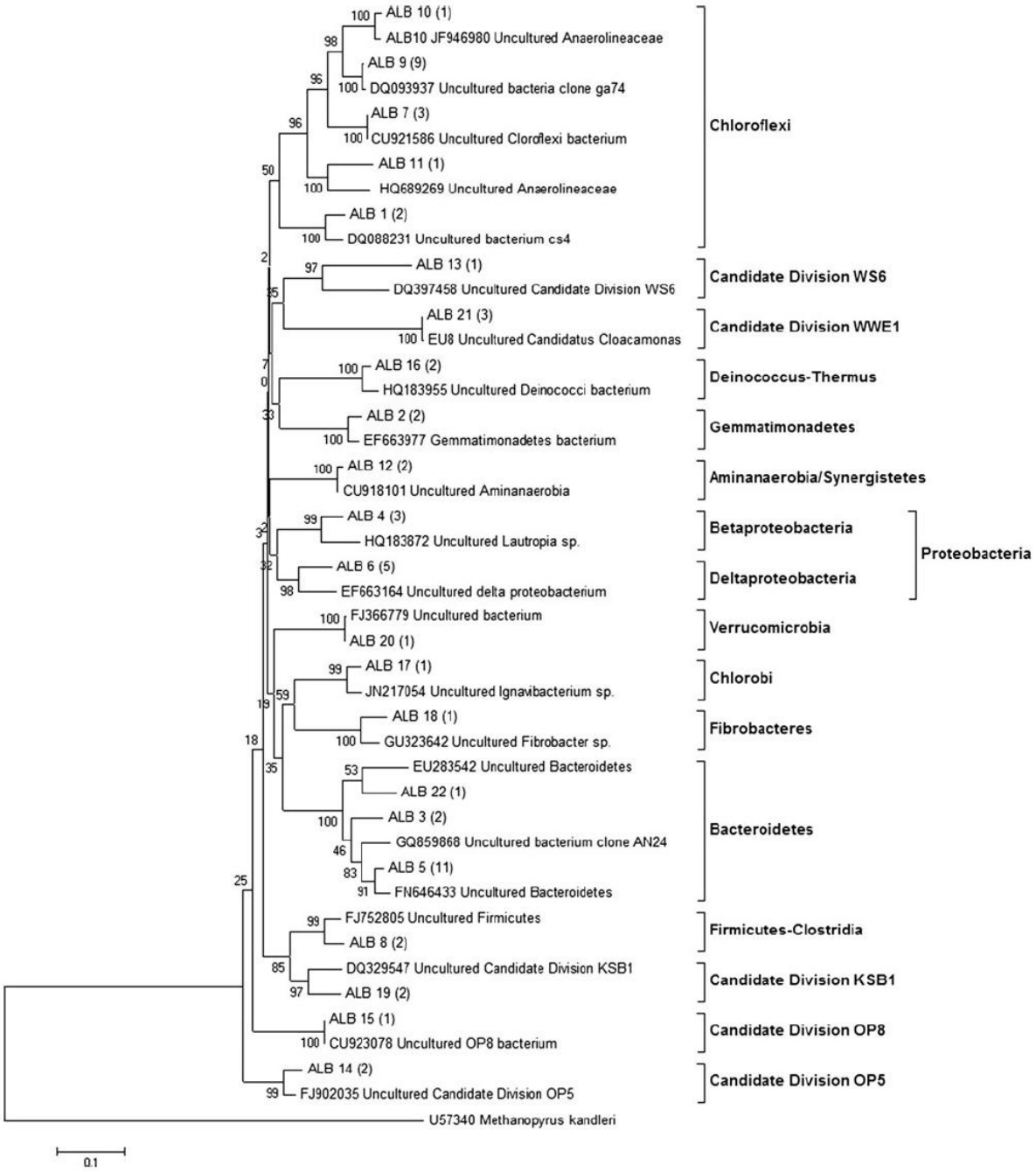

Fig. 3 Phylogenetic tree of the bacterial community, which was constructed using the neighbor-joining method. The numbers at the nodes indicate the percentages of occurrence in 1,000 bootstrapped trees. *Methanopyrus kandleri* (U57340) was used as the outgroup

culture-dependent approach, the bacterial community from this same sample (SAL) was characterized as the most phylogenetically diverse and as the second largest producer of biogas in the anaerobic digester that treated municipal solid waste—among the six inocula that were investigated. The present study extended the previous work (Cardinali-Rezende et al. 2011) and revealed an extremely diverse prokaryotic community, especially the *Bacteria* domain

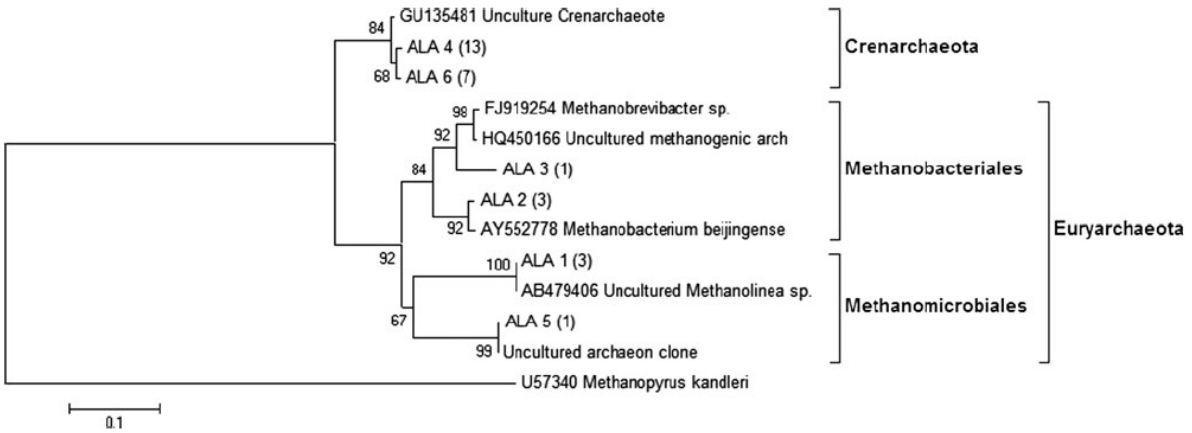

Fig. 4 Phylogenetic tree for the archaeal community, which was constructed using the neighbor-joining method. The numbers at the nodes indicate the percentages of occurrence in 1,000 bootstrapped trees. *Methanopyrus kandleri* (U57340) was used as the outgroup

that harbored 15 of the major lineages. The bacterial community associated with SAL was composed mainly of *Bacteroidetes*, *Chloroflexi* and *Proteobacteria*. The relative abundance of these phyla was similar to previous results that were obtained from anaerobic sludge (Rivière et al. 2009). Other studies have shown that carbohydrate-oxidizing *Bacteroidetes* and *Proteobacteria* predominate in the feces of pigs (Thompson and Holmes 2009) and wastewaters reactors (Figuerola and Erijman 2007). Representatives of the phylum *Chloroflexi*, which are filamentous green non-sulfur bacteria, have normally been found in diverse habitats such as municipal solid-waste digesters and activated sludges and have also been involved in the stabilization of flocs in wastewater digesters (Sekiguchi et al. 2001; Cardinali-Rezende et al. 2009).

In this study, candidate divisions WS6, OP5 and OP8 constituted a significant fraction of the bacterial 16S-rDNA clone libraries (Figs. 2, 3) and have been recovered from aquifers contaminated with hydrocarbons and from Yellowstone hot springs (Dojka et al. 1998; Hugenholtz et al. 1998). *Candidatus Cloacamonas* spp. belonging to the candidate division WWE1 are likely to be present in many anaerobic digesters and to be involved in the fermentation of amino acids, sugars, and carboxylic acids (Pelletier et al. 2008). The phyla that are less abundant, in particular, *Synergistetes*, *Deinococcus-Thermus*, *Verrucomicrobia*, *Chlorobi*, *Gemmatimonadetes* and *Fibrobacteres*, are ubiquitous to a wide variety of environments and play various roles in anaerobic digestion.

In relation to the *Archaea* domain, the phylum *Crenarchaeota* predominated in the SAL (Figs. 2, 4). Although previously considered present only in extreme habitats, 16S rRNA gene sequences have been recovered from low- to moderate-temperature environments: oceans, freshwater sediments, agricultural and forest soils, and rumen waste (Hershberger et al. 1996). *Crenarchaeota* is responsible for carbon fixation and ammonia oxidation in marine environments (DeLong et al. 1994), and its presence in anaerobic lagoons for treating swine waste could perhaps be explained by the ammonium concentration detected in the SAL sample (Table 1). Mesophilic *Crenarchaeota* affiliated with the *Desulfurococcales* order are anaerobic chemolithoautotrophic, fix carbon and use H_2 and reduced inorganic nitrogen as energy sources (Hallam et al. 2006).

Methane production in the anaerobic lagoon should be performed by hydrogenotrophic methanogens that are affiliated with the orders *Methanomicrobiales* and *Methanobacteriales* (Figs. 2, 4). The presence of hydrogenotrophic methanogens could be explained by the lowest acetate concentration and the great availability of other volatile fatty acids, such as butyrate, isobutyrate, isovalerate and valerate, in the SAL sample (Table 1). *Methanolinea* sp., also identified in cheese-waste anaerobic digesters, grow in H_2 and formate (Imachi et al. 2008). *Methanobacterium* spp. and *Methanobrevibacter* spp. grow in a H_2/CO_2 gas mixture, utilize formate, and some grow on simple alcohols. These microorganisms are common in mesophilic anaerobic digesters (Imachi et al. 2008; Cardinali-Rezende et al. 2009). *Methanobrevibacter* spp. are generally present in rumen wastes, and *Methanobacterium beijingense* is generally identified in anaerobic digesters (Cardinali-Rezende et al. 2009; Lin and Miller 1998). In the present study, the predominance of hydrogenotrophic methanogens suggests a high production of hydrogen by syntrophic bacteria during the organic acids degradation from swine waste. Furthermore, the syntrophic relationship between hydrogenotrophic methanogens and acetate-

oxidizing organisms could perhaps have been the main route of acetate degradation in this environment because *Methanosarcinales*, which are acetoclastic methanogens common in anaerobic digesters for treating swine waste, were not identified in this work. However, the aceticlastic pathway could be performed also by species affiliated with the lineage Arc I, which is a probably acetate consumer that can grow on formate and H_2/CO_2 and was early identified in sludge anaerobic digesters (Rivière et al. 2009). These results could explain why a MSW reactor operating with a SAL inoculum (Cardinali-Rezende et al. 2011) produced high levels of methane; the hydrogenotrophic methanogens that were mainly responsible for methane production in the solid-waste digesters predominated in the anaerobic lagoon that was investigated. In anaerobic lagoons for treating swine waste, a high ammonia concentration also could be concomitant with the increase of *Methanomicrobiales* representatives because these organisms are more resistant than aceticlastic methanogens, as has been observed in some mesophilic anaerobic digesters (Cardinali-Rezende et al. 2009; Schnurer et al. 1994; Angelidaki and Ahring 1993).

Conclusions

The bacterial community consisted mainly of the carbohydrate oxidizing bacteria that were affiliated with *Bacteroidetes*, *Chloroflexi* and *Proteobacteria*. This illustrates that these phyla could be mainly responsible for swine-waste treatment in anaerobic lagoons. The qPCR results demonstrated that the number of bacterial cells was higher than the number of archaeal cells in the SAL. Among the archaeal communities, *Crenarchaeota*, which are ammonia-oxidizing microorganisms, predominated, and methane was produced by hydrogenotrophic methanogens belonging to the *Methanomicrobiales* and *Methanobacteriales* orders. Many bacterial clones obtained in this study exhibited few similarities to known bacterial species. The results revealed a more comprehensive view of the complexity of the microorganisms that are involved in swine-waste degradation, as previously suggested by Cardinali-Rezende et al. (2011). In addition, this study indicates that the SAL could be used as an inoculum for MSW treatment because of community potential to degrade organic waste and to produce methane. However, the details of the functions of microbes in the waste's degradation in AL are not yet well defined, and further studies are thus necessary.

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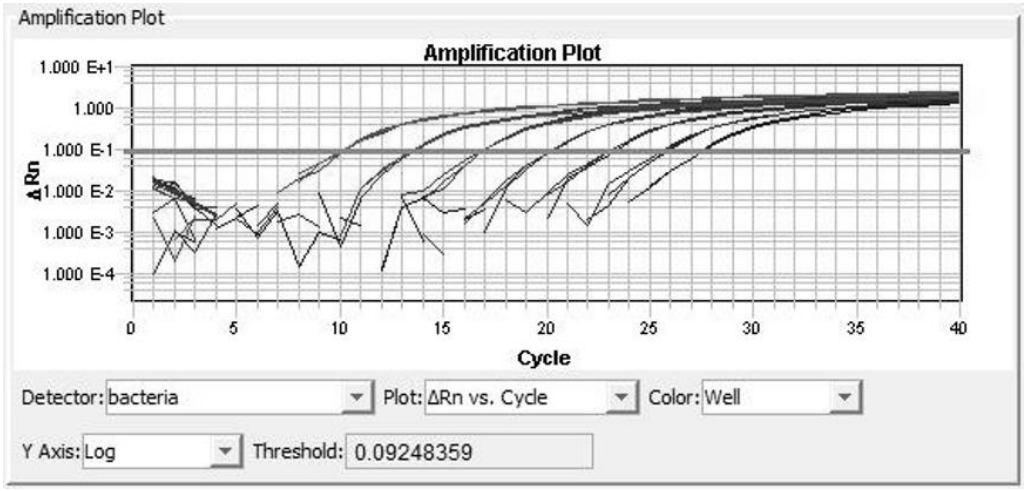
Supplementary Material

S1. Phylogenetic affiliation and distribution of the bacterial clones that were analyzed from the ALB library.

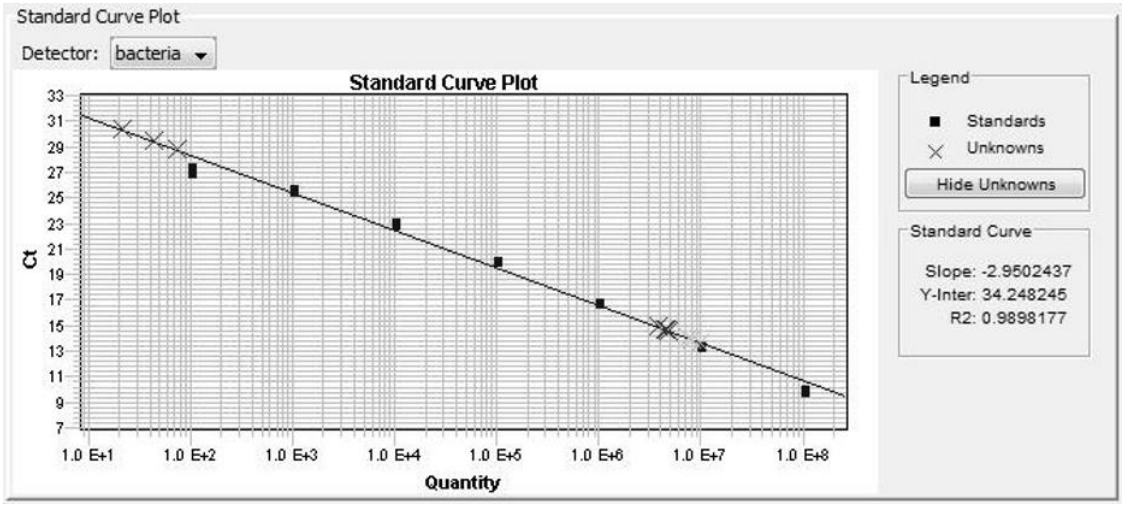
Phylogenetic group	OTU	Clones	Closest sequence/microorganism	Acession no.	Identity (%)	Habitat of closest relative
Chloroflexi	ALB_1	2	Uncultured bacterium clone cs4	DQ088231	97	Shuangcheng moat sediment
	ALB_7	3	Uncultured *Chloroflexi* bacterium	CU921586	98	Digester which treats municipal wastewater sludge
	ALB_9	9	Uncultured bacterium clone ga74	DQ093937	98	Rhizosphere
	ALB_10	1	Uncultured *Chloroflexi* bacterium	CU917947	99	Municipal wastewater sludge mesophilic anaerobic digester
	ALB_11	1	Uncultured unclassified bacterium	CU925480	99	Municipal wastewater sludge mesophilic anaerobic digester
Bacteroidetes	ALB_3	2	Uncultured bacterium clone 20	JN399114	99	Biogas plant
	ALB_5	11	Uncultured *Bacteroidetes* bacterium	FN646433	97	Limonene degrading methanogenic cultures
	ALB_22	1	Uncultured bacterium gene	AB559969	97	Marine sediments
Betaproteobacteria	ALB_4	3	Uncultured *Ralstonia* sp.	JN125279	95	Membrane biofilm reactor in drinking water
Deltaproteobacteria	ALB_6	5	Uncultured delta proteobacterium	EF663164	97	Water
Firmicutes-Clostridia	ALB_8	2	Uncultured *Firmicutes* bacterium clone NdGal47	FJ752805	95	Sediment from Canal S. Antoine, Gulf of Fos
Candidate Division WS6	ALB_13	1	Uncultured candidate division WS6	DQ397458	97	Hypersaline microbial mat
Candidate Division WWE1	ALB_21	3	Uncultured *Candidatus Cloacamonas* sp.	EU887773	99	Anaerobic digester
Candidate Division KSB1	ALB_19	2	Uncultured candidate division KSB1	DQ329547	90	Hypersaline microbial mat, Guerrero Negro
Candidate Division OP8	ALB_15	2	Uncultured candidate division OP8	CU923078	95	Municipal wastewater sludge mesophilic anaerobic digester
Candidate Division OP5	ALB_14	1	Uncultured OP5 bacterium	FJ902035	97	Biomat in the sediment of cenote La Palita
Chlorobi	ALB_17	1	Uncultured *Chlorobi* group	EU266920	97	Tar-oil contaminated aquifer sediments
Fibrobacteres	ALB_18	1	Uncultured *Fibrobacter* sp. clone	GU323642	95	Freshwater lake
Verrucomicrobia	ALB_20	1	Uncultured bacterium clone TS27_a03g04	FJ366779	99	Feces of adult twins and mothers
Synergistetes	ALB_12	2	Uncultured *Aminanaerobia* bacterium	CU918101	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
Gemmatimonadetes	ALB_2	2	Uncultured *Gemmatimonadetes* bacterium	EF663977	97	Grassland at the GASP KBS-LTER sampling
Deinococcus-Thermus	ALB_16	2	Uncultured *Deinococci* bacterium	FN598001	97	Leachate sediment

S2. Phylogenetic affiliation and distribution of the archaeal clones that were analyzed from the ALA library.

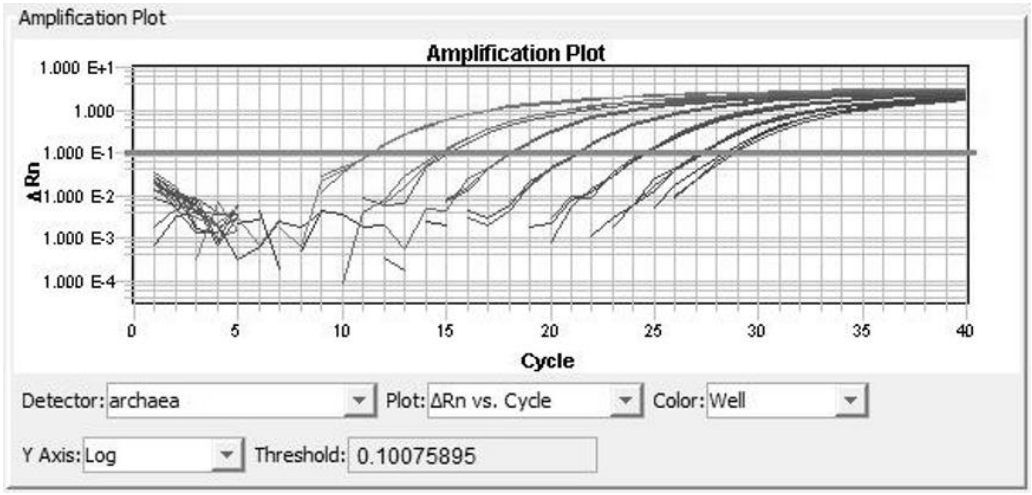
Phylogenetic group	OTU	Clones	Closest sequence/microorganism	Acession no.	Identity (%)	Habitat of closest relative
Crenarchaeota	ALA_4	13	Uncultured *Crenarchaeotes* archaeon	CU917220	99	Anaerobic digester which treats municipal wastewater sludge
	ALA_6	7	Uncultured *Crenarchaeote* clone Pav-sed-501	GU135481	99	Lake Pavin sediment
Euryarchaeota-Methanobacteriales	ALA_2	3	*Methanobacterium beijingense*	AY552778	99	Anaerobic digester
	ALA_3	1	Uncultured *Methanobrevibacter* sp.	FJ919254	96	Rumen
Euryarchaeota-Methanomicrobiales	ALA_1	3	Uncultured *Methanolinea* sp.	AB479406	100	Methanogenic propionate-degradation enrichment culture
Euryarchaeota- Arc I	ALA_5	1	Uncultured ArcI archaeon	CU916164	98	Mesophilic anaerobic digester which treats municipal wastewater sludge



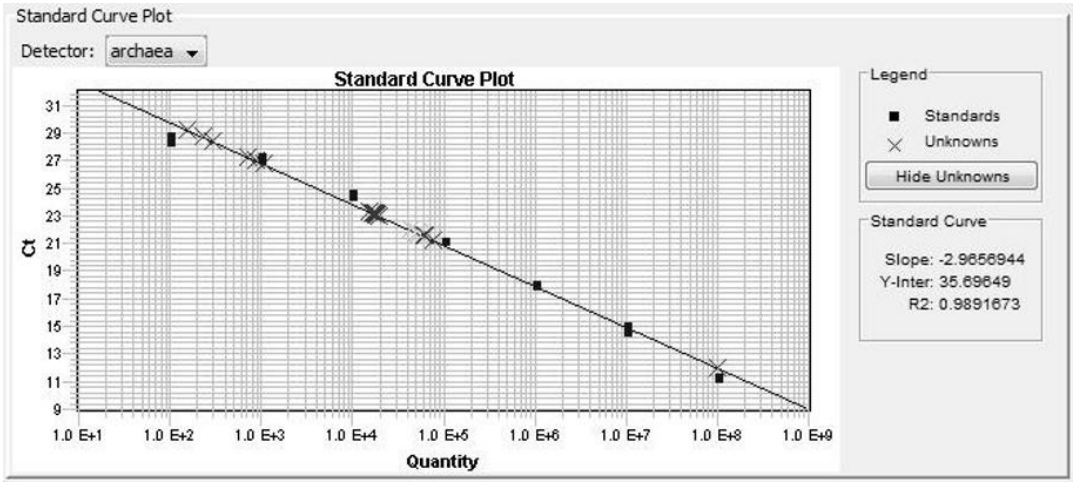
S3. Quantification of *E. coli* ATCC 25922 using qPCR. Relative fluorescence intensity of seven standard solutions of ATCC 25922 throughout the amplification cycles; Cq represents the threshold cycle number.



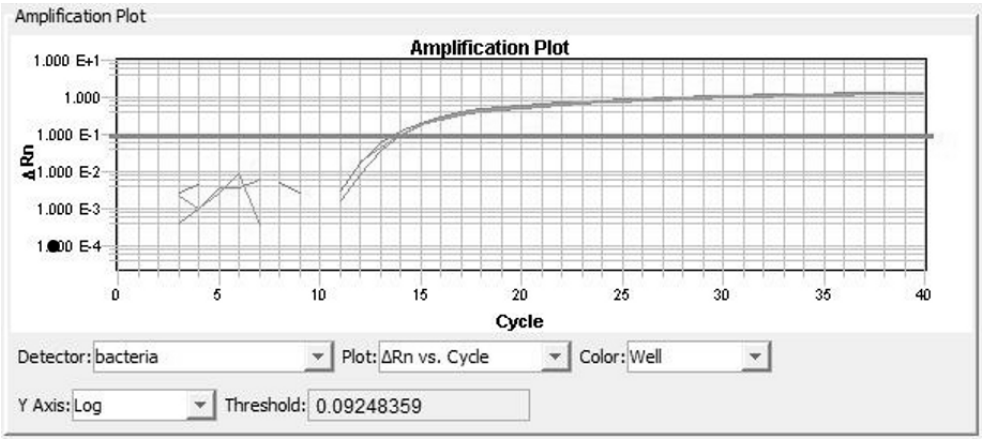
S4. Standard curve for the qPCR measurement of *E. coli* ATCC 25922 and bacterial 16S rDNA genes from the SAL sample.



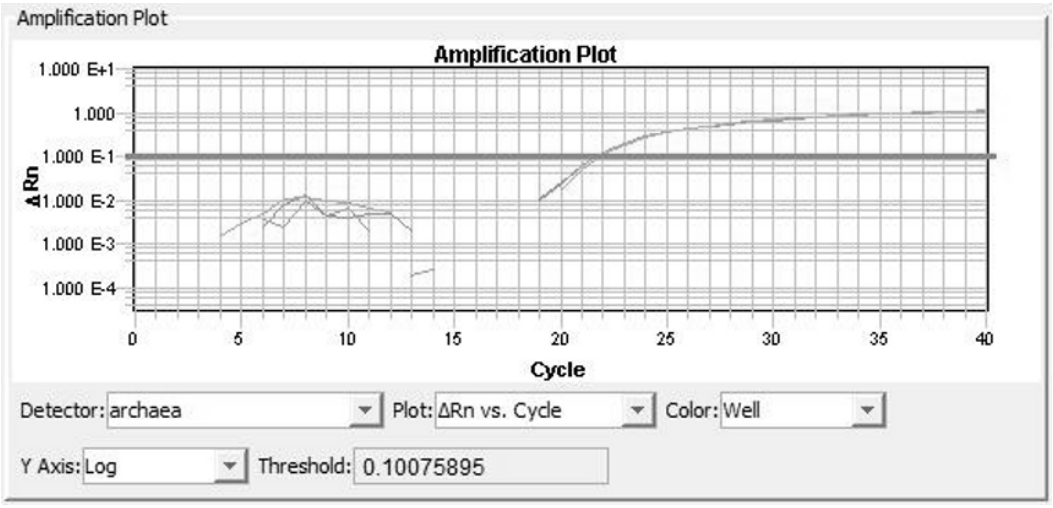
S5. Quantification of *Halococcus morrhuaea* ATCC 17082 using qPCR. Relative fluorescence intensity of seven standard solutions of ATCC 217082 throughout the amplification cycles; Cq represents the threshold cycle number.



S6. Standard curve for qPCR measurement of *Halococcus morrhuaea* ATCC 17082 and archaeal 16S rDNA genes from the SAL sample.



S7. Quantification of bacterial 16S rDNA genes from the SAL sample using qPCR. Relative fluorescence intensity of the sample amplification cycle; Cq represents the threshold cycle number.



S8. Quantification of archaeal 16S rDNA genes from the SAL sample using qPCR. Relative fluorescence intensity of the sample amplification cycle; Cq represents the threshold cycle number.

3.3 – CAPÍTULO III

Organic loading rate and food-to-microorganism ratio shape prokaryotic diversity in a demo-scale UASB reactor treating domestic wastewater

Artigo submetido.

Organic loading rate and food-to-microorganism ratio shape prokaryotic diversity in a demo-scale up-flow anaerobic sludge blanket reactor treating domestic wastewater

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Running title: Shifts in communities in UASB reactor

ABSTRACT

We investigated the microbial community in an up-flow anaerobic sludge blanket (UASB) reactor treating domestic wastewater (DW) during two different periods of organic loading rate (OLR) and food to-microorganism (F/M) ratio. 16S rDNA clone libraries were generated, and quantitative real-time PCR (qPCR) analyses were performed. Fluctuations in the OLR and F/M ratio affected the abundance and the composition of the UASB prokaryotic community, mainly at the species level, as well as the performance of the UASB reactor. The qPCR analysis suggested that there was a decrease in the bacterial cell number in the sludge sampled at rainy season (SR), when the OLR and F/M ratio were lower. However, the bacterial diversity was higher during this time, suggesting that the community became specialized in degrading more diversified substrates. The diversity and the abundance of the archaeal community were higher when the F/M ratio was lower. Shifts in the methanogenic community composition might have influenced the route of methane production, with methane produced by acetotrophic methanogens (sludge sampled at dry season, SD), and hydrogenothrophic, methylotrophic and acetotrophic methanogens (SR). This study revealed higher levels of bacterial diversity, metabolic specialization and COD removal efficiency of the DW UASB reactor during the rainy season,

INTRODUCTION

During the past two decades, anaerobic systems, such as the up-flow anaerobic sludge blanket (UASB) reactor, have been successfully applied and widely accepted for the treatment of industrial and domestic wastewaters, in many countries (Hammes et al. 2000). The anaerobic treatment of municipal wastewaters not only protects the environment but also produces considerable amounts of energy and a chemical oxygen demand (COD) removal of above 70% (Urban et al. 2007).The functioning of a UASB reactor depends on physical, chemical and biological parameters such as wastewater composition, organic loading rate (OLR), hydraulic retention time (HRT), concentration of volatile fatty acids (VFAs), pH, temperature, and the operating conditions as a whole. These parameters reflect the biological processes in the UASB reactor and are closely related to the relative abundance and composition of the prokaryotic communities and their metabolism, which affect the conversion of organic compounds into methane (Mahmoud et al. 2003). Previous studies have demonstrated that the domestic sewage undergoes variations due to cyclical nature of human activities, tourist seasons, runoff water and rainfall contributions, resulting in overloads in networks as well as in the treatment plants, among others (Metcalf and Eddy 1991). In addition, some studies have indicated a relationship between the organic load, pH, temperature and carbon source variations with bacterial abundance, community structure, and function (van Haandel and Lettinga 1994; Schmidt and Ahring 1997; Alves et al. 2000).

Many researchers have used molecular techniques based on the 16S rRNA gene for the identification and quantification of the microbial groups in anaerobic reactors that are operated at various temperatures (Levén et al. 2007) to treat different types of wastewater, such as industrial (Díaz et al. 2006), domestic (Lucena et al. 2011; Shin et al. 2011) and synthetic (Roest et al. 2005). However, studies on microbial community composition and diversity in full- and demo-scale domestic wastewater treatment systems are scarce (Lucena et al. 2011; Wan et al. 2011).

Therefore, we investigated a demo-scale UASB system for domestic wastewater (DW) treatment and the impact of different OLR and F/M ratiosratios, in the dry and rainy seasons, on the bacterial and archaeal communities composition and diversity. For this purpose, molecular analyses, including the sequencing of 16S rRNA gene clone libraries and quantitative real-time PCR (qPCR), were performed.

MATERIALS AND METHODS

Reactor conditions and chemical analyses

The mesophilic UASB reactor (demo-scale) used in this study is located at the Arrudas Wastewater Treatment plant, which receives the major part of the domestic wastewater (DW) of the urban area of Belo Horizonte city (Brazil). The reactor was fed on a small fraction of the wastewater (population equivalent to 300 inhabitants) taken from a chamber upstream the primary clarifiers of the full-scale plant, after being submitted to preliminary treatment for solids and grit removal. The main characteristics of the UASB reactor are: 4.8 m height with a useful volume of 16.8 m^3, hydraulic retention time (HRT) of 8 to 10 hours and OLR ranging from 0.8-1.2 (rainy season) to 1.2-1.6 (dry season) kg COD $m^{-3}d^{-1}$. The reactor was operated at room temperature ($\pm$ 25°C). During the operational period (May 2010 to February 2011), physical and chemical analyses were performed two to three times per week on the influent and the UASB effluent. The following parameters were determined: total suspended solids (TSS), volatile suspended solids (VSS), pH, temperature, chemical oxygen demand (COD), and biochemical oxygen demand (BOD). The concentrations of ammonium-N, acetate, propionate, butyrate, isobutyrate, valerate and isovalerate were also measured. All analyses were performed according to *Standard Methods* (APHA, 1992). Biogas composition was analyzed by using a gas chromatograph (Perkin-Elmer Autosystem XL) equipped with a thermal conductivity detector (TCD) and a 2m x 2mm inside diameter (ID) stainless-steel column packed with Poropak Q

(80/100 mesh). The temperatures of oven and detector were kept at 60°C and 150°C, respectively.

Sampling

Sludge samples (2 L) were taken from the mesophilic UASB reactor with sterilized bottles at four different points 50 cm from each other along the reactor depth. Further the samples were pooled in a single sample to better represent the composition of the whole microbial community in the reactor. The collections occurred in September of 2010 (sludge sampled from dry season - SD) and January of 2011 (sludge sampled from rainy season - SR), when the accumulated rainfall was 70.5mm and 720mm/month (http://www.climatempo.com.br/destaques/tag/belo-horizonte/), respectively. The samples were stored at −20°C until DNA extraction.

Nucleic acid extraction

DNA was extracted from 5 g (wet weight) of SD and SR samples using the MegaPrep Power Max™ Soil KIT (Mo Bio Laboratories, USA) according to the manufacturer's instructions. The total DNA obtained from the samples was quantified by measuring the absorbance at 260 nm using a NanoDrop Spectrophotometer (NanoDrop Technologies). The purity of the DNA was assessed by the ratios of A260/A280 and A260/A230. The isolated DNA was stored at −20°C until further processing.

PCR amplification and clone library construction

Bacterial and archaeal 16S rRNA gene fragments were amplified by touchdown PCR using conditions previously described by Cardinali-Rezende et al. (2009). The reactions were carried out in a Mini-cycler^{TM} PTC-100 (MJ Research Inc., Waltham, MA) with the bacterial-targeted primer set 8F (5′-AGAGTTT GATYMTGGCTCAG-3′) and 907R (5′-CCGTCAATTCMTTTRAGT-3′; Lane 1991) and the archaeal-targeted primer set Arc21F (5′-CCGGTTGATCCYGCCGGA-3′)

and Arc958R (5′-YCCGGCGTTGAMTCCAATT-3′; Delong 1992). The amplicons were visualized after electrophoresis in a 1% agarose gel stained with ethidium bromide and purified directly with the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cloning, the purified amplicons were cloned into the pJET1.2/blunt Cloning Vector from a CloneJET™ PCR Cloning Kit (Fermentas, EU) per the manufacturer's instructions and transformed into electrocompetent *Escherichia coli* DH5α. The plasmids containing 16S rDNA inserts were extracted using a standard alkaline lysis method (Sambrook et al. 1989), and the inserts were subsequently PCR-amplified with specific forward and reverse primers to the pJET1.2 vector for sequencing.

16S rRNA gene sequencing and phylogenetic analysis

The sequencing was performed in a MegaBace 1.000 capillary sequencer (Amersham Biosciences) using a DYEnamic ET dye terminator kit (Amersham Biosciences) according to standard protocols. The Classifier tool at the Ribosomal Database Project (RDP) and BLASTN were used to assign the 16S rRNA gene sequences from clone libraries to taxonomic levels. To accomplish this, the 16S rRNA gene sequences were base-called, checked for quality, aligned, and analyzed with Phred, version v.0.020425, Phrap, version v.0.990319 and Consed, version 12.0 software. The Bellerophon program (http://compbio.anu.edu.au/bellerophon/bellerophon.pl) was used to detect and omit chimeric DNA sequences. The vector sequences were removed using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Phylogenetic relationships were inferred by MEGA 4.1 software using the neighbor-joining method (Saitou and Nei 1987) and Kimura's 2-P model of sequence evolution. DOTUR software was used to assign the sequences to operational taxonomic units (OTUs) with a distance value of 0.03 (≥97% sequence similarity), which corresponds to the species level (Schloss and Handelsman 2005). The library coverage was calculated using the equation $C = 1-(n/N)$, where n denotes the

number of unique OTUs and *N* is the number of sequences analyzed in the library (Good 1953). The Chao1 and ACE estimators were applied for the species richness estimation, and the Shannon and Simpson indices were calculated for the diversity estimation with 97% confidence intervals. LIBSHUFF analysis for the difference between clone libraries was performed in the same way as described elsewhere (Reis et al. 2013). The nucleotide sequences generated were deposited in the GenBank database under the accession numbers JX301546 - JX301650 and JX301651 - JX301663 for the 16S rRNA gene sequences from *Bacteria* and *Archaea*, respectively.

Real-time PCR amplification (qPCR)

The quantification of the bacterial and archaeal communities was performed with qPCR using an ABI PRISM 7900HT SDS (Applied Biosystems, Foster City, CA, USA). The reactions had a final volume of 10 µL and consisted of 5 µL of SYBR Green PCR master mix (QIAGEN, USA), 0.5 µL of each primer (2.5 mM), 1 µL of the total DNA.

The qPCR amplification was performed with 40 cycles of 20 s for denaturation (95°C), 20 s at the annealing temperature (60°C) for the *Bacteria* and *Archaea* domains and 120 s for elongation (72°C). The amplifying primer sets of 338F (5'-TACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993) and 518R (Pauss et al. 1990) and 344FM (5'-ACGGGGCGCAGCAGG -3') (modified from Chan et al. 2002,) were used for the amplification of the 16S rRNA genes from the *Bacteria* and *Archaea*, respectively. A control reaction without template DNA was included in each real-time PCR assay. All DNA samples and the negative control were analyzed in triplicate.

The results were analyzed using an ABI PRISM 7900HT SDS (Applied Biosystems, Foster City, CA, USA) based on the melting curves generated. The relative quantitative analysis of bacteria and archaea communities was obtained from the amplification curves. C_t, which is the cycle where the maximum fluorescence crosses the log phase of amplification and the

amount of the amplicon is detected, was considered to compare the relative quantification of prokaryotic cells detected in the SD and SR samples.

RESULTS

Performance of UASB reactor

The physical and chemical characteristics of the DW before and after treatment, as well as those of the SD and -SR samples, are presented in Table 1.

Table 1. Physical-chemical analysis of domestic wastewater before and after the treatment and samples collected in the UASB reactor.

Parameters*	**Dry season**			**Rainy season**		
	Influent	**Effluent**	**SD**	**Influent**	**Effluent**	**SR**
TSS (mg/L)	200	62		238	65	
VSS (mg/L)	178	53		188	48	
pH	7.2	6.7		6.9	7.0	
Temperature (°C)	22	24		27	26.5	
BOD(mg/L)	193	105		63	66	
COD (mg/L)	516	256		363	136	
Alkalinity (mg CaCO3/L)	250.4	319.4		166.3	206.1	
Ammonium-N (mg/L)	37	47		22	29	
Propionate (mg/L)	-	-	5.56	-	-	2.38
Estimation of biogas production (m3/day)	2.0 - 4.0 m³/d			1.0 - 2.5 m³/d		
Estimation of methane production (kg/day)	1.5 - 3.0 kg/d			0.95 - 2.0 kg/d		
Biological load (food/microorganisms)	0.17 gCOD/gVS			0.12gCOD/gVS		

* **Influent:** domestic wastewater to be treated; **Effluent:** domestic wastewater treated; SD: sludge collected inside the reactor during dry season; SR: sludge collected inside the reactor during rainy season; Total suspended solids (TSS), volatile suspended solids (VSS), biochemical oxygen demand (BOD) and chemical oxygen demand (COD), biological load (is the relation between OLR (kgCOD/m3.d) applied to the reactor/average value of volatile solids-VS inside the reactor (kgVS). For the dry season the average OLR was 1.44kgCOD/m3.d and the average sludge mass (VS) was 140.2 KgVS. For the rainy season, the average OLR was 1.08kgCOD/m3.d and the average sludge mass (VS) was 148.52 KgVS. The estimation of biogas production and methane production was performed using a model based on total COD apllied to the reactor, according to Lobato et al. (2013).

Based on the influent and effluent COD concentrations (Fig. 1), the average COD removal efficiencies were 50 and 62%, for dry and rainy seasons, respectively.

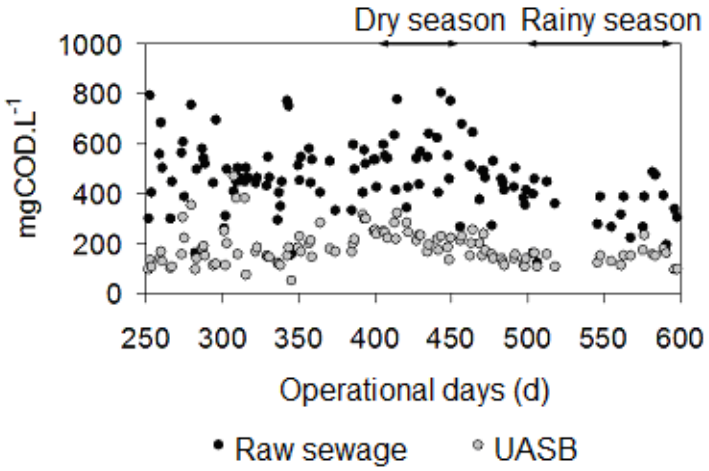

Fig. 1. Time course of total chemical oxygen demand (COD) for raw sewage and UASB effluent during the operational period investigated.

The OLR and the F/M ratio (gCOD/gVS.d) also varied between the seasons (Fig. 2), showing higher values in the dry season. This result indicates that the relation food/microorganism (F/M) varied and lower values were observed in rainy season. This could be due to the fact that sewage is more diluted in the rainy season (because of rainwater intrusion into the sewage). Although this is not a combined sewer overflow, the illegal rainwater drainage connections in sewers is frequently observed, and it has a significant influence on influent flow rate and OLR over the year.

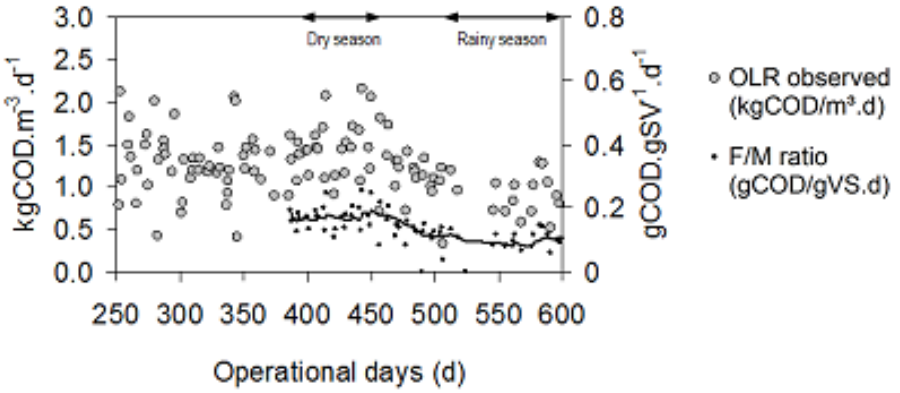

Fig. 2. Organic loading rate (OLR) applied and food-to-microorganism (F/M) ratio observed during the dry and rainy seasons.

The variation of OLR and F/M ratio resulted in different values for biogas and methane yield in the dry and rainy seasons (Table 1). The temperature (ranging from 20 to 28^{o}C) had no effect on the biological process, and the pH was steadily maintained at approximately 6.7, indicating a stable interaction between the acidogenic and methanogenic processes. The concentrations of VFAs, inside the reactor, were low (acetate, butyrate, isobutyrate, valerate and isovalerate < 2 mg/L), and the propionate concentration value determined in rainy season was lower than that observed in the dry season (Table 1). In the dry season, the ammonium-N effluent concentration increased, indicating that nothing has been oxidized in the reactor. In contrast, in the rainy season, a decrease in the ammonium-N effluent concentration was observed (Table 1), and this is due to the lower ammonium-N influent concentration because the sewage is more diluted in the rainy season.

Microbial diversity in the demo-scale UASB reactor

To investigate the diversity of bacterial and archaeal communities 16S rRNA gene clone libraries were constructed for sludge samples collected in rainy and dry seasons corresponding to different OLR and F/M ratio applied to the reactor. A total of 254 clones were analyzed. After removing chimeric and short sequences, 244 sequences were considered for the phylogenetic analysis. According to the coverage values of the archaeal libraries, most of the diversity was detected, mainly in the SD sample (Table 2). The bacterial SD and SR libraries represented the community diversity reasonably well (58–79% Good's coverage at ≥97% cut-off), suggesting that further sequencing of additional clones would have revealed additional diversity (Table 2).

Table 2. Sequence diversity and clone libraries estimates of the sludge samples.

Measurements	Bacteria		Archaea	
	SD	SR	SD	SR
Clones	91	113	22	28
Chimera	6	4	0	0
OTUs*	42	66	2	11
Shannon index diversity	3.4	3.95	0.45	1.76
Simpson index diversity	0.02	0.008	0.54	0.14
Chao 1 estimator	48.6	131.8	2	12.8
ACE estimator	51.4	130.6	2	13.5
Good's coverage (%)	79	58	100	75

*OTUs analyzed without chimera sequences.SD: sludge sampled from the reactor during dry season; SR: sludge sampled from the reactor during rainy season.

Phylogenetic analysis of the nucleotide sequences revealed a significant degree of bacterial and archaeal diversity in the reactor. The SR library was more diverse than the SD library, with more OTUs identified and the highest diversity index. The results demonstrate that temporal changes in the composition of the microbial community in the UASB reactor occurred from the dry season to the rainy season, and this could be related to the OLR and F/M ratio applied, which were

different in each season. Most of the sequences had high identity to those of uncultured organisms obtained from various environments, and some sequences were identified at the species level (Tables S1, S2 and S3).

Bacterial diversity

Proteobacteria, *Bacteroidetes* and *Firmicutes* (*Clostridia* class) phyla predominated in both libraries, but there were differences between the populations (Figs. 3, S1 and S2, Tables S1 and S2).

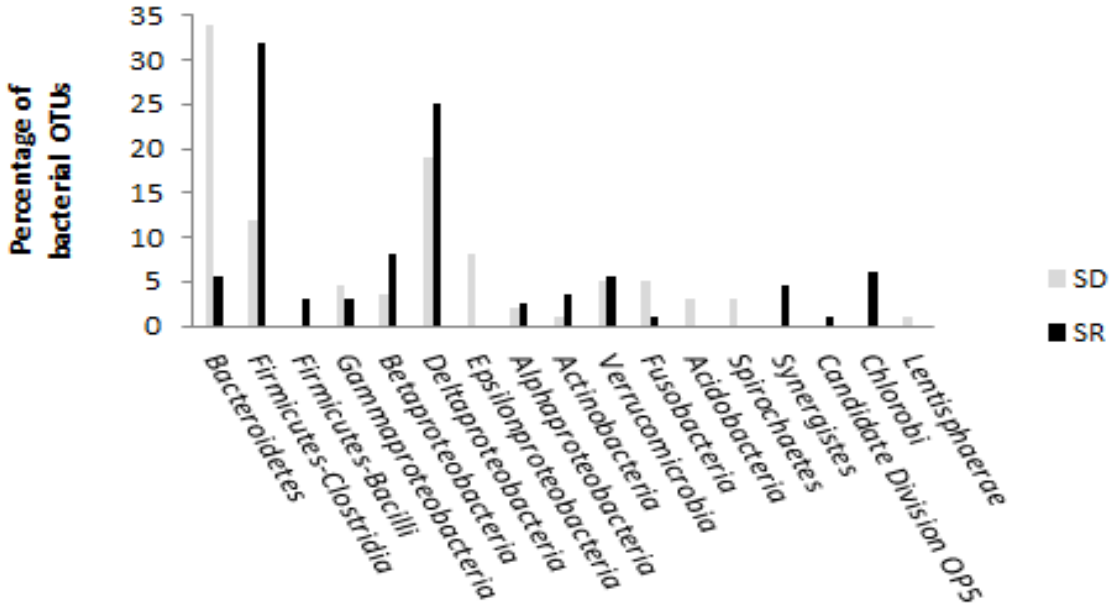

Fig. 3. Phylogenetic distribution of the bacterial 16S rDNA sequences that were generated from sludge samples collected during the dry (SD) and rainy (SR) seasons

The *Proteobacteria* was a major bacterial group and predominated under both climate conditions. All of the *Proteobacteria* classes were represented in the dry season, with δ-*proteobacteria* predominating (corresponding to 19% of the OTUs in the SD sample and 25% in the SR sample). β, α and γ-*proteobacteria* increased overall in the rainy season, but α-*proteobacteria* decreased whereas the ε-*proteobacteria* class disappeared. Many OTUs affiliated

with different proteobacterial classes were identified at the species level, and these belonged to 15 different families (Figs. 3 and S1, Tables S1 and S2). There was a decrease in the *Bacteroidetes* diversity from the dry season to the rainy season, and families such as *Porphyromonadaceae* (represented by *Paludibacter propionicigenes*) and *Marinilabiaceae* (presenting OTUs with 99% similarity to *Anaerophaga* sp.) disappeared (Figs. 3 and S1 and Tables S1 and S2). This disappearance was accompanied by an increase in the number of *Firmicutes-Clostridia* species and the emergence of *Bacilli* (*Streptococcaceae*) (Fig. S2). The *Clostridia* class was represented by OTUs belonging to the *Eubacteriaceae* and *Clostridiaceae* families in both samples. These OTUs presented 99% similarity to *Eubacterium* sp. and similarities of > 97% to *Clostridium* sp., *Clostridium bartlettii* and *Sarcina* sp. Some clostridial families were exclusive to the dry season (*Ruminococcaceae*) or the rainy season (*Peptostreptococcaceae*). Bacterial communities belonging to the *Actinobacteria, Caldiserica* (formally referred to as candidate division OP5)*,* and *Synergistetes* phyla emerged in the rainy season*. Acidobacteria* disappeared in the UASB reactor during the rainy season. Most phyla were represented by OTUs with similarities to the species level (Figs. 3, S1and S2, Tables S1 and S2).

Archaeal diversity

The archaeal 16S rRNA gene libraries revealed a predominance of sequences affiliated with the *Methanosarcinales* order in both seasons (Figs. 4 and S3, Table S3). Members of the *Methanosaetaceae* family were represented by all of the sequences retrieved from the dry season sample. In the rainy season, the emergence of sequences related to the *Methanosarcinaceae* family was observed, which predominated in relation to the *Methanosaetaceae* sequences. OTUs affiliated with *Methanosaetaceae* presented similarity > 97% to *Methanosaeta* sp. and 99% to *Methanosaeta concilli*. Among the *Methanosarcinaceae* members, representatives of *Methanomethylovorans* sp. predominated and only one OTU

showed 99% similarity to *Methanosarcina horonobensis*. The hydrogenotrophic methanogens were represented by the *Methanomicrobiales* order, but they were detected only during the rainy season, with one OTU with 95% similarity to *Methanolinea* sp.

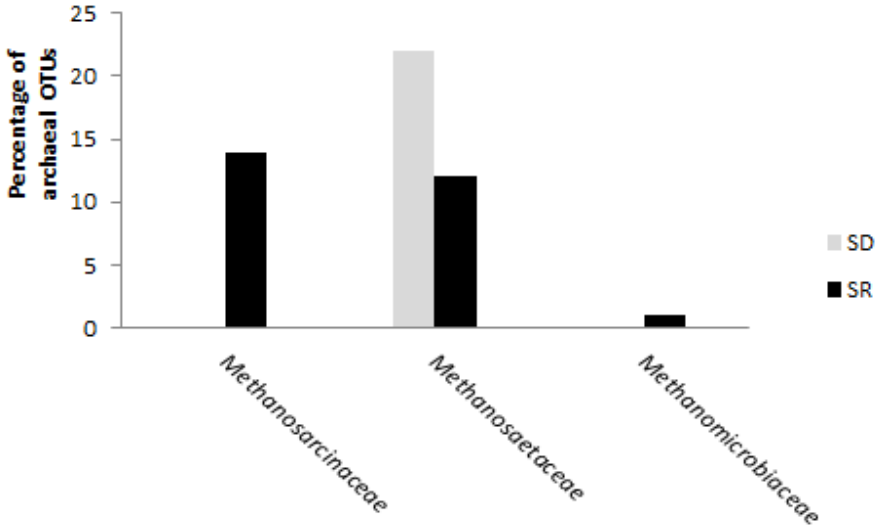

Fig. 4. Phylogenetic distribution of the archaeal 16S rDNA sequences recovered from the sludge samples in the dry (SD) and rainy (SR) seasons

Comparison of the bacterial and archaeal compositions between the clone libraries

LIBSHUFF statistics was applied to determine the significance of differences between the archaeal and bacterial clone libraries. The LIBSHUFF analysis revealed that the bacterial and archaeal communities compositions of each respective library differed significantly (SD, P = 0.002 and SR, P = 0.042 for each bacterial library combination) and (SD, P= 0.005 and SR, P = 0.01 for each archaeal library combination). The LIBSHUFF analysis also indicated that the SD bacterial and archaeal libraries were characterized by a deeper divergence than the SR bacterial and archaeal libraries, respectively.

Quantitative analysis of the microbial community

qPCR is an important tool for estimating the abundance of archaeal and bacterial cells providing a comprehensive culture-independent analysis of the microbial communities in the environment. In the present study, the qPCR analysis allowed a relative quantitative comparison 239 between bacterial and archaeal cells in the UASB sludge samples under different OLR and F/M ratio conditions. The results showed that changes in the abundance of the prokaryotic community occurred with the season. The Ct values demonstrate that the bacterial cell number decreased 2x from the dry season to the rainy season (Ct of 14 to 15), whereas the proportion of archaeal cells increased slightly (Ct of 26.5 to 26) in the reactor from the dry season to the rainy season, with the emergence and increase of new methanogens sequences (Table S3). These data are in accordance with the coverage values obtained for each library, considering the number of clones sequenced (Table 2). The archaeal primers used, which are normally used in metagenomic studies, were modified in this study and produced good amplification efficiencies for each PCR reaction. The melting curves of the bacterial and archaeal amplifications presented more than one peak; however, primer dimers were not observed. In the case of environmental samples (total DNA from a mixture of organisms), differences in the G + C content of genes from different prokaryotes may lead to the formation of more than one peak in the melting curve (Sharma et al. 2007).

DISCUSSION

Many studies that use approaches based on the cloning and sequencing of either functional or 16S rRNA genes, such as fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and qPCR, have been performed to analyze microbial communities in anaerobic reactors (Sanz and Kochling 2007). According to Nelson et al. (2011), who conducted a meta-analysis of all publicly available microbial 16S rRNA gene sequences found in anaerobic digesters, the cloning and sequencing of 16S rRNA genes has generally been favored over

other methods. In addition to the cloning and sequencing methods used in the present study, qPCR was applied to evaluate changes in the abundance of archaeal and bacterial communities in an UASB reactor operated during the dry and rainy seasons. The bacterial and archaeal communities changed from the dry season (high OLR and high F/M ratio) to rainy season (low OLR and low F/M ratio). The UASB community was more diverse during rainy season, with large numbers of OTUs and the absence and emergence of important but less abundant species, such as hydrogenotrophic methanogens (*Methanolinea* sp.) (Figs. 3, 4, S1, S2 and S3, Tables 2, S1, S2 and S3).

The bacterial communities in the SD and SR samples were mainly represented by fermentative species of the *Firmicutes*, *Proteobacteria* and *Bacteroidetes* phyla (Fig. 3; Tables S1 and S2). These results are in agreement with those of Riviére et al. (2009), who analyzed seven different anaerobic sludge digesters. Shifts in the composition of the *Firmicutes* families were observed (Figs. 3, S1 and S2, Tables S1 and S2). *Firmicutes*, which harbor syntrophic bacteria (often Clostridia), are degraders of alcohols from cellulosic substrates and volatile fatty acids (such as butyrate, propionate and acetate), and they produce H_2, which is further utilized by hydrogenotrophic methanogens (Hatori et al., 2000; Rivière et al. 2009). In the rainy season, the predominance of these groups was also accompanied by a decrease in the proportion of *Bacteroidetes* and *Proteobacteria* phyla and the dilution of DW going into the UASB reactor (Table 1). *Bacteroidetes*, which are proteolytic bacteria that predominated in the reactor during the dry season, are able to degrade proteins and ferment amino acids into acetate (Rivière et al. 2009). OTUs affiliated with the hydrolytically active *Proteobacteria* were represented by all classes in the dry season (Table S1). δ-*proteobacteria*, which predominated in the reactor in both seasons is a group that comprises sulfate-reducing bacteria and syntrophic bacteria, such as *Syntrophus* sp., which is an obligate syntrophic bacteria specialized in symbioses with methanogens (Guerrero et al. 1999), which were found in the reactor (Fig. 3, Tables S1 and S2). Furthermore, the presence of β-*proteobacteria*, which are the main consumers of propionate,

butyrate and acetate (Ariesyady et al. 2007), as well as ε- *proteobacteria* (*Arcobacter* sp.), which are acetate oxidizing bacteria commonly found in human and animal feces, may indicate that acetate oxidation also occurred in addition to the acetate degradation by *Methanosaeta* sp. in the reactor. The results suggest a syntrophic association between the acetate oxidizing bacteria and hydrogenotrophic methanogenic *Archaea* mainly in the rainy season, in which β-proteobacteria class was more highly represented and the emergence of *Methanomicrobiaceae* was observed (Petersen and Arhing 1991). These results could explain the low level of VFAs, such as acetate, in the UASB reactor and show that the abundance and activity of acetogenic bacteria and methanogenic *Archaea* were sufficient to degrade the acids produced during the DW treatment.

An increase in the number of OTUs belonging to *Actinobacteria* and *Verrucomicrobia* from the dry season to the rainy season was also observed (Fig. 3). Actinobacteria play an important environmental role in the decomposition of cellulose (Petković et al. 2006), and are also known for their ability to degrade xenobiotic compounds. This process has previously been reported in mesophilic anaerobic reactors (Cardinali-Rezende et al. 2009). Representatives of the *Verrucomicrobia*, which were identified early in other wastewater digesters, are lithoautotrophic bacteria and thermoacidophilic methanotrophs (Islam et al. 2008).

The minor number of bacterial cells detected by qPCR in the SR samples could be explained by the fact that the DW (influent to the reactor) was more dilute, which was evidenced by the lower OLR applied to the reactor and the lower F/M ratio (gCOD/gVS.d, Fig. 2) values observed when compared to the dry season (Fig. 2 and Table 1). In contrast, the diversity of the SR sample, according to the Simpson index, was 0.008, 2.5 larger than the Simpson value for the SD sample. This supports the view that the bacterial community was diversified and specialized in degrading more variable substrates when the relation F/M (i.e. gCOD/gSV.d) was lower, avoiding competition when there were less resources available. Evidence from the

literature suggests that the bacterial communities dominated by only a few groups are shaped by competitive interactions (Moyer et al. 1994; Zhou et al. 2002).

It is well known that in wastewater reactors, the aceticlastic methanogens are responsible for the decrease in COD in the liquid phase by the conversion of acetate to methane. In the present UASB reactor, the average COD removal efficiencies were 50 and 62%, for dry and rainy seasons, respectively. Thus, the highest mean value of COD removal efficiency observed in the rainy season, could suggest that the microbial activity in the rainy season was higher. The aceticlastic methanogens, which are affiliated with the Methanosaetaceae family, such as *Methansoaeta* sp., might be responsible for the acetate degradation in dry season, since a large number of *Methanosaeta* sequences were retrieved from the clone library from sludge sampled at dry season. In the rainy season, among the 27 sequences belonging to the *Methanosarcinales*, 12 were affiliated with *Methanosaetaceae* and 15 were affiliated with *Methanosarcinaceae*, suggesting that methane production was derived from other sources besides acetate. Members of *Methanosaeta concilli* exhibit high affinity for acetate and are responsible for acetate degradation when it is at a low concentration in anaerobic reactors (McMahon et al. 2001; Guerrero et al. 1999). According to Díaz et al. (2006), in a UASB reactor treating brewery wastewater there was a predominance of *Methanosaeta* sp. among the methanogenic *Archaea* ranging from 75% to 96% (Díaz et al. 2006).

In the rainy season, the competition for substrate by *Methanosarcinaceae* and *Methanosaetaceae* acetate oxidizing methanogen families may not have occurred because *Methanomethylovorans* sp. (*Methanosarcinaceae*) use methanol, and some species can also use dimethyl sulfide, methanethiol and methylamines for growth and methanogenesis, but do not use H_2-CO_2 or acetate (Roest et al. 2005). Only one OTU (SR) with 99% of similarity to *Methanosarcina horonobensis*, which are able to grow on methanol, dimethylamine, trimethylamine, dimethylsulfide and acetate but not on monomethylamine, H_2/CO_2, formate, 2-propanol, 2-butanol or cyclopentanol (Boone et al. 1993; Shimizu et al. 2010), was identified in

the reactor. The presence of these methanogens suggests that methanol, dimethylamine and dimethylsulfide substrates are likely available in the UASB reactor mainly in the rainy season.

In the UASB reactor, the low acetate and propionate levels, and consequently the possible hydrogen availability in the rainy season (Table 1) was accompanied by the emergence of hydrogenotrophic representatives affiliated with the *Methanomicrobiales* order (Fig. 4 and S3), such as *Methanolinea* sp., a hydrogen- and formate-utilizing methanogen already isolated from other wastewater UASB reactor (Lucena et al. 2011). *Methanomicrobiales* order and hydrogenotrophic methanogens in general play a key role in the overall process by maintaining the very low partial pressures of H_2 necessary for the functioning of the intermediated acetogenic bacteria, the syntrophic bacteria, which are responsible for the conversion of organic fatty acids, such as propionate, and alcohol intermediates to direct methane precursors (Pauss et al. 1990). Furthermore, the presence of hydrogenotrophic methanogens and the lower propionate concentration value observed in rainy season could suggest that propionate degradation efficiency was higher in the rainy season.

In the rainy season, the emergence of the hydrogenotrophic archaea *Methanolinea* sp. (Fig. 4) and the increase in the number of species belonging to the δ-*proteobacteria* and *Clostridia* sp. were followed by a decrease in the propionate concentration in the reactor (Table 1), which indicates a probable syntrophic association between these microorganisms as suggested by Imachi et al. (2008).

OLR and F/M ratio fluctuations affected the composition and abundance of the prokaryotic community, as well as the performance of the UASB reactor, suggesting that the microbial diversity and activity were higher when the F/M ratio values were lower. Similar results were found by Fernandes et al. (2013) in a full-scale sequencing batch reactor treating domestic wastewater under limited aeration. Shifts in the methanogenic community composition influenced the metabolic route of methane production, with methane produced by acetotrophic methanogens (SD), and hydrogenothrophic, methylotrophic and acetotrophic (SR). These

results suggest that the OLR and F/M ratio are important factors in promoting prokaryotic diversity in UASB reactors, leading to greater bacterial metabolic specialization and might have contributed to the higher COD removal efficiency observed in UASB reactor during rainy season.

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Supplementary material

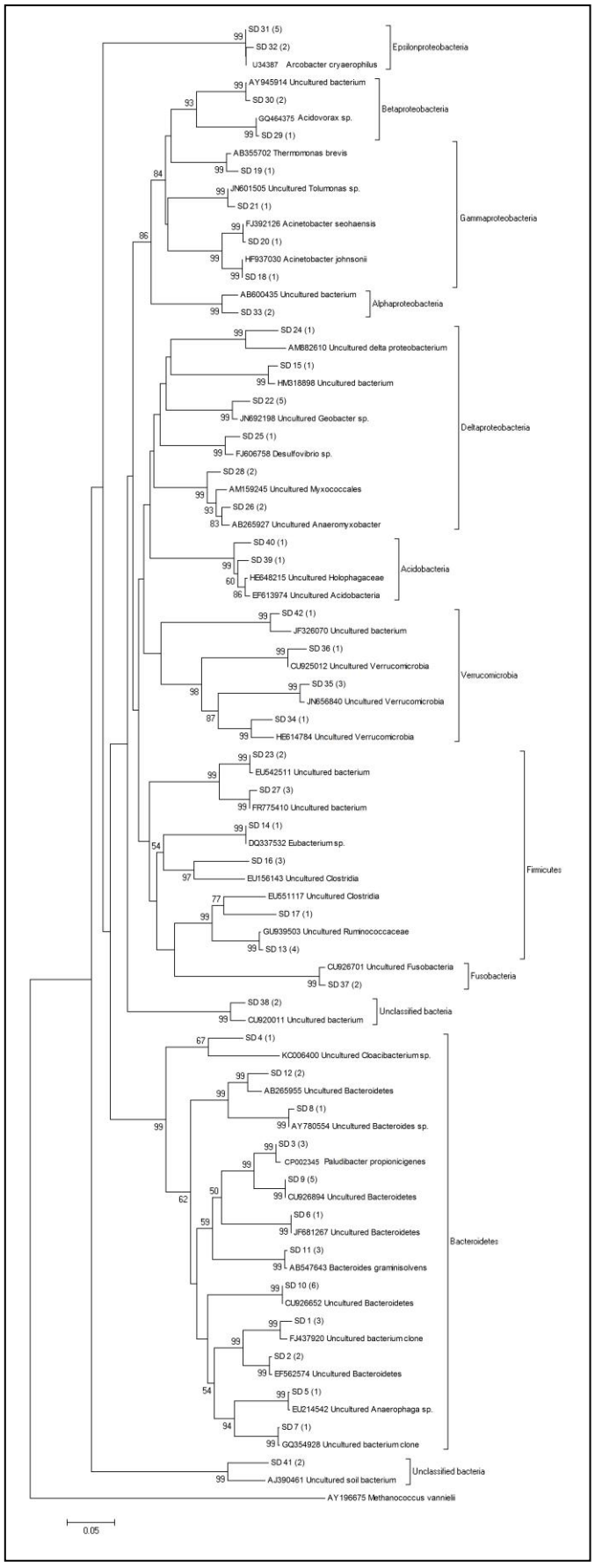

Figure S1. Phylogenetic tree of the bacterial community in the sludge sampled during the dry season (SD), which was constructed using the neighbor-joining method.

Figure S2. Phylogenetic tree of the bacterial community from sludge sampled during the rainy season (SR), which was constructed using the neighbor-joining method.

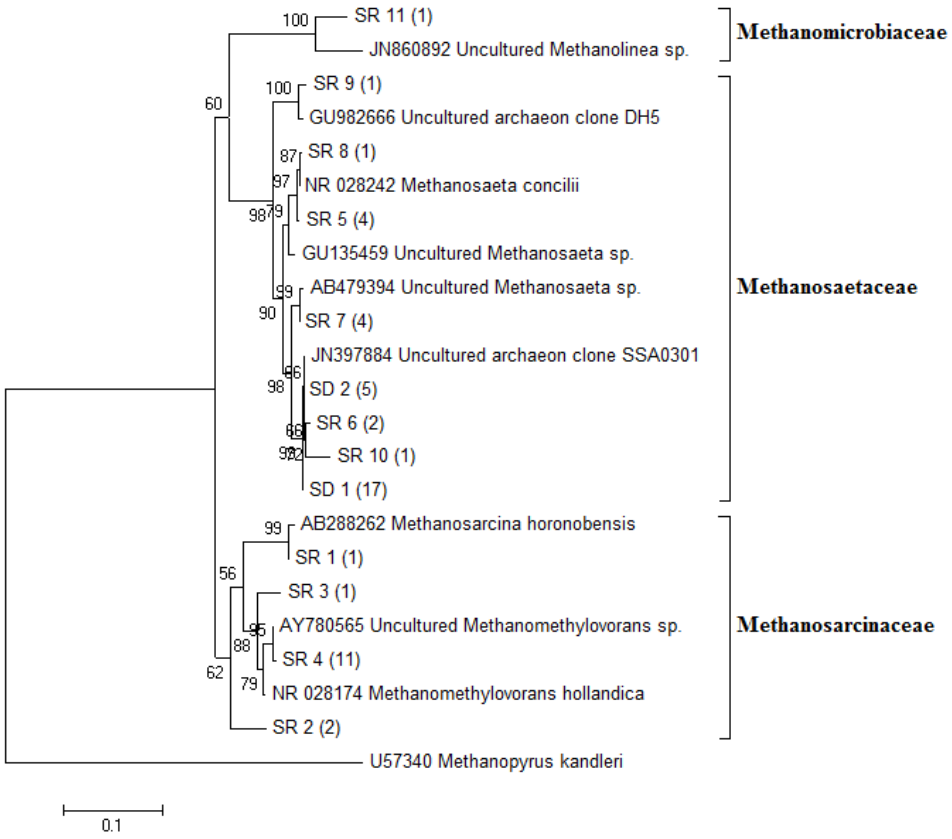

Figure S3. Phylogenetic tree of the archaeal community from sludge samples during the dry (SD) and rainy (SR) seasons, which was constructed using the neighbor-joining method.

Table S1. Phylogenetic filiations and distribution of bacterial clones analyzed from SD (sludge samples at Dry season)

Phylogenetic group	OTU	Closest sequence/microorganism	Order/Family	Acession no.	Identify (%)	Habitat of closest relative
Bacteroidetes	SD-1	Uncultured bacterium clone FGL12_B94		FJ437920	96	Green Lake at 25 m water depth
	2	Uncultured *Bacteroidetes* bacterium		EF562574	98	Paper pulp column
	3	*Paludibacter propionicigenes* WB4	*Porphyromonadaceae*	CP002345	97	Rice plant residue in anoxic rice-field soil
	4	Uncultured *Cloacibacterium* sp.		KC006400	96	Sludge-seeded bioreactor
	5	*Anaerophaga* sp	*Marinilabiaceae*	EU214542	99	DCM degradating envrionment
	6	Uncultured bacterium clone DC53		HM107047	99	Acidogenic anaerobic sludge fermenter
	7	Uncultured bacterium clone MS4-31		GQ354928	99	Spring
	8	Uncultured *Bacteroides* sp.	*Bacteroidaceae*	AY780554	99	Chlorinated ethene-degrading culture
	9	Uncultured *Bacteroidetes* bacterium	*Bacteroidales*	CU926894	99	Municipal wastewater mesophilic digester
	10	Uncultured *Bacteroidetes* bacterium	*Bacteroidales*	CU926652	100	Municipal wastewater mesophilic digester
	11	*Bacteroides graminisolvens*	*Bacteroidaceae*	AB547643	99	Type strain JCM 15093
	12	Uncultured *Bacteroidetes* bacterium		AB265955	95	Anoxic rice field soil
Firmicutes Clostridia	13	Uncultured *Ruminococcaceae* bacterium	*Ruminococcaceae*	GU939503	99	Adult fecal material
	14	*Eubacterium* sp.	*Eubacteriaceae*	DQ337532	99	Swine effluent holding pit
	16	Uncultured *Clostridia* bacterium		EU156143	95	Coffee Pots Hot Spring
	17	Uncultured Clostridia bacterium	*Clostridiaceae*	EU551117	90	Continuously stirred tank reactor
	23	Uncultured bacterium clone Er-LLAYS		EU542511	99	Sediment and soil slurry
	27	Uncultured bacterium partial	*Desulfobulbaceae*	FR775410	99	Anaerobic digester
Gammaproteobacteria	18	*Acinetobacter* sp. Ld3	*Moraxellaceae*	HQ659186	99	Potato field
	19	*Thermomonas brevis*	*Xanthomonadaceae*	AB355702	98	Activated sludge
	20	*Acinetobacter seohaensis*	*Moraxellaceae*	FJ392126	99	Surface of leaves
	21	Uncultured Tolumonas sp.	*Aeromonadaceae*	JN601505	99	biofilm in synthetic wastewater
Deltaproteobacteria	22	Uncultured bacterium clone AS-9	*Geobacteraceae*	HM749852	98	EGSB reactor
	15	Uncultured bacterium		HM318898	99	Skin, popliteal fossa
	24	Uncultured *deltaproteobacterium*		AM882610	90	Petrochemical treatment plant
	25	*Desulfovibrio* sp.	*Desulfovibrionaceae*	FJ606758	96	Toluene-degrading digested sludge
	26	Uncultured *Anaeromyxobacter* sp.	*Myxococcaceae*	AB265927	97	Anoxic rice field soil

Table S1. Continued

Phylogenetic group	OTU	Closest sequence/microorganism	Order/Family	Acession no.	Identify (%)	Habitat of closest relative
	SD-28	Uncultured *Myxococcales* bacterium	*Myxococcales*	AM159245	96	Rice rhizosphere
Betaproteobacteria	29	Uncultured *Acidovorax* sp.	*Comamonadaceae*	GQ464375	99	Frozen chicken storage container
	30	Uncultured bacterium clone DR-16	*Neisseriaceae*	AY945914	99	Sludge-seeded bioreactor
Epsilonproteobacteria	31	*Arcobacter cryaerophilus*	*Campylobacteraceae*	U34387	99	Gastric samples from swine
	32	*Arcobacter cryaerophilus*	*Campylobacteraceae*	AC34387	99	Gastric samples from swine
Alphaproteobacteria	33	Uncultured bacterium clone NW_97		AB600435	97	Iron-rich microbial mat
Verrucomicrobia	34	Uncultured *Verrucomicrobia*		HE614784	97	Sponge tissue
	35	Uncultured *Verrucomicrobia*		JN656840	99	Activated sludge
	36	Uncultured *Verrucomicrobia* bacterium	Opitutaceae	CU925012	96	Mesophilic anaerobic digester which treats municipal wastewater sludge
	42	Uncultured bacterium clone NP-B47		JF326070	95	Rice MFC anode
Fusobacteria	37	Uncultured *Fusobacteria* bacterium	*Fusobacteriaceae*	CU926701	98	Municipal wastewater mesophilic digester
Acidobacteria	39	Uncultured *Acidobacteria* bacterium	*Acidobacteriaceae*	EF613974	96	Paddy soil planted with OGM rice
	40	Uncultured *Holophagaceae* bacterium	*Holophagaceae*	HE648215	97	Tar-oil contaminated aquifer sediments
Unclassified bacteria	38	Uncultured NKB19 bacterium		CU920011	95	Municipal wastewater mesophilic digester
	41	Uncultured soil bacterium PBS-21	*Spirochaetales*	AJ390461	90	Bulk soil and rice roots of flooded rice

Table S2. Phylogenetic filiations and distribution of bacterial clones analyzed from SR (sludge sampled at rainy season)

Phylogenetic group	OTU	Closest sequence/microorganism	Order/Family	Acession no.	Identify (%)	Habitat of closest relative
Firmicutes	SR-1	Uncultured *Peptostreptococcaceae* bacterium	*Peptostreptococcaceae*	HQ730635	99	Extreme acid environment
	2	Uncultured *Peptostreptococcaceae*	*Peptostreptococcaceae*	HQ730632	98	Extreme acid environment
	3	Uncultured Firmicutes bacterium	*Peptostreptococcaceae*	CU927183	99	Municipal wastewater mesophilic digester
	4	Uncultured bacterium clone SGE26E	*Peptostreptococcaceae*	GU389988	99	Anaerobic digester treating feedstock
	6	Uncultured *Clostridium* sp.	*Clostridiaceae*	EF221807	97	Anaerobic reactor
	7	Uncultured bacterium clone	*Clostridiaceae*	FR691506	99	Mahananda river sediment
	8	Uncultured *Clostridium* sp.	*Clostridiaceae*	GQ868425	99	Pig stable bioaerosols
	9	*Clostridium* sp.	*Clostridiaceae*	FJ424481	99	Slaughterhouse waste
	10	Uncultured Firmicutes bacterium	*Clostridiaceae*	CU918169	99	Municipal wastewater mesophilic digester
	11	Uncultured *Sarcina* sp.	*Clostridiaceae*	FJ957597	99	Spacecraft associated clean room
	12	*Clostridium bartlettii* DSM 16795	*Clostridiaceae*	NR_027573	99	Human feces
	14	Uncultured bacterium clone 5	*Clostridiaceae*	FJ462126	97	Mesophilic anaerobic reactor fed with effluent from the chemical industry
	15	*Eubacterium* sp. BBDP17	*Eubacteriaceae*	DQ337524	98	Swine effluent holding pit
	16	*Eubacterium sp.*	*Eubacteriaceae*	DQ337524	98	Swine effluent holding pit
	17	Uncultured *Eubacterium* AA05	*Eubacteriaceae*	AF275916	97	Anaerobic reactor
	18	*Streptococcus bovis* strain FMA766	*Streptococcaceae*	HQ721264	99	Healthy human colon
	20	Uncultured bacterium clone HFV01_120		GU103200	99	Human feces
	64	*Clostridium* sp.	*Clostridiaceae*	FJ424481	98	Slaughterhouse waste
Bacteroidetes	13	Uncultured *Bacteroidetes* bacterium	*Bacteroidales*	CU926100	99	Municipal wastewater mesophilic
	19	Uncultured bacterium	*Bacteroidales*	FR691517	99	Mahananda river sediment
	21	Uncultured *Bacteroidetes* bacterium	*Bacteroidales*	CU926100	98	Municipal wastewater mesophilic digester
	22	Uncultured *Bacteroidetes* bacterium	*Bacteroidales*	CU926100	99	Municipal wastewater mesophilic digester
	23	Uncultured *Bacteroidetes* bacterium	*Bacteroidales*	CU926894	99	Municipal wastewater mesophilic digester
	30	*Bacteroides termitidis*	*Bacteroidales*	M58678	98	Genomic DNA ATCC 33386
Caldiserica	5	Uncultured Caldiserica	*Caldisericaceae*	AY693837	94	Anaerobic sludge
	24	Uncultured Caldiserica	*Caldisericaceae*	AY693837	92	Anaerobic sludge

Table S2. Continued

Phylogenetic group	OTU	Closest sequence/microorganism	Order/Family	Acession no.	Identify (%)	Habitat of closest relative
Chlorobi	SR-26	Uncultured *Chlorobi* bacterium		EF205450	90	Geothermal spring mat
	27	Uncultured bacterium clone 5S5		DQ663997	99	Petroleum contaminated aquifer
Verrucomicrobia	28	Uncultured *Verrucomicrobia* bacterium		CU921474	98	Municipal wastewater mesophilic digester
	29	Uncultured *Verrucomicrobia* bacterium		CU925564	96	Municipal wastewater mesophilic digester
	30	Uncultured bacterium clone t30d60H49		FM956301	98	Rice field soil
Fusobacteria	31	*Bacteroides termitidis*	*Fusobacteriaceae*	M58678	98	Genomic DNA ATCC 33386
Gammaproteobacteria	32	*Pseudoxanthomonas taiwanensis*	*Xanthomonadaceae*	AB210278	99	Activated sludge
	33	*Acinetobacter* sp.	*Moraxellaceae*	X95305	99	Genomic DNA strain BEM 59
	34	Uncultured *Gammaproteobacteria* bacterium		CU923949	99	Municipal wastewater mesophilic digester
Betaproteobacteria	35	*Acidovorax delafieldii*	*Comamonadaceae*	GQ284437	99	Sediment sample of natural spring
	36	Uncultured *Acidovorax* sp.	*Comamonadaceae*	JF460940	99	Drinking water
	37	*Diaphorobacter* sp. bk_62	*Comamonadaceae*	HQ538669	99	Bulking activated sludge
	38	*Brachymonas denitrificans*	*Comamonadaceae*	EU434434	97	Effluent of an antibiotic production wastewater treatment plant
	39	*Neisseria* sp.	*Neisseriaceae*	JF496558	99	Genomic DNA
	40	Uncultured *Thiomonas* sp.	*Burkholderiales*	FJ536894	99	Municipal wastewater mesophilic digester
Deltaproteobacteria	41	Uncultured *Deltaproteobacteria*		CU922057	99	Municipal wastewater mesophilic digester
	42	Uncultured delta *proteobacterium* clone		FJ485013	97	Biomat in El Zacaton at 114m depth
	43	Uncultured *Deltaproteobacteria* bacterium	*Syntrophobacteraceae*	CU921479	96	Mesophilic anaerobic digester which treats municipal wastewater sludge
	44	*Syntrophorhadus aromaticivorans*	*Syntrophorhabdaceae*	NR_041306	93	UASB granular sludge
	45	Uncultured *Syntrophorhabdus* sp.	*Syntrophorhabdaceae*	JN809561	99	Anaerobic bioreactor
	46	*Syntrophorhabdus aromaticivorans*	*Syntrophorhabdaceae*	AB212873	93	Syntrophic phenol-degrading strain
	47	Uncultured *Deltaproteobacteria* bacterium	*Syntrophaceae*	CU922073	99	Municipal wastewater sludge mesophilic digester
	48	Uncultured *Syntrophaceae* bacterium	*Syntrophaceae*	FN679121	99	Switzerland:Lake Geneva
	49	Uncultured *Syntrophus* sp.	*Syntrophaceae*	GU112190	99	Slurry OF anaerobic fermentation of pig manure
	50	Uncultured anaerobic bacterium clone B-1V	*Syntrophaceae*	AY953187	99	Anaerobic swine lagoon
	51	Uncultured bacterium clone B98		HQ640575	90	Partial nitrifying-ANAMMOX municipal wastewater reactor
	52	Uncultured *Desulfobacteraceae* bacterium	*Desulfobacteraceae*	FJ516927	97	Upper sediment
	53	Uncultured bacterium gene	*Desulfobacteraceae*	AB355082	96	Sediment, Manzallah Lake
	54	Uncultured *Deltaproteobacteria* bacterium		CU922107	90	Municipal wastewater mesophilic digester

Table S2. Continued

Phylogenetic group	OTU	Closest sequence/microorganism	Order/Family	Acession no.	Identify (%)	Habitat of closest relative
Deltaproteobacteria	SR-55	Uncultured sediment bacterium clone 285p-13		FN553659	93	Termite gut
Alphaproteobacteria	56	*Rhizobium* sp. PY13	*Rhizobiaceae*	DQ096643	98	Activated sludge from wastewater treatment plant
	57	*Agrobacterium tumefaciens*	*Rhizobiaceae*	AF508094	98	Denitrifying and phenol-degrading organism
	58	Uncultured alpha proteobacterium clone R7-90		JF808782	99	Activated sludge in a membrane bioreactor
Actinobacteria	59	*Propionibacteriaceae* bacterium WR032	*Propionibacterineae*	AB377179	99	Reactor of cattle farm waste
	60	*Dietzia aerolata*	*Dietziaceae*	FM995533	97	Air of a duck barn
	61	*Gordonia soli* strain CC-AB07	*Gordoniaceae*	AY995560	97	SOIL
	62	*Gordonia effusa*	*Gordoniaceae*	AB162799	97	Type strain IFM 10200
Synergistetes	63	Uncultured *Aminanaerobia* bacterium	*Synergistaceae*	CU918588	99	Municipal wastewater mesophilic digester
	64	Uncultured *Aminanaerobia* bacterium		CU923978	98	Municipal wastewater mesophilic digester
	65	Uncultured *Aminobacterium* sp.	*Synergistaceae*	EU887774	97	Anaerobic digester
	66	Uncultured bacterium clone GP_1aaa02a11		EU473160	97	Giant panda feces

Table S3. Phylogenetic filiations and distribution of archaeal clones analyzed from sludge samples at Dry (SD) and Rainy (SR) season samples

Phylogenetic group	OTU	Closest sequence/microorganism	Family	Acession no.	Identify (%)	Habitat of closest relative
Methanosarcinales	SD-1	Uncultured *Methanosaeta* sp.	*Methanosaetaceae*	GU135459	97	Sedimento of lake Pavin
	SD -2	Uncultured *Methanosarcinales* archaeon	*Methanosaetaceae*	CU916103	99	Municipal wastewater mesophilic digester
Methanosarcinales	SR-1	*Methanosarcina horonobensis*	*Methanosarcinaceae*	AB288262	99	Deep subsurface groundwater
	2	*Methanomethylovorans* sp.	*Methanosarcinaceae*	EF174501	96	Ruoergai wetland
	3	*Methanomethylovorans hollandica*	*Methanosarcinaceae*	NR_028174	96	Freshwater sediment
	4	Uncultured Methanomethylovorans sp	*Methanosarcinaceae*	AY780565	99	Chlorinated ethene-degrading culture
	5	Uncultured *Methanosaeta* sp.	*Methanosaetaceae*	JN052768	99	Anaerobic digester
	6	Uncultured archaeon clone SSA0301-14	*Methanosaetaceae*	JN397884	99	Spring pit
	7	Uncultured *Methanosaeta* sp.	*Methanosaetaceae*	AB479394	99	Mesophilic methanogenic granular sludge
	8	*Methanosaeta concilii* GP-6	*Methanosaetaceae*	CP002565	99	*Methanosaeta concilii* GP-6
	9	Uncultured archaeon clone DH5	*Methanosaetaceae*	GU982666	98	Municipal sewage EGSB bioreactor 12°C
	10	Uncultured *Methanosarcinales* archaeon		CU917358	97	Municipal wastewater mesophilic digester
Methanomicrobiales	11	*Methanolinea* sp.	*Methanomicrobiaceae*	CU917018	95	Thermophilic anaerobic alkane-biodegrading

4 – DISCUSSÃO GERAL

A digestão anaeróbica é uma boa solução para o tratamento de uma variedade de resíduos produzidos pelo homem, como RSU, esgotos sanitários e resíduos suínos de frigoríficos. AD ocorre a partir da interação de vários grupos de bactérias fermentativas (hidrolíticas e acidogênicas) e acetogênicas com as arqueias metanogênicas hidrogenotróficas e acetoclásticas para a degradação da matéria orgânica e produção do biogás, composto principalmente por CH_4 e CO_2 (Welander e Metcalf, 2005). Este tratamento é economicamente e socialmente favorável, uma vez que o biogás produzido poderá ser aproveitado como energia ou comercializado como créditos de carbono; o resíduo tratado, por ser rico em sais, poderá ser utilizado como adubo orgânico. Além disso, o resíduo será tratado em compartimentos fechados (reatores anaeróbicos) ou em lagoas anaeróbicas de estabilização, que por serem sistemas controlados, não acarretarão danos ao meio ambiente e nem ao homem (Chynoweth et al. 2001; Foresti, 1997), tornando a digestão anaeróbica auto-sustentável e promissora.

Portanto, neste trabalho, várias técnicas moleculares baseadas no gene de rRNA 16S foram utilizadas e associadas para identificação filogenética (ARDRA, clonagem-sequenciamento e DGGE) e a quantificação (qPCR, FISH e CARD-FISH) das comunidades de bactérias e arqueias e de suas mudanças durante a digestão anaeróbica dos diferentes resíduos, em diferentes períodos do tratamento: no *start-up* e em condições estáveis do reator anaeróbico, em escala industrial, em tratamento o RSU (Madri – Espanha); nos períodos de seca e chuva do reator anaeróbico UASB, em escala piloto, em tratamento de esgoto sanitário (Belo Horizonte – MG); e do resíduo suíno tratado em uma lagoa anaeróbica de estabilização.

A análise comparativa das sequências do rDNA 16S revelou ocorrência similar no nível de filo de algumas comunidades de procariotos entre os diferentes sistemas. Entretanto, no nível de espécie e em relação a sua abundância, diferenças foram observadas nos reatores e na lagoa anaeróbica assim como nos mesmos sistemas, mas em diferentes condições operacionais e ambientais. Entre as comunidades bacterianas identificadas nas bibliotecas de clones, as bactérias fermentadoras predominaram nos três sistemas anaeróbicos. Bactérias acidogênicas, que degradam proteínas e produzem acetato, afiliadas ao filo *Bacteroidetes* (Holmes et al. 2007) predominaram na lagoa anaeróbica, no reator UASB – na estação seca –, e no reator em tratamento de RSU, durante todo o período de amostragem, *start-up* e em condições estáveis. *Firmicutes* foram representados, principalmente, pela classe *Clostridia* em todos os sistemas, sendo que no reator UASB, na estação de chuva, este táxon predominou sobre *Bacteroidetes*, indicando mudanças nas comunidades bacterianas, da estação seca para

a chuvosa. Na estação chuvosa, observou-se também o surgimento de bactérias pertencentes à classe *Bacilli* no reator UASB, a qual foi também identificada durante no *start-up* do reator em tratamento de RSU. *Firmicutes* abrigam bactérias sintróficas que degradam celulose e AGVS, como butirato e seus análogos, e produzem H_2 utilizados pelas arqueias metanogênicas hidrogenotróficas para a produção de metano (Riviére et al. 2009). A predominância de *Firmicutes*, principalmente *Clostridiaceae*, foi acompanhada pelo surgimento de arqueias hidrogenotróficas afiliadas à ordem *Metanomicrobiales: Methanolinea* sp. no reator UASB e na lagoa anaeróbica, e pela predominância de *Methanoculleus bourgensis* no *start-up* e em condições estáveis do reator em tratamento de RSU. *Proteobacteria*, outro filo geralmente envolvido na digestão anaeróbica em reatores (Rivière et al. 2009; Cardinali-Rezende et al. 2009), foi responsável, principalmente, pela oxidação de carboidratos, em conjunto com representantes de *Bacteroidetes*, nos três sistemas. Representantes de *Proteobacteria* foram identificados em grande número no reator UASB, sendo as cinco classes representadas com a predominância de δ-*proteobacteri*a, também identificada na lagoa anaeróbica, em conjunto com as espécies de β-*proteobacteria,* degradadoras de propionato, butirato e acetato (Ariesyadi et al. 2007). Em relação ao reator em tratamento de RSU, identificou-se apenas γ-*proteobacteria* no *start-up*. *Chloroflexi*, muito comum em reatores em tratamento de esgoto sanitário e de RSU (Sekiguchi et al. 2001; Rivière et al. 2009; Cardinali-Rezende et al. 2009), foi identificado apenas na lagoa anaeróbica, sendo predominante dentre as bactérias envolvidas na digestão anaeróbica do resíduo suíno. Outros filos citados anteriormente foram específicos a certos tratamentos, ou mesmo comuns entre eles, mas ocorreram em pequeno número abrangendo, principalmente, bactérias responsáveis pelas primeiras etapas da digestão anaeróbica, hidrólise e acidogênese. Mudanças nas comunidades bacterianas foram observadas nos reatores anaeróbicos em tratamento dos diferentes resíduos, como consequência de mudanças operacionais e ambientais. Durante o tratamento do RSU, do *start-up* até alcançar condições estáveis, mudanças na composição da comunidade dos procariotos e um aumento no número de células de bactérias e arqueias foram, como consequência do carregamento do reator com mais RSU. No reator UASB em tratamento de esgoto sanitário, sob condições de seca e chuva, observou-se uma diluição na quantidade de substratos na estação chuvosa e, consequentemente, uma diminuição na quantidade de células bacterianas, que se tornaram mais especializadas em degradar diferentes substratos, aumentando assim sua diversidade na estação chuvosa. Nos três sistemas, a quantidade de células bacterianas quantificadas por qPCR e também por FISH e CARD no reator em tratamento de RSU foi maior que a de arqueias.

No reator em tratamento de RSU e na lagoa anaeróbica em tratamento de resíduo suíno a produção de metano predominou a via de degradação do H_2, CO_2 e formiato pelas arqueias hidrogenotróficas: *Methanomicrobiales* (*Methanoculleus* sp. no reator RSU e *Methanolinea* sp. na lagoa anaeróbica) e *Methanobacteriales (Methanobrevibacter* sp*.* em ambos sistemas, e *Methanobacterium* sp. na lagoa anaeróbica) e sua relação sintrófica com as bactérias que degradam acetato nos dois sistemas e oxidam propionato no reator anaeróbico em tratamento de RSU. As arqueias metanogênicas acetoclásticas não foram identificadas na lagoa anaeróbica, entretanto arqueias pertencentes à *Arc I*, que usam H_2, CO_2 e formiato para seu crescimento, podem estar envolvidas na degradação do acetato (Riviére et al. 2009) na lagoa anaeróbica. No reator em tratamento de RSU, *Methanosarcinales* (*Methanosarcina* sp., *Methanomicroccocus* sp. e *Methanosaeta* sp.) foram identificadas e quantificadas por qPCR e apresentaram um menor número de células em relação às arqueias hidrogenotróficas, sendo que *Methanosaeta* sp. foi detectada apenas por técnicas mais sensíveis, como qPCR e FISH. Além dos representantes da ordem *Methanomicrobiales,* o filo *Crenarchaeota* também predominou na lagoa anaeróbica e no reator em tratamento de RSU, em condições estáveis, isto é, completamente cheio de resíduo. Este grupo foi anteriormente identificado em ambientes marinhos onde a concentração de amônia é alta, e possivelmente está envolvido na degradação de nitrogênio inorgânico como fonte de energia (DeLong et al. 1994; Hallam et al. 2006). Similaridade entre as comunidades de arqueias presentes nestes dois sistemas poderia explicar a grande produção de biogás observada em um reator anaeróbico em escala laboratorial em tratamento de RSU, onde o lodo dessa mesma lagoa anaeróbica foi utilizado como inóculo (Cardinali-Rezende et al. 2010).

Em contraste, a estes dois sistemas anaeróbicos, no reator UASB em tratamento de esgoto sanitário, a produção do metano foi predominantemente realizada pela degradação do acetato por *Methanosaetaceae* (*Methanosaeta* sp.) nas estações de seca, e por arqueias acetoclásticas e metilotróficas *Methanosarcinaceae* (*Methanometylovorans hollandica* e *Methanosarcina honorobensis,*) na estação de chuva, com uma pequena quantidade de metano sendo produzida por arqueias hidrogenotróficas *Methanomicrobiales*, tais como *Methanolinea* sp.. Esta espécie foi também identificada na lagoa anaeróbica, como citado anteriormente. De acordo com os resultados observou-se que ocorreram mudanças nas comunidades de arqueias nos períodos de seca e chuva no reator UASB em tratamento de esgoto sanitário. Em contraste às comunidades bacterianas o número de arqueias aumentou no período de chuva.

Observou-se nos diferentes sistemas que as técnicas de qPCR, FISH e CARD-FISH são boas ferramentas para a quantificação de procariotos e de suas respectivas mudanças durante o processo de digestão anaeróbica dos resíduos. Estas técnicas permitiram maior sensibilidade na quantificação e detecção de grupos de procariotos, mesmo diante do pequeno número presente nas amostras, os quais ainda não haviam sido identificados pelas técnicas de clonagem-sequenciamento e DGGE. Apesar da sensibilidade, ocorreram problemas na utilização das técnicas de FISH e CARD-FISH no processo de hibridização das comunidades de procariotos com sondas específicas nas amostras de RSU coletadas no reator anaeróbio. Esta ocorrência pode ser devido à presença de inibidores nessa amostra. Em relação à eficiência do tratamento dos resíduos nestes sistemas anaeróbicos, a estabilidade foi mantida e a degradação da matéria orgânica obtida com sucesso, confirmados pela eficiência de degradação de sólidos voláteis no reator em tratamento de RSU, além da remoção da DBO no reator UASB e na lagoa anaeróbica.

Como observado nos diferentes sistemas de tratamento anaeróbico neste trabalho e de acordo com outros trabalhos, vários aspectos biológicos, físico-químicos, operacionais ou ambientais – tipo do resíduo, inóculo a ser adicionado, concentração de matéria orgânica, taxa de carregamento orgânico, tempo de retenção, disponibilidade de mistura do resíduo em tratamento, concentração de AGVs, concentração de amônia, pH, temperatura, condições de operação, fatores sazonais (chuva e seca) –; estão diretamente relacionados à diversidade filogenética das comunidades de procariotos e sua abundância, com sua atividade metabólica na conversão da matéria orgânica dos diferentes resíduos nos diferentes sistemas anaeróbico (Mahmoud et al. 2003; Tang et al. 2007).

5 – CONCLUSÕES

Este trabalho revelou mudanças na composição filogenética, na abundância das comunidades de procariotos e na via de produção de metano, durante a digestão anaeróbica de resíduos orgânicos doméstico, suíno e de esgoto sanitário, como consequência das alterações microbiológicas, operacionais e sazonais nos sistemas: reatores anaeróbicos RSU e UASB, e lagoa anaeróbica. A comunidade bacteriana da lagoa anaeróbica revelou a mais alta diversidade filogenética, no nível de filo, enquanto foi menor no reator RSU. Diferentes comunidades bacterianas foram identificadas, e como observado anteriormente em outros trabalhos, os filos *Firmicutes*, *Bacteroidetes*, *Proteobacteria* e *Chloroflexi* predominaram entre as bactérias identificadas. Observou-se arqueias *Euryarchaeota* em todos os sistemas e *Crenarchaeota* no reator RSU e lagoa anaeróbica. Observou-se, ainda, como já relatado na literatura, que a produção de metano em reatores RSU foi efetuada, predominantemente, pela arqueias hidrogenotróficas. Deve-se enfatizar que na lagoa anaeróbica o metano foi produzido exclusivamente por arqueias hidrogenotróficas. Em contraste, no reator UASB a produção do metano foi realizada predominantemente pelas arqueias acetoclásticas e metilotróficas. Observou-se que a diversidade filogenética das comunidades de procariotos e sua abundância estão diretamente associadas a sua atividade metabólica. Avanços no entendimento da ecologia e fisiologia destes micro-organismos, principalmente das arqueias metanogênicas, é essencial para o desempenho de sistemas anaeróbicos de tratamentos mais eficazes.

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7 - ANEXO

7.1 - Artigos completos publicados em periódicos

1- Cardinali-Rezende, Juliana ; Debarry, Renan B. ; Coltur ato, Luis F. D. B. ; Carneiro, Eduardo V. ; Chartone-Souza, Edmar ; Nascimento, Andrea M. A. 2009. Molecular identification and dynamics of microbial communities in reactor treating organic household waste. Applied Microbiology and Biotechnology, v. 84, p. 777-789, 2009.

2 - Juliana Cardinali-Rezende, Ana M. M. Moraes, Luis F. D. B. Colturato, Eduardo V. Carneiro, Ivanildo E. Marriel, Edmar Chartone-Souza and Andréa M. A. Nascimento**. 2010.** Phylogenetic and physiological characterization of organic waste-degrading bacterial communities. World Journal of Microbiology and Biotechnology. Volume 27, Number 2, 245-252, DOI: 10.1007/s11274-010-0449-9

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ENVIRONMENTAL BIOTECHNOLOGY

Molecular identification and dynamics of microbial communities in reactor treating organic household waste

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Abstract The prokaryotic diversity associated with organic household waste (OHW), leachate (start-up inoculum), and mesophilic anaerobic digestion processes in the degradation of OHW for 44 and 90 days was investigated using a culture-independent approach. Bacterial and archaeal 16S rRNA and *mcr*A gene clone libraries were constructed from community DNA preparations. Bacterial clones were affiliated with 13 phyla, of which *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were represented in all libraries, whereas *Actinobacteria*, *Thermotogae*, *Lentisphaerae*, *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Synergistetes*, *Spirochaetes*, *Deferribacteres*, and *Deinococcus-Thermus* were exclusively identified in a single library. Within the *Archaea* domain, the *Euryarchaeota* phylum was the only one represented. Corresponding sequences were associated with the following orders of hydrogenotrophic methanogens: *Methanomicrobiales* (*Methanoculleus* genus) and *Methanobacteriales* (*Methanosphaera* and *Methanobacterium* genera). One archaeal clone was not affiliated with any order and may represent a novel taxon. Diversity indices showed greater diversity of *Bacteria* when compared to methanogenic *Archaea*.

Keywords 16S rRNA gene · *mcrA* gene · Clone library · Anaerobic reactor · Organic household waste · Leachate

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Introduction

Millions of tons of household solid waste (HSW) are produced daily worldwide, and this has become a serious environmental, social, and public health problem for urban communities due to a lack of available areas for its disposal. Thus, one of the greatest challenges of contemporary society is to reduce and treat HSW.

Currently, HSW treatment has gained relevance due to an increased interest in renewable energy sources, as it combines the removal of organic pollutants and energy conservation in the form of biogas production (Lastella et al. 2002). Anaerobic digestion has been extensively and successfully applied to organic waste treatment over the past decades (Abegglen et al. 2008; Fernández et al. 2008). It takes place in four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, and is the consequence of a series of metabolic interactions among bacterial and archaeal groups. The first group of microorganisms secretes enzymes which hydrolyze polymers to monomers, such as glucose, amino acids, lactate, and ethanol. These are subsequently converted by acidogenic and acetogenic bacteria to H_2 and volatile fatty acids. Finally, methanogenic *Archaea* convert H_2, formate, and acetate to CH_4 and CO_2.

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ORIGINAL PAPER

Phylogenetic and physiological characterization of organic waste-degrading bacterial communities

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Abstract One of the greatest challenges in contemporary society is to reduce and treat household solid waste. The choice of inoculum to be used for start-up in reactors that degrade organic waste is critical to the success of organic waste treatment. In this study, the functional diversity, phylogenetic identification, and biogas production of bacterial communities from six inoculum sources were investigated. We used BIOLOG EcoPlates to evaluate the metabolic abilities of the bacterial communities, followed 16S rRNA gene sequence analysis to determine the phylogenetic affiliation of the bacteria responsible for carbon consumption. We observed great diversity in the physiological profiles. Of the six inocula tested, the sludge from an upflow anaerobic sludge blanket reactor (SRU) contained the most diverse, metabolically versatile microbiota and was characterized by the highest level of biogas production. By contrast, the sludge of the anaerobic lagoon (SAL) showed the worst performance in BIOLOG EcoPlates assays, but it exhibited the most diversity and generated the second largest amount of biogas. The bacterial isolates retrieved from BIOLOG EcoPlates were characterized as aerobic and/or facultative anaerobic, and were mainly Gram-negative. Phylogenetic analysis revealed that the isolates belonged to three major phyla: Proteobacteria, Firmicutes and Actinobacteria, represented by 33 genera. Proteobacteria exhibited the most diversity. The distribution of the bacterial genera differed considerably among the six inocula. *Pseudomonas* and *Bacillus*, which are able to degrade a wide range of proteins and carbohydrates, predominated in five of the six inocula. Analysis of the bacterial communities in this study indicates that both SRU and SAL microbiota are candidates for start-up inocula in anaerobic reactors. These start-up inocula must be studied further in order to identify their practical applications in degrading organic waste.

Keywords BIOLOG EcoPlate · 16S rRNA gene · Inoculum · Organic waste

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Introduction

The collection and disposal of household municipal solid waste (MSW) are rapidly becoming a serious and unsolved problem for urban communities worldwide. It is estimated that about 125 thousand tons of MSW are generated daily in Brazil (IBGE 2004, http://www.ibge.gov.br), and India (Agarwal et al. 2005) and Spain (Macé et al. 2005) generate

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7.2 - Doutorado Sanduíche

- Doutorado sanduíche desenvolvido no Departamento de Biologia Molecular da Universidad Autónoma de Madrid, Espanha sob a coordenação do pesquisador Dr. José Luís Sanz Martín.
- Período: - Pesquisadora Geneticista Molecular de Microorganismos – CLT - DEFLOR / LTDA (21/03/2009 a 22/03/2010)

- Bolsista CAPES: (03/05/2011 a 03/10/2011)

7.3 - Projetos de pesquisa com financiamentos aprovados

1- Produção de biogás através da biometanização da fração orgânica do resíduo municipal (FORM) aceito pelo Fundo Nacional de Desenvolvimento Científico e Tecnológico - FNDCT / subvenção econômica 2007;

2- Prospecção molecular de procariotos em inóculos para uso em biodigestores anaeróbios no tratamento de matéria orgânica do Lixo aceito pela FAPEMIG Edital Universal 2008;

3- Análise funcional de uma biblioteca metagenômica e quantificação em tempo real de arquéias metanogênicas e genes envolvidos na degradação anaeróbia do lixo para otimização de um biorreator anaeróbio aceito pela FAPEMIG - Edital Resíduos Sólidos 2008.

7.4 - Produção Técnica

1. Cardinali-Rezende, J; Colturato, L. F. ; Pereira,R,A. ; Andréa Maria Amaral Nascimento ; Chartone-Souza, Edmar . Produção de biogás através da biometanização da fração orgânica do resíduo municipal. 2010. (Relatório de pesquisa).

7.5 - Participações em Encontros, Simpósios e Congressos

1- X Latin American Workshop and Symposium on Anaerobic Digestion. 2011. Ouro Preto.

2- 4th Congress of European Microbiologists. 2011. Genebra.

3- VI Fórum de Microbiologia Prof. Ênio Cardillo Vieira. 2010. UFMG

4- I Simpósio de Genética e Biotecnologia da UFMG. 2008.

5- Semana de Biologia da Universidade Metodistade Minas Izabela Hendrix. 2008.

6- II Amostra de Biotecnologia e Meio Ambiente da UNA. 2008.

7.6 - Trabalhos apresentados em Encontros, Simpósios e Congressos: Apresentação oral

1- J. Cardinali, L.F. Colturato, J.C. Araújo, C.A.L. Chernicharo, J.L. Sanz, A.A. Nascimento. Seasonal changes in the microbial community in an UASB reactor treating domestic wastewater. X Latin American Workshop and Symposium on Anaerobic Digestion. 2011. Ouro Preto (Simpósio).

2- Cardinali-Rezende, J ; Renan Barbosa Debarry ; Felipe Correia(2 ; COLTURATO, L. F. ; Eduardo Vieira Carneiro ; Edmar Chartone-Souza ; Andréa Maria Amaral Nascimento . Identificação Molecular dos Procariotos presentes no lodo de um biorreator anaeróbio. 2008. (Apresentação de Trabalho/Simpósio).

7.8 - Trabalhos apresentados em Encontros, Simpósios e Congressos: Poster

1- J. Cardinali, L.F. Colturato, T.F. Colturato, E. Chartone-Souza, A.A. Nascimento, J.L. Sanz. Prokaryote Diversity and Dynamics in a Full-Scale Municipal Solid Waste Anaerobic Digester. **X Latin American Workshop and Symposium on Anaerobic Digestion. 2011. Ouro Preto.**

2. Cardinali-Rezende, Juliana ; SANZ, J. L. ; Edmar Chartone-Souza ; Nascimento, Andrea M. A. . HIGH PROCARYOTIC DIVERSITY IN SLUDGE OF AN ANAEROBIC LAGOON. In: **4th Congress of European Microbiologists, 2011, Genebra.**

3. Cardinali-Rezende, Juliana ; SANZ, J. L. ; Andréa Maria Amaral Nascimento . GREAT DIVERSITY OF BACTERIA AND ARCHAEA IN ANAEROBIC DIGESTER TREATING HOUSEHOLD SOLID WASTE. In: **4th Congress of European Microbiologists, 2011, Genebra.**

4. Cardinali-Rezende, J ; SANZ, J. L. ; Edmar Chartone souza ; Andréa Maria Amaral Nascimento . Procaryotic biodiversity in anaerobic digester treating municipal solid waste. In: **III International Conference on Environmental, Industrial and Applied Microbiology, 2009, Lisboa.**

5. Cardinali-Rezende, J ; Renan Barbosa Debarry ; Felipe Correia(2 ; Luís Felipe Colturato ;

Eduardo Vieira Carneiro ; Edmar Chartone-Souza ; Andréa Maria Amaral Nascimento . Identificação Molecular dos procariotos presentes no lodo de um biorreator anaeróbio. In: I Simpósio de Genética e Biotecnologia da UFMG, 2008, Belo Horizonte. **I Simposio de Genética e Biotecnologia da UFMG, 2008.**