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# INFLUÊNCIA DAS CONDIÇÕES AMBIENTAIS NAS COMUNIDADES MICROBIANAS EM ZONAS DE WETLANDS CONSTRUÍDOS TRATANDO ÁGUAS CINZA

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Faculdade de Engenharias, Arquitetura e Urbanismo e Geografia

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Tese apresentada para obtenção do grau de Doutor no Programa de Pós-Graduação em Tecnologias Ambientais da Fundação Universidade Federal de Mato Grosso do Sul, área de concentração: *Saneamento Ambiental e Recursos Hídricos*.

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# Sumário

# RESUMO

*Os wetlands construídos (CW) vêm se expandindo mundialmente como uma forma eficiente de tratamento descentralizado de água cinza. Com esse avanço, alguns problemas de aplicação dessa tecnologia começam a surgir: entupimentos e liberações de gases como metano e sulfeto de hidrogênio. Esses gases podem ser convertidos por vias microbiológicas em zonas de oxidação, porém, pouco se conhece sobre condições ambientais e comunidades microbianas em CWs tratando água cinza. Dessa forma, o funcionamento de um CW melhorado (EvaTAC) tratando água cinza clara (ACc) foi monitorado por meio da combinação de análises físico químicas, sequenciamento de DNA de alto rendimento e métodos estatísticos, visando entender a influência das condições ambientais na comunidade de bactérias e arqueias existentes nas diferentes zonas do sistema. Os resultados demostraram que potencial redox e a diversidade e riqueza da comunidade microbiana aumentaram ao longo do fluxo de ACc e foram inversamente proporcionais aos parâmetros que representam presença de matéria orgânica. As zonas de entrada foram caracterizadas pela presença de grupos específicos de microrganismos (menor diversidade e riqueza), que desempenharam função importante para a estabilidade do sistema. Por outro lado, as zonas de saída do sistema apresentaram maior diversidade e riqueza de microrganismos e foram relacionadas à maior abundância de bactérias e arqueias envolvidas nos processos de oxidação do metano e sulfeto. Assim, este estudo sugere a ocorrência de zonas de redução e oxidação no EvaTAC e a existência de correlações entre condições ambientais e comunidades microbianas envolvidas principalmente nos ciclos biogeoquímicos do carbono e enxofre.*

**Palavras chave:** *Águas cinza, condições ambientais, sequenciamento de alto rendimento, comunidade microbiana, digestão anaeróbia.*

## ABSTRACT

*Constructed wetlands (CW) have been expanding worldwide as an efficient form of decentralized treatment of greywater. With this advancement, some problems of application of this technology begin to appear: clogs and releases of gases like methane and hydrogen sulfide. These gases can be converted by microbiological pathways into oxidation zones, however, little is known about the environmental conditions and microbial communities within the CW treating greywater. Thus, the operation of an improved CW (EvaTAC) treating light greywater (ACc) was monitored by combining physical chemical analyzes, high-throughput DNA sequencing and statistical methods to understand the influence of environmental conditions on the community of bacteria and archaea in the different zones of the system. The results showed that the redox potential and microbial community diversity and richness increased along the ACc flux and were inversely proportional to the parameters that represent the presence of organic matter. The inlet zones were characterized by the presence of specific groups of microorganisms (lower diversity and richness), which played an important role in the stability of the system. On the other hand, the outlet zones of the system presented greater diversity and richness of microorganisms and were related to the higher abundance of bacteria and archaea involved in the processes of oxidation of methane and sulfide. Therefore, this study suggests the occurrence of reduction and oxidation zones in the EvaTAC and the existence of correlations between environmental conditions and microbial communities involved mainly in the biogeochemical cycles of carbon and sulphur.*



**Keywords**: *Greywater, Environmental conditions, High-throughput sequence, Microbial diversity, anaerobic digestion*.

## 1.1 Organização da Tese

A Tese está organizada em 3 capítulos. O **Capítulo 1** (Introdução Geral) traz o estado da arte, a justificativa do trabalho e a caracterização dos problemas relacionados ao tema do trabalho, bem como os objetivos específicos da pesquisa. O **Capítulo 2** discute os microrganismos identificados em um *wetlands* construído melhorado (EvaTAC) implantado em escala real em uma residência com 3 moradores e suas relações com as condições ambientais predominantes nas zonas do sistema. O **Capítulo 3** discute a relação entre os processos microbiológicos realizados por arqueias e bactérias e as condições ambientais de diferentes zonas de um EvaTAC implantado em escala piloto na Universidade Federal do Mato Grosso do Sul. Por fim, o **Capítulo 4** traz as conclusões gerais da pesquisa.

# CAPITULO 1 – INTRODUÇÃO GERAL

## 1.2 Antecedente e Estado da Arte

### 1.2.1 Saneamento Básico e Tratamento Descentralizado

São de conhecimento comum os benefícios que a melhora da qualidade de água e do saneamento traz à saúde pública (JOINT MONITORING PROGRAM, 2015). Doenças relacionadas a fontes não seguras de água, saneamento deficiente e falta de higiene sanitária são causas comuns de enfermidades e mortes por todo o mundo (ESREY et al., 1991). Preocupados com esse quadro, no ano de 2000, a Organização das Nações Unidas (ONU) propôs, nos Objetivos de Desenvolvimento do Millenium (7c), reduzir pela metade a proporção de pessoas que não têm acesso a fontes de água potável e saneamento básico até o fim de 2015, utilizando como base, os dados do ano de 1990. O acesso à fonte de água potável foi alcançado no ano de 2010, com mais de 90% da população sendo atendida, já os esforços para o acesso em saneamento básico falharam, deixando 2,4 bilhões de pessoas sem acesso às instalações sanitárias eficientes (WHO, 2014).

No Brasil, segundo dados do Sistema Nacional de Informação sobre Saneamento (SNIS), 55% da população urbana é atendida com rede de coleta e tratamento de esgoto. Analisando esses dados, verifica-se que cerca de 147 milhões de pessoas, 71% da população total, não estão conectadas em sistemas coletivos de esgotamento sanitário, uma vez que o país apresenta população total de 206 milhões de pessoas, sendo 63% urbana e 37% rural (CARLOS MIRANDA; KÊNIA SANTOS; PATRÍCIA PORTO, 2013). Na maioria dos países em desenvolvimento, sistemas centralizados de tratamento de esgoto existem somente em grandes cidades, ao passo que pequenas e médias cidades geralmente descarregam seus esgotos domésticos sem tratamento no ambiente (MATOS et al., 2014). Nesse sentido, a descentralização, em associação com incentivos do governo local, é cada vez mais reconhecida como um caminho potencialmente adequado para reduzir o percentual da população mundial sem acesso à água potável e ao saneamento básico (BIEKER; CORNEL; WAGNER, 2010), bem como é vista como ferramenta para aumentar a eficiência do tratamento de esgoto, possibilitando a recuperação e reuso do esgoto doméstico tratado (MASSOUD; TARHINI; NASR, 2009). Dessa maneira, a

descentralização é considerada como um dos meios para se atingir uma das metas do desenvolvimento do milênio. (LIBRALATO; GHIRARDINI; AVEZZÙ, 2012).

A legislação brasileira determina, através da Lei Nacional de Saneamento Básico (n.° 11.445), sancionada em 2007 e regulamentada através do Decreto n.º 7217/2010, que todas as edificações permanentes urbanas devem ser conectadas às redes públicas de abastecimento de água e de esgotamento disponível. Porém, prevê que na ausência de redes públicas de saneamento básico, serão admitidas soluções individuais de abastecimento de água e de afastamento e destinação final dos esgotos sanitários, assim abrindo precedentes para instalações de tecnologias descentralizadas.

As tecnologias descentralizadas de tratamento de esgoto vêm se destacando dos modelos centralizados principalmente devido à economia de água e energia e ao reuso de nutrientes. Os modelos centralizados apresentam como maior custo os sistemas de coleta (entre 80 a 90% do total), principalmente pelos gastos com energia elétrica para o bombeamento de esgoto até a estação de tratamento (LIBRALATO; GHIRARDINI; AVEZZÙ, 2012). Por sua vez, sistemas descentralizados são instalados próximos às fontes geradoras de efluente, evitando assim, dispêndios expressivos com sistemas de coleta e os altos gastos de energias (BROWN; JACKSON; KHALIFÉ, 2010; HO; ANDA, 2006; RAUCH et al., 2003). Outra importante comparação entre os sistemas é o elevado volume de água potável necessário para manter o sistema de coleta de esgotamento sanitário funcionando (LIBRALATO; GHIRARDINI; AVEZZÙ, 2012; MATOS et al., 2014), enquanto em modelos descentralizados é possível operar com separação de contaminantes na fonte, facilitando o tratamento e o potencial reuso (BORSUK et al., 2008), e refletindo assim, em maior economia de água.

Um dos primeiros e mais comum sistema descentralizado utilizado para tratamento de esgoto doméstico foi o tanque séptico. No entanto, esse sistema nos dias atuais é considerado como tratamento preliminar e deve ser conectado com um tratamento subsequente, pois implantado de forma isolada ou mal dimensionado pode trazer saturamento e contaminação do solo, com sérios riscos à saúde das pessoas da região (WANG et al., 2016). Dessa forma, a escolha da tecnologia de tratamento descentralizada tem que ser eficiente e confiável, com baixo custo para construção, gestão e manutenção, e que apresente autossuficiência e aceitação pelas partes interessadas e o público em geral (CHUNG et al., 2008; MASSOUD; TARHINI; NASR, 2009; VAN AFFERDEN et al.,

2010). Nos últimos anos, muitos projetos de saneamento focado em recurso vêm respeitando essa linha e têm sido implantados com sucesso em diferentes áreas urbanas e rurais com baixa densidade demográfica (WANG et al., 2016).

### 1.2.2 Saneamento focado em recurso

O saneamento focado em recurso, conforme descreve a International Water Association (IWA) está embasado em 5 critérios: os sistemas de tratamento precisam ser economicamente e socialmente aceitáveis, tecnicamente e institucionalmente apropriados, e proteger o ambiente e os recursos naturais. Dessa forma, tem como objetivo imitar os processos ecológicos naturais para tratamento dos resíduos gerados na vida humana, tratando esses materiais da forma mais ecológica e econômica possível, evitando danos ao meio ambiente e a saúde das pessoas (WANG et al., 2016). Esse conceito aborda uma visão holística e interdisciplinar, abrangendo higiene, abastecimento de água, conservação de recursos, paisagismo e planejamento urbano (WERNER et al., 2009)

Assim, o enfoque principal dessa forma de saneamento é o aumento da disponibilidade hídrica pela economia de água, a proteção dos recursos hídricos pelo não lançamento de esgoto (tratado ou não) nos cursos d'água, e a reutilização de forma racional de todos os nutrientes presentes nas excretas (HÉBERT, 2004), sendo a eficiência de tratamento e reutilização de nutrientes facilitada muitas vezes nesse processo devido à separação na fonte.

### 1.2.3 Separação do esgoto doméstico na fonte

Mesmo não sendo considerado como um pré-requisito para o saneamento focado em recurso (WANG et al., 2016), a separação do esgoto doméstico na fonte é um passo importante para simplificar o tratamento domiciliar (OTTERPOHL, 2001). Em uma classificação simples, as águas residuárias podem ser separadas em águas negras e águas cinza. A água cinza (AC) é a porção que não recebe a contribuição dos efluentes do vaso sanitário (ERIKSSON et al., 2002; OTTOSON; STENSTRÖM, 2003), podendo ainda ser classificada em água cinza clara (ACc), quando não recebe a contribuição do efluente da pia da cozinha e de máquinas de lavar pratos.

A água cinza, além de representar a maior fração do esgoto doméstico, podendo chegar a 75% do volume total (LI; WICHMANN; OTTERPOHL, 2009), é a que contém menor concentração de matéria orgânica, nutrientes e patógenos quando comparada com as águas negras, que representa maior risco de contaminação a saúde pública (PRÜSS et al., 2002). Comparando ainda as porções de AC, observa-se que as águas cinza escuras contêm níveis de DQO e sólidos suspensos mais elevados que as águas cinza claras (LI; WICHMANN; OTTERPOHL, 2009). Porém, mesmo com essas visões gerais, ainda é muito difícil ter uma caracterização comum para as águas cinza, uma vez que suas características são muito particulares, dependendo do número e distribuição de idade dos usuários, do estilo e dos padrões de vida e de uso de água e dos hábitos sociais e culturais, que envolvem os tipos de produtos utilizados (sabonetes, pasta de dente, shampoos, detergentes, etc) (GHAITIDAK; YADAV, 2013).

A tabela 1 apresenta algumas caracterizações de águas cinza encontradas na literatura. Com ela é possível verificar que os parâmetros analisados são bem específicos para a finalidade de cada estudo, dificultando ainda mais qualquer comparação entre os efluentes. A tabela ainda apresenta características apenas das águas cinza clara, nas quais os estudos são bem mais restritos.

Tabela 1 - Estudos com caracterizações de águas cinza e águas cinza clara.

| Parâmetros | Zipf et al., 2016 | Barisçi e Turkay, 2016 | Matos et al., 2014 | Stasinakis et al., 2013 | Bani-Melhem e Smit, 2012 | Leal et al.,2011 | Friedler e Gilboa, 2008 | Poh et al.,2015 | Philip e Ramprasad, 2016 |
|---|---|---|---|---|---|---|---|---|---|
| | Fonte de dados | | | | | | | | |
| | Águas Cinza | | | | | | Águas Cinza Clara | | |
| | Dados primários (pia do banheiro do Campus da Universidade) | Dados primários (Águas cinza de alojamentos públicos) | Revisão Bibliográfica* | Dados primários (Águas Cinza de residências) | Dados primários (Águas Cinza do Campus de Universidade) | Dados primários (32 residências) | Dados primários (Águas Cinza Clara de 14 residências) | Dados primários (Águas Cinza Clara de 1 residência) | Dados primários (Águas cinza clara do alojamento do campus da Universidade) |
| pH | 7,7 | 7,62 | 8,9 | 9,03 | 7,6 | - | - | 6,13 | 8,34 |
| Potencial Redox | - | | 204,6 | - | - | - | - | - | - |
| Turbidez | 35,8 | 53,4 | - | - | 133 | - | 33 | - | - |
| DQO ($mg.L^{-1}$) | 145,8 | 229 | 770,0 | 1178 | 463 | 724 | 148 | 445 | - |
| Sólidos Suspensos Totais ($mg.L^{-1}$) | - | 33,5 | 85,0 | 542 | 78 | - | - | 81 | 320 |
| NO3- (mg.L-1) | - | 0,375 | 4,0 | - | 0,93 | 0,77 | - | - | 17,84 |
| Nitrogênio Total ($mg.L^{-1}$) | - | 11,1 | - | - | - | 26,3 | - | - | 28,82 |
| Sulfato ($mg.L^{-1}$) | - | 155,8 | 130,0 | - | - | - | - | - | - |
| Sulfeto ($mg.L^{-1}$) | - | | - | - | - | - | - | - | - |
| Enxofre ($mg.L^{-1}$) | - | | - | - | - | 20 | - | - | - |
| Fósfato ($mg.L^{-1}$) | - | 0,311 | - | - | 0,53 | 2,36 | - | - | 3,84 |
| Ferro ($mg.L^{-1}$) | - | | 0,6 | - | - | 0,74 | - | - | - |
| Coliformes Totais (NMP/100 mL) | 1,8 x 10^5 | - | 1,3 x 10^8 | - | 4,3 x 10^5 | - | 3,8 x 10^4 | 1,10 x 10^8 | - |
| Surfactantes ($mg.L^{-1}$) | 8,3 | - | - | - | 6,45 | 41,1 | - | - | - |

* Foram analisadas as literaturas das referências bibliográficas, para não se considerar dados repetitivos.
- O parâmetro não foi analisado no estudo.

** Referências da tabela (ANTONOPOULOU; KIRKOU; STASINAKIS, 2013; BANI-MELHEM; SMITH, 2012; GILBOA; FRIEDLER, 2008; HERNÁNDEZ LEAL et al., 2011; MATOS et al., 2014; RAMPRASAD; PHILIP, 2016; TEH et al., 2015; ZIPF; PINHEIRO; CONEGERO, 2016)

No entanto, mesmo a água cinza sendo a fração com menores níveis de poluentes do esgoto doméstico, considerando matéria orgânica, nutrientes e patógenos, LI; WICHMANN; OTTERPOHL (2009), em seu estudo de revisão de tecnologias para tratamento de águas cinza, concluíram que processos físicos e biológicos realizados de forma individual não são suficientes para garantir uma adequada redução de matéria orgânica, nutrientes e surfactantes. Segundo os autores a solução mais econômica e viável para o tratamento de águas cinza é a combinação de processos biológicos com filtrações físicas e desinfecção.

### 1.2.4 *Wetlands* Construídos

De acordo com estudo de revisão realizado por Arden e Ma (2018), os sistemas frequentemente usados para o tratamento descentralizado de águas cinza são os *wetlands* construídos (CW). Essa tecnologia vem ganhando espaço (BRIX, 1999; PAULO et al., 2013; VYMAZAL, 2005), por combinar técnicas simples sem necessitar de habilidades operacionais e altos gastos de energia (VYMAZAL, 2010) e apresentar baixos custos de implementação (WANG et al., 2016). Além disso, a versatilidade de se implantar *wetlands* construídos em níveis domésticos pequenos/individuais, bem como nos níveis de comunidade, tornam-no opção preferida para o tratamento de águas cinza (ARUNBABU et al., 2015). Nesses sistemas, a remoção de contaminantes depende de uma combinação de tecnologias, incluindo sedimentação, filtração, precipitação, absorção pelas plantas e principalmente por processos microbiológicos (FAULWETTER et al., 2009; WU et al., 2014). Essa variedade de processos podem ser controlada e intensificada de acordo com o tipo de *wetlands* construídos implantado, que pelo seu modo de operação, configuração hidráulica e hidrologia podem ter seus fluxos classificados em vertical (RAGUSA et al., 2007), horizontal superficial (GAO et al., 2000), horizontal subsuperficial (GARCIA et al., 2005; VYMAZAL, 2005) e horizontal de descarga zero (GREGERSEN; BRIX, 2001).

#### 1.2.4.1 *Wetlands* construídos de fluxo horizontal superficial

Nessa configuração, o efluente a ser tratado é disposto na porção inicial do sistema, que irá percolar através do material filtrante, com a declividade do leito. Esse tipo de *wetlands* é geralmente considerado anóxico, com uma fina camada aeróbia na

superfície devido à aeração passiva da água (BRIX, H; KADLEC, R.H.; KNIGHT, R.L., VYMAZAL, J.; COOPER, P.; HABERL, 2000).

1.2.4.2 *Wetlands* construídos de fluxo horizontal subsuperficial

Essa configuração de CWs apresenta zonas de oxidação e de redução, porém em uma situação global é geralmente considerado um sistema anóxico (BRIX, H; KADLEC, R.H.; KNIGHT, R.L., VYMAZAL, J.; COOPER, P.; HABERL, 2000) com o potencial redox usualmente aumentando da entrada para a saída do sistema, devido à progressiva biodegradação de poluente (VYMAZAL, 2005). Nesse *wetlands,* o esgoto irá passar por regiões anaeróbias (fundo), anóxicas e aeróbias na camada mais superficial pela presença de plantas (KADLEC, ROBERT; KNIGHT, ROBERT, 2000).

1.2.4.3 *Wetlands* construídos de fluxo vertical subsuperficial

Esse tipo de configuração é geralmente considerado por ser altamente aeróbio, com o fluxo de águas escoando verticalmente sobre a matriz plantada, permitindo condições não saturadas e com excelente transferência de oxigênio (BRIX, H; KADLEC, R.H.; KNIGHT, R.L., VYMAZAL, J.; COOPER, P.; HABERL, 2000), o que mantém o sistema com condições redox elevadas, favorecendo os processos aeróbios de remoção de matéria orgânica e de nitrificação e prejudicando processos anaeróbios como a desnitrificação e redução de sulfato (LI; WICHMANN; OTTERPOHL, 2009; STURMAN et al., 2008; VYMAZAL, 2007).

1.2.4.4 *Wetlands* construídos visando descarga zero

Essa configuração, menos conhecida, se assemelha ao sistema horizontal de fluxo subsuperficial, no entanto todo o nutriente presente no meio é aproveitado pelas plantas e a água é evapotranspirada para atmosfera, tornando-o assim um sistema de descarga zero de efluente. Como esse CW não apresenta saída, grande áreas superficiais são necessárias, variando em uma faixa de 200 a 300 $m^2$ para uma residência unidomiciliar (GREGERSEN; BRIX, 2001). GREGERSEN; BRIX (2001), estudando na Dinamarca sistemas de descarga zero com área superficial variando de 150 a 500 $m^2$ preenchidos com solo e três espécies da planta *Salix Viminalis,* concluíram que um dos mais

importantes aspectos para o bom desempenho desses tipos de sistemas é a capacidade de evapotranspiração das plantas utilizadas e a quantidade de chuva incidente no local.

Esse modelo visando à descarga zero é também proposto por PAULO et al. (2013) na utilização de Tanque de Evapotranspiração para o tratamento descentralizado de águas negras. As vantagens desse tipo de configuração é a mínima manutenção requerida, mas, por não apresentarem descarga de efluentes, esses sistemas estão mais sujeitos a colmatação (GREGERSEN and BRIX, 2001).

### 1.2.5 Condições Ambientais em *wetlands* construídos

A eficiência global de CWs para tratamento de esgoto doméstico é determinada pela combinação da configuração hidráulica, tipo de vegetação, tipo de meios filtrantes e processos microbiológicos (BUTTON et al., 2016; FAULWETTER et al., 2009). Os três primeiros fatores são os principais determinantes das condições ambientais, que são influenciadas diretamente pelo pH, potencial redox, temperatura, oxigênio dissolvido e condutividade do sistema, que, por sua vez, juntamente com as características do efluente, determinam os processos microbiológicos dentro de um *wetland* construído.

#### 1.2.5.1 Configuração hidráulica e modo de operação

Cada tipo de configuração hidráulica e modo de operação podem promover uma condição redox específica ou uma larga variedade de condições redox alternadamente. (FAULWETTER et al., 2009). Nesse sentido, as configurações hidráulicas mais utilizadas para sistemas descentralizados e reuso de águas cinza são os de fluxo subsuperficial, pois trazem menores problemas decorrentes de odor, vetores ou exposição pública, do que os de fluxo superficial (YANG; CHANG; HUANG, 2001). Os *wetlands* de fluxo subsuperficial podem ter dois modelos: vertical e horizontal. Fortes comparações entre ambos ainda é muito discutido na literatura, principalmente em relação à eficiência de remoção de poluentes.

RAMPRASAD; PHILIP, (2016) comparando *wetlands* de fluxo subsuperficial horizontal e vertical no tratamento de águas cinza clara de um hotel estudantil, verificaram, para ambas as configurações, eficiência acima de 90% para o parâmetro

Demanda Química de Oxigênio (DQO) e acima de 80% para Demanda Bioquímica de Oxigênio (DBO) e Sólidos suspensos totais (SST). ARUNBABU et al., (2015), tratando águas cinza sintética em *wetlands* construídos de fluxo subsuperfical horizontal com plantas da espécie *Axonopus Compressus,* demonstraram eficiência de 93% e 95,4% para turbidez e DQO respectivamente. Por sua vez, AHMED; AZMA; SAPHIRA (2014), tratando águas cinza de uma residência familiar em protótipos de *wetlands* construídos de fluxo subsuperficial vertical, alcançaram uma eficiência de 84,6% e 81,4% para DQO e DBO respectivamente. Dessa forma, pode-se inferir que, para a remoção de cargas orgânicas e de sólidos, esses dois tipos de sistemas apresentam eficiências bem semelhantes.

Por outro lado, os *wetlands* construídos de fluxo vertical sempre foram conhecidos por sua melhor taxa de areação e consequentemente seu melhor desempenho nos processos de nitrificação (PROCHASKA; ZOUBOULIS; ESKRIDGE, 2007), sendo a limitação de oxigênio na subsuperfície um dos principais fatores limitantes do tradicional fluxo horizontal, especialmente quando o objetivo é a nitrificação e subsequentemente remoção de nitrogênio total (BRIX; SCHIERUP, 2013). No entanto, ADRADOS et al. (2014), pelo fato de alguns microrganismos aeróbios terem sido encontrados em *wetlands* construídos de fluxo horizontal, sugerem que esse sistema embora na maioria do tempo seja saturado, apresentam oxigênio suficiente para permitir a proliferação desses grupos de microrganismos, com a subsequente possibilidade de nitrificação no sistema, sendo a disponibilidade de oxigênio provavelmente devido à areação pelas plantas e ao fato da parte superior da camada filtrante normalmente permanecer insaturada. CHEN et al. (2016), tratando esgoto doméstico sintético em *wetlands* construídos de fluxo subsuperficial horizontal, demonstraram que esses sistemas podem ainda ter maiores níveis de oxigênio dissolvido quando operados em regime de batelada, resultando em maior eficiência na degradação de nutrientes como a amônia, que chegou a 95,2% em regimes de batelada contra 80% em sistemas operados com regime de fluxo contínuo. RAMPRASAD; PHILIP (2016), tratando águas cinza clara em CW de fluxo horizontal, demonstraram eficiência de 95,5% na remoção de nitrogênio total. Assim, esses estudos recentes estão criando novas perspectivas quanto a eficiência da nitrificação para *wetlands* de fluxo subsuperficial horizontal.

Outro ponto interessante é que a efetividade de CWs para tratamento de efluentes sempre esteve direcionada principalmente pela determinação do desempenho do tratamento considerando os ciclos do carbono, nitrogênio, fósforo e sólidos suspensos (KADLEC; WALLACE, 2008). Porém, as transformações do ciclo do enxofre, como a redução microbiológica do sulfato e a reoxidação de compostos reduzidos de enxofre, podem ter um impacto significante na eficiência de sistemas de tratamento de efluentes (AIDA et al., 2015; HAO et al., 2013; ZHOU et al., 2015a), principalmente em *wetlands* construídos (CHEN et al., 2016a; STEIN et al., 2007; WIESSNER et al., 2010; WU et al., 2013). Merece ainda mais atenção principalmente quando o efluente é a água cinza, uma vez, que diversos produtos químicos utilizados no banho contêm em seus componentes o elemento enxofre. Dessa forma, a maior flexibilidade de mudanças de potencial redox encontrada em CW de fluxo subsuperficial horizontal tende a dar maior vantagem a essa configuração na transformação do enxofre, auxiliando a performance do sistema.

#### 1.2.5.2 Plantas

Os principais papeis das plantas em *wetlands* construídos de fluxo subsuperficial horizontal são a provisão de substrato (raízes e rizomas) para o crescimento de bactérias, perda de oxigênio radial (difusão de oxigênio das raízes para a rizosfera), captação de nutrientes e isolamento de biofilme (VYMAZAL, 2007). Assim, elas não estão somente ligadas à absorção de poluentes, mas também aumentam a diversidade ambiental da rizosfera (BRIX, 1997), que é caracterizada como um micro ambiente ao redor das superfícies das raízes (HILTNER, 1904), sendo um dos locais onde as interações entre plantas e microrganismos acontecem em CWs (LLOYD et al., 2004). As zonas de rizosferas em *wetlands* construídos de fluxo subsuperficial horizontal se apresentam como uma alternativa interessante para melhorar a oxigenação do sistema e consequentemente a degradação de poluentes específicos. As taxas de liberação de oxigênio advindas das plantas em CWs são bastante variáveis, sendo reportada uma faixa entre 0,014 e 12 $g/m^2/d$ (NIVALA et al., 2013a). Essa variação é totalmente plausível, uma vez que a liberação de oxigênio pelas plantas pode ser afetada pela sua espécie, condições redox, biomassa, pH, concentração de oxigênio, características químicas do meio, temperatura, intensidade de luz, umidade e velocidade do vento (ZHANG; WU; HU, 2014). Nesse sentido, diversos autores estão estudando os efeitos que diferentes

espécies de plantas em CW provocam na rizosfera e demonstrando que a quantidade e diversidade de microrganismos encontradas nesses microambientes são interdependentes das plantas escolhidas (BUTTON et al., 2016; LIU et al., 2016; ZHANG; WU; HU, 2014). GARCÍA et al., (2005), tratando esgoto doméstico em *wetlands* construídos no nordeste da Espanha, demonstraram maior eficiência na remoção de DQO, DBO e amônia em CWs de 27 cm do que em CWs de 50 cm de profundidade, sugerindo que os resultados alcançados foram motivados pelo fato do efluente no *wetlands* mais raso ser forçado a passar pelas zonas de raízes.

#### 1.2.5.3 Meios Filtrantes

A microbiota mais estável em *wetlands* construídos é encontrada no biofilme associado às raízes das plantas e/ou aderidos a superfície do meio filtrante (FAULWETTER et al., 2009). Essa complexa comunidade microbiana criada por interações com as águas residuárias é a principal responsável pelo desempenho da degradação da matéria orgânica no sistema (SLEYTR et al., 2009). Pesquisas estão demonstrando que a eficiência de remoção de matéria orgânica em sistemas de leito fixo é diretamente relacionada ao meio filtrante escolhido para imobilização dos microrganismos (YANG et al., 2004a), sendo que os meios filtrantes com alta porosidade apresentam melhor eficiência do que materiais menos porosos (INCE; KASAPGIL INCE; DONNELLY, 2000). Nesse sentido, uma enorme variedade de meios filtrantes, como escória de alto forno (RENMAN et al., 2009), espuma de poliuretano (SARTI et al., 2006), bucha vegetal (YANG et al., 2004b), bambu (FENG et al., 2008), carbono vegetal (GARCIA et al., 2008), brita (BAPTISTA et al., 2003), cerâmica (PAULO et al., 2013), cascalhos ricos em ferro (CHEN et al., 2016a), entre outros vêm sendo testados para fixação da biomassa.

Estudos comparativos no uso de diferentes meios filtrantes foram realizados em diferentes trabalhos. GARCIA et al. (2008) compararam o desempenho de 4 diferentes meios filtrantes (espuma de poliuretano, carbono vegetal, polietileno de baixa densidade reciclado e pedra pomes) aplicando esgoto doméstico em reatores anaeróbios, e YANG et al. (2004a) compararam a performance de 4 diferentes meios filtrantes (filtro de carvão, pedra de lã, bucha vegetal e espuma de poliuretano) aplicando ácido acético. Ambos verificaram que os meios filtrantes mais porosos, como a espuma de poliuretano, têm

maiores vantagens sobre os menos porosos. No entanto, a avaliação de diferentes meios filtrantes em sistemas de condições redox variáveis, como *wetlands* construídos, ainda é bastante recente, existindo uma falta de informação da diversidade e das mudanças na comunidade microbiana em longas operações tratando esgoto doméstico (KRASNITS et al., 2009). ROZARI; GREENWAY; HANANDEH, (2016), tratando esgoto doméstico em *wetlands* de fluxo subsuperficial vertical preenchidos com areia com diferentes quantidades de carvão vegetal, demonstraram que a adição de maiores quantidades de carvão encoraja a atividade microbiana, porém verificaram que a redução do fósforo é inversamente proporcional a adição de carvão. LIU et al. (2014), testando zeólito, areia de quartzo, rochas vulcânicas e argila biológica como meios filtrantes em protótipos de CWs tratando esgoto doméstico sintético, composto por amônia, DQO e fósforo, concluíram que a abundância de bactérias específicas teve relação com o meio filtrante testado, porém a diversidade microbiana foi semelhante nos meios. CHEN et al. (2016) tratando esgoto doméstico em *wetlands* construídos, demonstraram que a utilização de cascalho rico em ferro, com adição de diferentes quantidades de matérias orgânicas decompostas, como meio filtrante, pode formar um ecossistema propício para tratamento de sulfato, sulfeto e nitrogênio.

Além de servir como base para comunidade microbiana, o uso de meios filtrantes em *wetlands* construídos é também um importante fator para determinar condições ambientais (como potencial redox) dentro do sistema (FAULWETTER et al., 2009). A combinação e proximidade de diferentes processos redox são importantes para eficácia global de remoção de poluentes e estão entre as principais vantagens do tratamento de águas residuais com CWs, em comparação com outras tecnologias (WU et al., 2013).

### 1.2.6 Comunidades microbianas em *wetlands*

Até recentemente, pesquisas em *wetlands* construídos estavam voltadas principalmente para os aspectos de configurações hidráulicas, com a principal preocupação sendo os conceitos de entrada e saída de carga orgânica. Ainda no início do século 21, as zonas internas de atividade de comunidades microbianas eram vistas como uma caixa preta (KUSCHK et al., 2003). No entanto, os principais avanços no entendimento da função central dos microrganismos nos ciclos biogeoquímicos dentro dos CWs tornaram o detalhamento do conhecimento da diversidade funcional e

propriedades metabólicas das comunidades microbianas fatores indispensáveis para melhorar o desempenho e configuração desses sistemas (BOUALI et al., 2013; BRIX; SCHIERUP, 2013; DENG et al., 2011; GARRIDO et al., 2014; TRUU; JUHANSON; TRUU, 2009). Além disso, informações sobre as estruturas e diversidade das comunidades de microrganismos têm sido consideradas bastante importantes para o entendimento da relação entre condições ambientais e funções do ecossistema (PERALTA; AHN; GILLEVET, 2013; SIMS et al., 2013).

A expansão desse conhecimento só foi permitida recentemente devido à evolução dos estudos moleculares baseados nas análises de sequência de genes do RNAr16S (BOUALI et al., 2013). A disponibilidade de sequenciamento de alto rendimento (sequenciamento de parte do gene RNAr16S) permite uma análise rápida da comunidade microbiana em nível maior de detalhamento (INCEOĞLU et al., 2011), possibilitando a identificação das complicadas estruturas bacterianas aderidas a meios filtrantes em sistemas de *wetlands* construídos (ANSOLA; ARROYO; SÁENZ DE MIERA, 2014; LIGI et al., 2014; ZHONG et al., 2014). A aplicação dessas tecnologias tem apresentado bons resultados para análise da comunidade bacteriana, mas seu uso ainda é bastante recente para a identificação de arqueias em CWs. HE et al. (2015) foi o pioneiro a identificar comunidade de arqueias, por meio de sequenciamento de alto rendimento (plataforma Illumina), em sistema piloto de wetlands construídos preenchidos com cascalho tratando esgoto domestico de uma comunidade rural. Posteriormente, LONG et al. (2016) utilizando a mesma técnica de identificação, analisaram a comunidade de arqueias em CW para tratar águas poluídas de um rio. Dessa forma, verifica-se que com o uso de técnicas de sequenciamento de alto rendimento pode ser possível entender, em maior profundidade e detalhes, como são as transformações biológicas realizadas pela comunidade microbiana e a degradação de nutrientes e poluentes em CW (BAI et al., 2014).

Com essas novas tecnologias, muitos autores começaram a abrir essa caixa preta e a estabelecer algumas considerações sobre as comunidades microbianas dentro dos *wetlands* (ANSOLA; ARROYO; SÁENZ DE MIERA, 2014; BOUALI et al., 2013; CHEN et al., 2016a; HE et al., 2016; ZHANG et al., 2015a; ZHI et al., 2015). Os resultados em comum encontrados nesses estudos demonstram a existência de uma variabilidade espacial da comunidade microbiana, sendo a profundidade e as zonas de entrada e saída dos sistemas fatores determinantes para essas diferenças. Verificou-se

também que o grupo de bactérias do filo Proteobactérias são predominantes em CWs. HE et al. (2015), tratando efluente de uma comunidade rural em protótipos CWs de fluxo vertical, demonstraram que a diversidade de microrganismos diminuiu com a profundidade, sendo que a comunidade de bactérias foi principalmente composta por Proteobactérias, Chloroflexi e Firmicutes, enquanto Euryachaeota e Thaumarchaeota foram dominantes na comunidade das arqueias. BOUALI; ZRAFI; BAKHROUF, (2013), por sua vez, analisando a comunidade de microrganismos das zonas de rizosfera (0-20cm) e do fundo (50-100cm) de um *wetland* construído de fluxo horizontal subsuperficial preenchido com cascalho, assim como HE et al. (2015), verificaram que a diversidade de microrganismos diminui da rizosfera para o fundo, assumindo que a maior parte do processo de degradação biológica dos poluentes ocorreu na camada superior, onde existia maior presença de oxigênio e nutrientes. O grupo de bactérias predominante na camada da rizosfera e do fundo foram as Proteobactérias com clones afiliados a microrganismos aeróbios para a camada superior e anaeróbios para camada inferior do sistema. Por sua vez, CHEN et al. (2016), tratando esgoto doméstico em *wetlands* contruídos preenchidos com cascalho ricos em ferro, verificaram que a liberação de oxigênio realizada pelas plantas nas zonas de rizosfera pode diminuir a eficiência de redução de sulfato, que foi principalmente realizada por Bactérias Redutoras de Sulfato (BRS). Já os compostos reduzidos de enxofre foram oxidados principalmente por vias anóxicas com utilização de nitrato como aceptor de elétrons, por bactérias oxidadoras de enxofre) desnitrificantes.

Na análise dos microrganismos encontrados nos CW, observa-se que as arqueias podem participar de vários processos biogeoquímicos como metanogênese, oxidação de amônia e redução de sulfato (ZHANG et al., 2015b), e podem também contribuir na remoção de nitrogênio (BOUALI et al., 2012). O filo *Proteobactérias* inclui um nível muito elevado de bactérias com diversidade metabólica que abrangem os ciclos do carbono, nitrogênio e enxofre (GILLIS et al., 2006). Por sua vez, o filo Chloroflexi, representado principalmente pela familia Anaerolineaceae é comum em ambientes naturais com condição anóxica e sendo também encontrados em ambientes artificias, como sistemas de tratamento biológico, inclusive tratando esgoto doméstico (YAMADA; SEKIGUCHI, 2009), e em reatores *anammox* (Fernandes et al., 2018; Pereira et al., 2014) podendo ainda também estar relacionado à oxidação de compostos reduzidos de enxofre (BERNARDES et al., 2016).

Dessa forma, verifica-se que para o tratamento de esgoto doméstico, a

comunidade científica começa a acumular conhecimento quanto à comunidade microbiana nesses sistemas. No entanto, as pesquisas relacionadas à identificação da composição e diversidade microbiana em sistemas tratando águas cinza ainda são escassas. HYLANDER et al. (2014) foram os pioneiros na identificação da comunidade microbiana, utilizando técnicas de sequenciamento de alto rendimento estudando filtros biológicos preenchidos com cascas de árvore, carvão biológico e areia no tratamento de água cinza sintética. Os resultados do estudo demonstraram que a diversidade microbiana é diferente em cada meio filtrante e diminui com a profundidade do filtro.

A mais comum e efetiva técnica para analisar a saúde ou a condição biológica de um *wetland* é a identificação da comunidade microbiana, seguida pela avaliação de sua condição em relação aos parâmetros físico químicos submetidos ao sistema (SIMS et al., 2012). BUTTON et al. (2015), tratando esgoto doméstico em diferentes configurações de CW e aplicando análises de correlação, verificaram que o oxigênio dissolvido apresentava correlação negativa com a atividade microbiana, podendo inferir que as zonas aeróbias tenderam a maiores atividades e que a turbidez apresentava correlação positiva com a presença de microrganismos, inferindo que quanto maior a turbidez maior a presença de substratos para os microrganismos. ZHI; SONG; DUAN, (2015) tratando águas de um rio contaminado com efluentes industriais e de agricultura, demonstraram, com técnicas estatísticas de correlação, que a abundância de bactérias oxidadoras de amônia é diretamente proporcional ao pH, e que o gene *amoA* apresenta correlação com pH, oxigênio dissolvido, amônia e nitrato. Assim, o conhecimento das condições ambientais do meio, representadas por fatores físico químicos, são essenciais para o entendimento das funções microbiológicas e consequentemente do desempenho de um CW, podendo exercer forte influência no funcionamento do ecossistema (MICHAEL R. BROOKER, 2013). CAVIGELLI; PHILIP ROBERTSON, (2000), estudando a significância funcional da composição de uma comunidade de bactérias desnitrificantes, concluíram que organismos encontrados em nichos específicos, com características definidas (nutrientes, pH, potencial redox, entre outros), irão crescer melhor e se adaptar em ambientes com similaridade dessas características.

Nesse contexto, é importante salientar que a taxonomia não implica explicitamente funcionalidade entre microrganismos, e estudos detalhados, relacionando genes funcionais à taxonomia, devem ser realizados para atingir esse nível de identificação (URICH et al., 2008). No entanto, alguns organismos compartilham mais

semelhanças filogenéticas do que outros, como, por exemplo, os metanogênicos e metanotróficos, e são bem mais fáceis de determinar em uma base taxonômica (NAZARIES et al., 2013). Nessa linha de raciocínio, a tabela 2 demonstra uma variedade de gêneros de microrganismos que estão sendo relacionados a ciclos biogeoquímicos específicos em sistemas de tratamento de efluentes.

Tabela 2. Gêneros de microrganismos e seus metabolismos e funções potenciais.

| Grupo | Gênero | Referência | Grupo | Gênero | Referência |
|---|---|---|---|---|---|
| Hidrolíticas, Fermentativas, Sintróficas e Acetogênicas (HFSA) | *Aminivibrio* | (Militon et al., 2015; Morató et al., 2014; Veeravalli et al., 2016; Wang et al., 2018) | Metanogênicas | *Methanosaeta* | (Chen et al., 2013; Enzmann et al., 2018; Sun et al., 2018; Zhang et al., 2018) |
| | *Propioniovibrio* | | | *Methanosarcina* | |
| | *unidentified Synergistaceae* | | | *Methanomethylovorans* | |
| | *Cloacibacillus* | | | *Methanospirillum* | |
| | *Brevibacillus* | | | *Methanobrevibacter* | (Enzmann et al., 2018; Jing et al., 2013; Thauer et al., 2008) |
| | *Aminomonas* | | | *Methanobacterium* | |
| | *Aminiphilus* | | Metanotróficas | *Methylobacter* | (Osborne and Haritos, 2018; Prasse et al., 2015; Truu et al., 2009; Urakawa et al., 2017; Bao et al., 2014) |
| | *VadinCA02* | | | *Methylocaldum* | |
| | *Lactivibrio* | | | *Methylococcus* | |
| | *Thermovirga* | | | *Methylomicrobium* | |
| | *[Eubacterium]rectale_group* | (Tang et al., 2011; Wang et al., 2018; Zeigler, 2016; Zhou et al., 2018) | | *Methylomonas* | |
| | *Faecalibacterium* | | | *Methylosinus* | |
| | *Paenisporosarcina* | | | *Candidatus_Koribacter* | (Kielak et al., 2016; Koch et al., 2008) |
| | *Christensenellaceae R-7_group* | | | *Candidatus Methylomirabilis* | (Ettwig et al., 2010) |
| | *Ruminococcaceae. UCG-002* | | | *Pleomorphomonas* | (Bao et al., 2014; Dedysh et al., 2003) |
| | *Roseburia* | | | | |
| | *[Eubacterium]_coprostanoligenes_group* | | | | |
| | *Acinetobacter* | (Adrados et al., 2014; He et al., 2016; Hylander et al., 2014) | Metilotróficas | *Badryrhizobium* | (Bao et al., 2014; Mäkipää et al., 2018) |
| | | | | *Methylobacterium* | (Knief et al., 2010; Tang et al., 2011; Urakawa et al., 2017) |
| | *Microbacterium* | (Anderson et al., 2011; Wang et al., 2018) | | *Methylophilus* | |
| | *vadinBC27_wastewater-sludge_group* | (Adrados et al., 2014; Dai et al., 2017; Yan et al., 2018) | | *Methyloversatilis* | |
| | *Prevotella_9* | | | *Candidatus Methylospira* | (Ho et al., 2016) |
| | *Bacteroides* | | Nitrificantes | *Nitrosospira* | (Fan et al., 2016; Pelissari et al., 2017a) |
| | *Sediminibacterium* | | | *Nitrosomonas* | |
| | *Rhodopseudomonas* | (Adessi et al., 2016; Koku et al., 2002; Mckinlay and Harwood, 2011; Tang et al., 2011) | | *Nitrobacter* | (Zuma et al., 2009) |

| Grupo | Gênero | Referência | Grupo | Gênero | Referência |
|---|---|---|---|---|---|
| Hidrolíticas, Fermentativas, Sintróficas e Acetogênicas (HFSA | *Rhodoplanes* | (Anderson et al., 2011; Sun et al., 2018) | Nitrificantes | *unidentified Nitrospiraceae* | (Zhong et al., 2014) |
| | *Syntrophus* | (Kersters et al., 2006; Zhang et al., 2018) | | | |
| Degradadoras de Compostos Aromáticos (ACD) | *Brevibacterium* | (Kielak et al., 2016) | | *Candidatus Nitrosotalea* | (Bouali et al., 2013; Zhang et al., 2018) |
| | *Arthrobacter* | | | *Candidatus Nitrososphaera* | |
| | *Alcaligenes* | (He et al., 2016; Zhou et al., 2018) | | *Candidatus Nitrosoarchaeum* | |
| | *Alicycliphilus* | | | *unidentified Thaumarchaeota* | |
| | *Ideonella* | | Denitrificantes | *Opitutus* | (Pelissari et al., 2017b) |
| | *Ramlibacter* | | | *Dechloromonas* | (Adrados et al., 2014; Sun et al., 2018; Wu et al., 2016) |
| | *Massilia* | | | *Thauera* | |
| | *Mycobacterium* | (Chipasa and Mędrzycka, 2006; Seo et al., 2009) | | *Denitratisoma* | (Qian et al., 2015; Rodríguez-Martínez et al., 2016) |
| | *Rhodococcus* | | | *Zoogloea* | |
| | *Staphylococcus* | (Tommaso et al., 2002) | | *Propionivibrio* | |
| | *Novosphingobium* | (Gan et al., 2013; Kersters et al., 2006; Takeuchi et al., 2001) | | *Azospira* | |
| | *Sphingomonas* | | | *Variovorax* | (Zhong et al., 2014) |
| | *Sphingobium* | | | *Acidovorax* | |
| | *Sphingopyxis* | | | *Hydrogenophaga* | |
| | *Erythrobacter* | | | *Pseudomonas* | (Hylander et al., 2014) |
| | *Pseudoxanthomonas* | (He et al., 2016) | | *Bosea* | (Bao et al., 2014) |
| Bactérias Redutoras de Sulfato (BRS) | *Desulfomonile* | (Aida et al., 2014; Hao et al., 2014) | | *Xanthobacter* | (Kersters et al., 2006) |
| | *Desulfobacca* | | | *Candidatus_Competibacter* | (McIlroy et al., 2014) |
| | *Smithella* | | *Fixadoras de Nitrogênio* | *Rhizobium* | (Aya et al., 2015) |
| | *Desulforegula* | (Chen et al., 2016; Stein et al., 2007; Sturman et al., 2008) | Bactérias oxidadoras de Enxofre (BOS) | *Thiobacillus* | (Chen et al., 2016; Pokorna and Zabranska, 2015) |
| | *Desulfovibrio* | | | *Roseobacter_clade_CHAB-I-5_lineage* | (Luo et al., 2013) |
| | *Desulfomicrobium* | | | *Sulphuricurvum* | (Chen et al., 2016) |
| | *Synthrophobacter* | (Plugge et al., 2011; Urakawa et al., 2017) | | *Rhodoblastus* | (Luo et al., 2013; Pfennig, 1975; Urakawa et al., 2017; Urakawa and Bernhard, 2017) |
| | *Desulforhabdus* | | | *Rhodobacter* | |
| | *Desulfomonile* | | | *Sulfolobus* | (Pokorna and Zabranska, 2015; Urakawa et al.,2017) |
| | *Desulfovirga* | | | *Beggiota* | |
| | *[Desulfobacterium]_catecholicum_group* | (Fawzy et al., 2015) | | *Thiothrix* | |
| | | | | *Chlorobium* | |

| Grupo | Gênero | Referência | Grupo | Gênero | Referência |
|---|---|---|---|---|---|
| Bactérias Redutoras de Sulfato (BRS) | *Desulfitobacterium* | (Villemur et al., 2006; Urakawa et al., 2017) | Bactérias oxidadoras de Enxofre (BOS) | *Choroherpeton* | (Pokorna and Zabranska, 2015; Urakawa et al.,2017) |
| | | | | *Prosthecochloris* | |
| | | | | *Chloroflexi* | (Bernardes et al., 2016; H. Urakawa and Bernhard, 2017) |
| Bactérias Redutoras de Ferro | *Geobacter* | (Sun et al., 2018; Xu et al., 2017) | Fotossintéticas | *Cyanobacteria - unidentified Chloroplast* | (Kong et al., 2017) |
| | *Geothrix* | (Kielak et al., 2016) | | *Prochlorococcus* | (Brown et al., 2009) |

### 1.2.7 Comunidade Microbiana e os Ciclos Biogeoquímicos

Para tornar o tema mais complexo, grupos de bactérias podem estar associados com funções simultâneas em diferentes ciclos, como por exemplo, do carbono, nitrogênio e enxofre (CAO et al., 2017). O ciclo do carbono é, sem dúvida, o que contém maior número de microrganismos envolvidos, uma vez que a maioria das bactérias, principalmente as heterotróficas, necessitam de carbono orgânico para formação de tecido celular (SAEED; SUN, 2012). Nesse sentido, este tópico não tem a intenção de exaurir a discussão sobre composição e função de microrganismos nos mais abrangentes ciclos biogeoquímicos, mas sim descrever determinados comportamentos que algumas bactérias e arqueias vem demonstrando dentro de sistemas de tratamento de efluentes.

#### 1.2.7.1 Processo de hidrólise e fermentação

A degradação anaeróbia em *wetlands* é um processo realizado em duas etapas por microrganismos anaeróbios heterotróficos. Na primeira etapa, bactérias formadoras de ácido convertem matéria orgânica em novas células, ácidos orgânicos e álcoois (SAEED; SUN, 2012). De forma simplificada, essa primeira fase é realizada nas seguintes etapas: polímeros são hidrolisados para monômeros, e lipídios são hidrolisados para ácidos graxos de cadeia longa (AGCLs) e glicerol. Os AGCLs são absorvidos e transportados dentro das membranas das células microbianas e, uma vez lá dentro, são

degradados em ácido acético, hidrogênio e $CO_2$ por bactérias acetogênicas sintróficas (MA et al., 2015; THAUER et al., 2008).

O gênero *Acinetobacter* e o filo Synergistetes têm sido reportados por ser responsável por esses passos iniciais, o primeiro agindo na degradação de lipídios para ácidos graxos de cadeias longas (CHIPASA; MĘDRZYCKA, 2006; MA et al., 2015) e o segundo na degradação de proteínas para ácidos graxos (MILITON et al., 2015). Nos passos sequenciais, a classe Bacilli, Clostridia dentro do filo Firmicutes e os genêros *Syntrophus* e *Syntrophobacter* dentro da família Syntrophobacteraceae estão sendo apontados como responsáveis pela degradação de butirato e propionato em acetato, hidrogênio e gás carbônico (LIU; CONRAD, 2017; NELSON; MORRISON; YU, 2011; WANG et al., 2018) e, dessa forma, trabalham em sintrofia com os microrganismos envolvidos no processo de metanogênese e sulfetogênese (NOBU et al., 2015; ZHANG et al., 2018). Os genêros *Syntrophus* e *Syntrophobacter* dentro da família Syntrophobacteraceae é um exemplo do envolvimento de microrganismos em dois ciclos biogeoquímicos, pois a realização da degradação de propionato por essa bactéria está relacionada à redução de sulfato, quando este está presente no meio (LIU; CONRAD, 2017). Outro microrganismo que pode atuar nessa etapa final da fermentação é a espécie *Rhodopseudomonas Palustris,* que está sendo indicada como participante na degradação de compostos orgânicos (principalmente, piruvato, lactato, malato e sucinato) para produção de hidrogênio (ADESSI et al., 2016; TANG; TANG; BLANKENSHIP, 2011). O segundo passo da digestão anaeróbia consiste nos processos de metanogênese e sulfetogênese.

#### 1.2.7.2 Processo de metanogênese

O processo de metanogênese é realizado por microrganismos conhecidos como arqueias metanogênicas (AM). Essas arqueias, estritamente anaeróbias, pertencem ao filo Euryarchaeota que utilizam $CO_2$ e $H_2$ e/ou pequenas moléculas orgânicas, como acetato, formiato, metanol e metilaminas para formação de metano (ENZMANN et al., 2018; SAEED; SUN, 2012; THAUER et al., 2008). Recentes pesquisas têm demonstrado que esses microrganismos não estão restritos apenas ao filo Euryarchaeota, podendo também estar presentes em dois novos filos denominados Bathyarchaeota (ENZMANN et al., 2018) e Verstraetearchaeota (VANWONTERGHEM et al., 2016).

No entanto, as principais ordens descritas dessas arqueias são pertencentes ao filo Euryarchaeota: Methanococcales, Methanobacteriales, Methanosarcinales, Methanomicrobiales, Methanopyrales, Methanocellales e Methanomassiliicoccales (SAKAI et al., 2008). Essas ordens são responsáveis por três pincipais tipos de metanogênese: hidrogenotrófica, acetotrófica e metilotrófica, que se diferem pelo doador de elétrons para o processo de produção de metano. A metanogênese hidrogenotrófica é realizada a partir do $H_2$ e do $CO_2$, e basicamente todas as ordens citadas, com excessão da *Methanomassiliicoccales,* a realizam. A formação do metano a partir do acetato, denominada acetotrófica, pode ser encontrada apenas na ordem *Methanosarcinales.* Por fim, a metanogênese a partir de compostos metilados (metanol, metilaminas ou tióis metilados*)* é encontrada nas ordens Methanomassiliicoccales, Methanobacteriales and Methanosarcinales (ENZMANN et al., 2018; THAUER et al., 2008).

Em sistemas de tratamento de efluentes os tipos mais encontrados são as arqueias hidrogenotróficas e as acetotróficas (HE et al., 2015; O'FLAHERTY et al., 1998; THAUER et al., 2008; YAN et al., 2018). No grupo das hidrogenotróficas podemos destacar os genêros: *Methanobacterium, Methanobrevibacter e Methanospirillum*. No grupo das acetotróficas: *Methanosarcina e Methanosaeta*, porém o genêro *Methanosarcina* pode usar também o $H_2 + CO_2$ para produção de metano, já a *Methanosaeta* são arqueias exclusivamente acetotróficas. É ainda importante destacar que alguns membros pertencentes a *Methanobacterium* e *Methanosarcina* podem sobreviver em ambientes com exposição de oxigênio (HE et al., 2015; KIENER; LEISINGER, 1983), e dessa forma, se adaptar até mesmo nas camadas superiores dos *wetlands* construídos.

#### 1.2.7.3 Processo de sulfetogênese

A outra forma de realizar as etapas finais da degradação anaeróbia é por meio da sulfetogênese. A sulfetogênese é realizada por bactérias conhecidas como redutoras de sulfato (BRS), e, na presença de sulfato, são apontadas como responsáveis por contribuir de 20 a 60% na redução de DQO em *wetlands* e outros ambientes anaeróbios (FAULWETTER et al., 2009; JØRGENSEN, 1982; STEIN et al., 2007; STURMAN et al., 2008). Os procariotos redutores de sulfato constituem um grupo diverso de bactérias anaeróbias comuns na natureza e desempenham um papel essencial no ciclo do carbono

e do enxofre. As BRS utilizam principalmente sulfato, a forma mais oxidada de enxofre, como o aceptor de elétrons na oxidação de produtos originados da hidrólise e fermentação anaeróbia, tais como, aminoácidos, açúcares, ácidos de cadeias longas, ácidos graxos, compostos aromáticos, lactato, butirato, propionato, acetato e hidrogênio (CHEN et al., 2016a; MUYZER; STAMS, 2008; PLUGGE et al., 2011; WIESSNER et al., 2010). Alguns autores demonstram que as BRS podem ainda utilizar sulfito, tiossulfato, enxofre elementar, nitrato, nitrito e compostos reduzidos de ferro como aceptor de elétrons (AIDA et al., 2015; HAO et al., 2014; HE et al., 2015; QIAN et al., 2015).

Três tipos principais de conversão biológica do enxofre são envolvidos no crescimento heterotrófico de BRS: a) oxidação completa de ácidos intermediários para dióxido de carbono, b) oxidação incompleta de ácidos intermediários para acetato e c) degradação sintrófica de partículas orgânicas, como o crescimento fermentativo na presença de propionato e etanol (COLLERAN; FINNEGAN; LENS, 1995; HAO et al., 2014). As BRS mais conhecidas como oxidadoras incompletas são os gêneros *Desulfomicrobium, Desulfovibrio e Desulfobulbus,* as mais conhecidas como oxidadoras completas são *Desulfobacterium, Desulfobacter e Desulfococus* (QIAN et al., 2015) e como organismos sintróficos são *Syntrophobacter, Syntrophaceae e Syntrophorhabdaceae* (PLUGGE et al., 2011).

No entanto, é preciso esclarecer que o crescimento de oxidadoras incompletas, como a *Desulfovibrio*, em adição ao $H_2$-$CO_2$, requer 2 compostos de carbono, como o acetato, como fonte de carbono (COLLERAN; FINNEGAN; LENS, 1995), dessa forma, mesmo sendo relatado que as BRS tem maior afinidade por lactato e propionato do que hidrogênio, metanol, etanol e acetato (TAYLOR; LIAMLEAM; ANNACHHATRE, 2010), elas acabam também utilizando substratos de menor afinidade para redução do sulfato. LU et al. (2018), estudando a composição da comunidade microbiana em reatores UASB tratando efluente de amido, verificaram que o aumento de *Desulfovibrio* reduziu a produção de metano, por outro lado o aumento de *Syntrophobacter,* que pode degradar propionato para acetato e $CO_2$ e assim atuar em sintrofia com arquéias acetotróficas (PLUGGE et al., 2011), foi diretamente proporcional ao aumento de arqueias metanogênicas e consequentemente ao aumento da produção de metano.

Assim, quando não estão trabalhando em sintrofismo, arqueias metanogênicas e bactérias redutoras de sulfato são conhecidas por competir pela disponibilidade de

carbono em faixas similares de potencial redox (OMIL et al., 1998). Estudos demonstram que as BRS geralmente vencem as arqueias metanogênicas, por vários motivos: BRS se adaptam melhor e resistem a variações de cargas orgânicas (DAMIANOVIC; FORESTI, 2009; HANSEN, 1994), ao sulfeto livre no meio (HAO et al., 2014; MIZUNO; LI; NOIKE, 1998; STEIN et al., 2007) e, como demonstrado, apresentam maior afinidade a uma variedade de substratos (KRISTJANSSON; SCHÖNHEIT, 1983; LOVLEY; KLUG, 1983; THAUER et al., 2008). Outro fator que pode determinar a vencedora dessa competição é a relação DQO/Sulfato do meio, que em média geral, mostram que BRS levam vantagem nas relações abaixo de 2 e desvantagem nas acima de 2 (CHOI; RIM, 1991; LU et al., 2018; MIZUNO; LI; NOIKE, 1998). Porém, HU et al. (2015), verificando o efeito da relação DQO/Sulfato em UASB tratando efluentes sintéticos contendo sulfato, concluíram que a competição entre BRS e AM não depende apenas de um único fator, como a relação DQO/Sulfato, mas sim de uma combinação de fatores.

#### 1.2.7.4 Processo de oxidação do metano

Um dos principais produtos da degradação anaeróbia é o metano ($CH_4$), gás produzido pelas arqueias metanogênicas, no processo de metanogênese. O metano é o segundo mais importante gás do efeito estufa na atmosfera após o dióxido de carbono ($CO_2$), e dessa forma, a mitigação de suas emissões se tornou uma preocupação global para minimização das mudanças climáticas (DENMAN et al., 2007). A maior fonte de emissão de metano, representando cerca de 19% do total, são os *wetlands* (DENMAN et al., 2007). Nesse sentido, microrganismos metilotróficos, capazes de oxidar o metano a dióxido de carbono tem sido estudados (HE et al., 2015; HO et al., 2016; MÄKIPÄÄ et al., 2018; ZHANG; JIAO; LU, 2018).

As metilotróficas são bactérias que usam compostos orgânicos sem ligações C-C como doadores de elétrons e como fontes de carbono (MADIGAN et al., 2014). Algumas metilotróficas, chamadas metanotróficas, podem utilizar o metano como fonte de carbono, e assim são um caso especial das metilotróficas (URAKAWA; BERNHARD, 2017). Existem três tipos principais de metanotróficas: tipo I (ex: *Methylomonas* e *Methylobacter*), tipo II (ex: *Methylosinus* e *Methylocystis)* e tipo X (*Methylococcus*). A metanotrófica tipo I (recentemente reagrupado como tipo Ia) são adaptadas a condições ambientais com altas taxas de oxigênio e baixa quantidade de metano, o tipo II prefere

alta quantidade de metano e baixa quantidade de oxigênio e o tipo X (recentemente reagrupado como tipo Ib) possui características de ambos os tipos (URAKAWA; BERNHARD, 2017).

No entanto, o processo bioquímico da conversão de $CH_4$ para $CO_2$ não é tão simples e não envolve apenas as metanotróficas. Essa conversão é realizada de modo resumido nos seguintes passos: conversão de $CH_4$ para metanol, de metanol para formaldeído, de formaldeído para formiato, de formato para o $CO_2$ e são mediados respectivamente pelas seguintes enzimas: metano monoxigenase, metanol desidrogenase, formaldeído desidrogenase e formiato desidrogenase (HANSON; HANSON, 1996). Nesse sentido uma série de microrganismos está envolvida no processo de conversão do metano para $CO_2$. HO et al., (2016), estudando a interação biótica em comunidades microbianas, verificaram, por análises estatísticas de network, a coocorrência de metilotróficas, organismos que oxidam o metanol (ex: *Methylotenera, Methylobacterium, Methylobacillus, Methylohalomonas*), com as metanotróficas, sugerindo que as metilotróficas se alimentam do metanol derivado da oxidação do metano. BAO et al. (2014), investigando a comunidade de metanotróficas, em campos de arroz, encontraram a enzima metanol desidrogenase na bactéria do genêro *Bradyrhizobium* e verificaram sua coocorrência com as metanotróficas. Por fim, KIELAK et al. (2016), estudando os genes e genomas do filo Acidobactéria, verificaram que esses microrganismos (ex: *Candidatus Koribacter*) utilizavam exopolissacarídeos produzidos pelas metanotróficas como fonte de carbono.

1.2.7.5 Processo de oxidação do enxofre

Outro produto resultante da digestão anaeróbia é o sulfeto, que é produzido pelas bactérias redutoras de sulfato, no processo de sulfetogênese. O sulfeto é tóxico para maioria dos microrganismos e sua oxidação é um notável serviço para o ecossistema (URAKAWA; BERNHARD, 2017). A oxidação microbiológica é o principal processo de retirada de sulfeto em *wetlands* com condições anóxicas e anaeróbias (WU et al., 2013). O processo microbiológico de oxidação de compostos reduzidos de enxofre é principalmente realizado pelas bactérias oxidadoras de S (BOS), que transformam o sulfeto, sulfito, tiossulfato e enxofre elementar para sulfato, usando oxigênio ou nitrato como aceptor de elétrons (FAULWETTER et al., 2009). O desenvolvimento do

entendimento sobre as BOS ainda é incompleto nos *wetlands*, devido as ineficientes detecções de espécies nesses sistemas, que geralmente estão presentes em baixa abundância (CHEN et al., 2016a).

A remoção biológica de sulfetos é realizada principalmente por dois tipos de bactérias: fototróficas e quimiotróficas (POKORNA; ZABRANSKA, 2015; URAKAWA; BERNHARD, 2017). As bactérias quimiotróficas oxidadoras de sulfeto usam a energia de compostos inorgânicos reduzidos contendo enxofre (sulfeto de hidrogênio, tiossulfatos, sulfitos, elementar enxofre) e em alguns casos também de compostos orgânicos de enxofre (ex: metanotiol, sulfeto dimetil, disulfeto dimetil) (WU et al., 2013). Em uma visão tecnológica, a remoção biológica de sulfeto realizada pelas quimiotróficas (*Thiobacillus, Sulfobus, Thermothrix, Beggiota e Thiothrix*) é a mais apropriada (POKORNA; ZABRANSKA, 2015). Por outro lado, a gama de microrganismos que podem realizar a oxidação de compostos de enxofre de forma fototrófica é bastante elevada, incluindo: bactérias purpuras sulfurosas (Gammaproteobacteria), bactérias não sulfurosas (Alphaproteobacteria e Betaproteobacteria), bactérias verdes sulfurosas (Chlorobi), bactérias verdes não sulfurosas (Chloroflexi) e algumas outras como, *Heliobacteria* (Firmicutes), *Chloracidobacterium* (Acidobacteria) (MADIGAN et al., 2014; URAKAWA; BERNHARD, 2017).

As bactérias purpuras sulfurosas (ex: *Chromatium*, *Thiospirillum, Thiocystys*) oxidam o sulfeto de hidrogênio a enxofre orgânico e geralmente são encontradas na presença de oxigênio (POKORNA; ZABRANSKA, 2015). As bactérias não sulfurosas (*Rhodoblastus, Rhodobacter, Rhodospirillum*) são os microrganismos metabolicamente mais versáteis de todos e desempenham um papel importante nos *wetlands*. No ciclo do enxofre utilizam baixas concentrações de sulfeto de hidrogênio como doador de elétrons para a redução de dióxido de carbono (URAKAWA; BERNHARD, 2017) e estudos apontam seu envolvimento no processo de oxidação de metano (OSBORNE; HARITOS, 2018; ZHOU et al., 2015b). As bactérias verdes oxidadoras de S (*Chlorobium, Chloroherpeton, Prosthecochloris)* utilizam sulfeto como doador de elétrons para oxida-lo primeiramente a enxofre elementar e posteriormente a sulfato e algumas, podem ainda, utilizar compostos orgânicos como doadores de elétrons (POKORNA; ZABRANSKA, 2015; URAKAWA; BERNHARD, 2017). Por fim, as bactérias verdes não sulfurosas (ex: *Chloroflexi*) possui características fotossintéticas híbridas de bactérias verdes sulfurosas

e bactérias roxas, podendo crescer rapidamente sobre uma larga variedade de fontes de carbono (URAKAWA; BERNHARD, 2017). BERNARDES et al. (2016), tratando água cinza em sistemas piloto de *wetlands* com descarga zero, sugeriram que as *Chloroflexi*, em adição a degradação de matéria orgânica, podem oxidar compostos de enxofre em ambientes anóxicos.

### 1.2.8 Poblemáticas de Wetlands Construídos

As vantagens atribuídas aos *wetlands* construídos pela facilidade técnica operacional e pela baixa manutenção requerida começaram a ser mais discutidas na última década, pois alguns autores vêm demonstrando que a interação entre processos de tratamento, características dos efluentes e longos tempos de detenção hidráulica podem resultar, em longos períodos de operação, em um gradual entupimento do substrato que pode afetar negativamente o desempenho hidráulico e de remoção de poluentes em CW e definitivamente reduzir a vida útil do sistema (BARBAGALLO et al., 2016; BRIX; ARIAS, 2005; CASELLES-OSORIO et al., 2007; COOPER; GRIFFIN; COOPER, 2005; KADLEC; WALLACE, 2008; NIVALA; ROUSSEAU, 2009; PEDESCOLL et al., 2011; SAMSÓ et al., 2016). O entupimento é um fenômeno complexo derivado da retenção de partículas orgânicas e inorgânicas, precipitação de poluentes, formação de biofilme e crescimento das raízes das plantas (KNOWLES et al., 2011), sendo caracterizado como um dos maiores problemas operacionais de *wetlands* construídos de fluxo horizontal subsuperficial (KADLEC; WALLACE, 2008; NIVALA et al., 2012). Nesse sentido, na tentativa de reduzir esse inconveniente, reatores anaeróbios vêm sendo testados como tratamento preliminar para reduzir as cargas de sólidos submetidas à CW tratando esgoto doméstico (PEDESCOLL et al., 2011; RUIZ et al., 2010).

Outro problema frequente encontrado em CW está relacionado à presença do gás sulfeto ($H_2S$), que normalmente causam problemas de cor, gosto e principalmente odor aos efluentes (CHEN et al., 2016a). Esse mau cheiro pode afetar outro benefício dos CWs que está ligado ao seu valor estético pela presença de plantas ornamentais, as quais integram o sistema às áreas verdes proporcionando o paisagismo e o desenvolvimento urbano (BRISSAUD, 2007).

### 1.2.9 Sistema EvaTAC

Como águas cinza de diversas fontes são geralmente difíceis de tratar em um CW de fase única, sistemas híbridos de *wetlands* construídos em séries de vários tipos estão ganhando maiores interesses (VYMAZAL, 2005). Seguindo esse raciocínio e se atentando para as problemáticas discutidas, foi proposto por esse grupo de pesquisa, o sistema denominado EvaTAC, que é formado por uma câmara de evapotranspiração e tratamento (CEvaT) conectada em série com um *wetlands* construído de fluxo horizontal subsuperficial (CW-FHSS). A inovação tecnológica desse sistema é a CEvaT, que é a inserção de uma câmara de digestão anaeróbia (Cdig) dentro de um *wetlands* construído. A Cdig é o compartimento que recebe o efluente e tem a função de absorver a carga de sólidos, através da retenção, sedimentação e digestão da maior parte da biomassa, que predominantemente ocorre na entrada do *wetlands* (KADLEC; WALLACE, 2008), evitando assim grandes choques à camada de microrganismos que estão aderidas aos meios filtrantes que circundam a Cdig e complementam o sistema. Além disso, por não ser conectada com as raízes das plantas, a Cdig pode manter uma zona mais anaeróbia dentro da CEvaT, a qual é essencial para certos tipos de microrganismos na degradação microbiológica (LIU et al., 2016), principalmente para remoção de sulfato (STEIN et al., 2007; WU et al., 2013).

Posteriormente, o *wetland* construído conectado em série pode dar maior qualidade ao efluente e se tornar um promissor ecossistema artificial para controle de odores, uma vez que a maioria do sulfeto produzido nas camadas anaeróbias pode ser imobilizado ou dissipado nas camadas desse sistema (CHEN et al., 2016a).

## 1.3 Objetivo

### 1.3.1 Objetivo Geral

- Estudar as diferentes zonas existentes em um *wetland* construído melhorado, denominado sistema de Evapotranspiração e Tratamento de Águas Cinza (EvaTAC), verificando as correlações entre condições ambientais e as comunidades microbianas.

### 1.3.2 Objetivos específicos

- Identificar a comunidade microbiana e as condições ambientais de diferentes zonas de um EvaTAC implantado em escala real para o tratamento de água cinza clara, verificando possíveis correlações entre as zonas e suas condições ambientais com a estrutura microbiana identificada.

- Correlacionar a comunidade de bactérias e arqueias de um EvaTAC implantado em escala piloto com as condições ambientais e os processos de degradação biológica de poluentes ao longo do fluxo de águas cinza clara, com atenção especial para as fases de redução e oxidação do ciclo do carbono e do enxofre.

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# CAPÍTULO 2 - Relationship between microbial community and environmental conditions in a constructed wetland system treating greywater

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## Abstract

Knowledge of the diversity of microbial communities and their relationship with biogeochemical cycles is a key factor in understanding and improving the performance of constructed wetlands (CW) systems, allowing improvements in design and operation methods to reach the desired effluent quality. Assemblies of microbial groups in CWs are driven by several factors, including the environmental conditions of the mesocosm and substrate availability. This study combined physicochemical analyses, high-throughput DNA sequencing and statistical methods to gain an insight into the influence of operational conditions on the bacteria and archaea communities in a modified constructed wetland system treating raw-light greywater. The EvaTAC system is composed of an upflow evapotranspiration and treatment tank (CEvaT) which has an inbuilt anaerobic chamber (AnC), followed by a horizontal subsurface flow constructed wetland (HSSF-CW). Samples were collected after five years of system operation in both units: sludge from the AnC, coarse gravel from the CEvaT and fine gravel from the CW. The results showed that system operated predominantly in anaerobic conditions, with redox potential (Eh) increasing from the inlet (-364.1 mV) to the outlet (-240.4 mV) zone. The Eh was the environmental condition that most influenced the microbial community diversity and richness, and, as well as Eh, increased along the light greywater (LGW) flow. Conversely, the chemical oxygen demand (COD) decreased, suggesting negative correlation among these factors. The clustering analyses of microbial community showed that, besides environmental condition, the media filter and related depth could also be drivers of microbial community composition. Proteobacteria and Synergistetes phyla were detected at the bottom layer of CEvaT, indicating the accomplishment of the first steps of anaerobic digestion. The next layer was characterised by the presence of *Desulfovibri*o, *Syntrophobacter* and *Methanobacterium*, microorganisms responsible for the sulfidogenesis and methanogenesis processes. Finally, in the CW zone, the community composition was dominated by microorganisms involved in the methane oxidation, like *Methylosinus*, *Bradyrhizobium* and *Candidatus Koribacter*. This study explored the composition of bacterial and archaeal communities, and is an initial step in elucidating microbial diversity conversions in CW systems treating greywater, which is important knowledge to steer the conversion pathway and establish the ideal environmental conditions for the development of desired microbial community in these systems.

**Keywords**: Wetland, Domestic wastewater, Filter Media, High-throughput sequence, Microbial diversity

**Esse artigo foi aceito com "major review" como Bernardes et al., 2019, "Relationship between microbial community and environmental conditions in a constructed wetland system treating greywater", na Ecological Engineering.*

## 2.1 Introduction

Household and personal care activities such as dish washing, laundry and showering generate large quantities of organic and inorganic material, resulting in polluted water, referred to as greywater (GW) (HYLANDER et al., 2014), which comprises approximately 70% of domestic sewage (HERNÁNDEZ LEAL et al., 2011). Greywater is domestic wastewater that does not include toilet flush water (ERIKSSON et al., 2002; OTTOSON; STENSTRÖM, 2003; VYMAZAL, 2005), and is classified as light GW (LGW) when it does not contain the GW generated in kitchen sinks and dishwashers (BERNARDES et al., 2016).

Constructed wetlands (CWs) are often used for the decentralized treatment of GW (ARDEN; MA, 2018).This technology has increased in popularity (BRIX, 1999; PAULO et al., 2013; VYMAZAL, 2005) for combining simple techniques that do not require operational skills, and having low energy (VYMAZAL, 2010) and implementation costs (WANG et al., 2016). The versatility of implementing CW at household as well as community levels makes it a preferred treatment option for greywater (ARUNBABU et al., 2015).

The global diffusion of CW has suggested to some authors that the interaction between treatment processes, effluent characteristics and long periods of system operation could result in a gradual substrate clogging, which will negatively affect hydraulic performance and pollutant removal in CWs and definitely reduce the life of the system (BARBAGALLO et al., 2016; COOPER; GRIFFIN; COOPER, 2005; KADLEC; WALLACE, 2008; NIVALA; ROUSSEAU, 2009; PEDESCOLL et al., 2011). Clogging is a complex phenomenon caused by the retention of organic and inorganic particles, pollutant precipitation, biofilm formation and plant root growth (KNOWLES et al., 2011), and is characterised as one of the major operational problems of horizontal subsurface flow constructed wetlands (KADLEC; WALLACE, 2008; NIVALA et al., 2012). In order to reduce this problem, anaerobic reactors have been tested as a preliminary treatment to reduce the loads of solids submitted to CWs treating domestic sewage (PEDESCOLL et al., 2011; RUIZ et al., 2010).

Our research group proposed a combined-compact system for the onsite treatment of greywater. The system, called EvaTAC (evapotranspiration and treatment of

greywater), is composed of an evapotranspiration and treatment tank (CEvaT) with an inbuilt anaerobic chamber (AnC) connected in series with a horizontal subsurface flow constructed wetland (HSSF-CW). The innovation of this system is the AnC within a constructed wetland. The AnC is the compartment that receives the raw GW and has the function of absorbing organic and hydraulic loads, through the retention, sedimentation and digestion, which, according to Kadlec and Wallace (2009), would predominantly occur at the inlet portion of the CWs. The GW flows through perforated holes around the AnC to the filter media. Previous studies have already demonstrated that AnC is an attractive alternative to replace septic or sedimentation tank usually used as pre-treatment. In a recent study, da Silva et al. (2017) performing 24-h and 8-d profiles in a full scale EvaTAC system, demonstrated the capacity of the AnC to equalise daily variations of flow and organic load, attenuating peak load and stabilising the system, even when receiving $COD_{total}$ as high as 900 $mg.L^{-1}$. In bench scale, Magalhães Filho et al. (2018) found that the flow distribution pattern provided by the AnC within the CEvaT combined with the plants in the system, helped to maintain the hydraulic conductivity stable, possibly reducing clogging in the HSSF-CW filter medium. These abilities would be helpful in the development of microbial communities, due to the lower water flow and consequently lower influx of electron acceptor for the next zones, resulting in the appearance of different microorganism groups (HE et al., 2015). Furthermore, as it receives higher organic loads, this zone is more anaerobic, which is essential for some microbiological degradation (LIU et al., 2014), mainly for sulphate degradation (STEIN et al., 2007; WU et al., 2013). Lastly, the absence of a filter medium in this zone will enable a sludge structure to form from suspended microbial communities, and may also be important to the diversity of microorganisms in the whole system (He, Guan, Luan & Xie, 2016; Long et al., 2016).

The vast majority of studies in the last decade have focused on nutrient removal processes, and very little is known about microbial communities (URAKAWA; DETTMAR; THOMAS, 2017), however, with advances in technology such as high-throughput DNA sequencing, recent research have investigated the microbial community structure (AGUILAR et al., 2019; AN et al., 2019; CAO et al., 2017; GARRIDO et al., 2014; PELISSARI et al., 2017; ZHANG; JIAO; LU, 2018) and begun to detect spatial variation inside the system and relationships between environmental conditions using statistical correlation analysis (HE et al., 2016; HUA et al., 2018; WU et al., 2016;

ZHANG et al., 2018). Knowledge of the microbial community is becoming an indispensable tool with which to improve the performance and design of these systems (URAKAWA; DETTMAR; THOMAS, 2017). Nevertheless, advances in the identification and correlation of microbial communities in wetlands treating greywater remain rare, with few studies investigating the bacterial community (DING et al., 2014; HYLANDER et al., 2014; JONG et al., 2010; RODRÍGUEZ-MARTÍNEZ et al., 2016). Moreover, a gap remains on the knowledge related to archaeal community in CW systems treating GW. The aim of this study was therefore to use high-throughput DNA sequencing to provide an insight into the microbial community involved in the process of greywater degradation in an EvaTAC system, linking the environmental conditions to effects on the diversity and structure of archaeal and bacterial communities.

## 2.2 Material and Methods

### *2.2.1 System description*

The full scale EvaTAC system in this study served a three-person household located in Campo Grande, Mato Grosso do Sul, Brazil (20°31' S,54°39' W) and was in continuous operation receiving LGW for five years. The EvaTAC system is composed of two subsystems: an upflow evapotranspiration and treatment tank (CEvaT) with an inbuilt anaerobic chamber (AnC), followed by a horizontal subsurface flow constructed wetland (HSSF-CW). The AnC was made of a fiberglass pipe, with 0.5 m diameter and 2.0 m length, with a useful volume of 392.7 L. It was perforated along all its extension and top part of its circumference, with the lowest perforations at 0.4 m from the bottom, as sludge was expected to settle in the bottom part. The diameter of the holes was 1 cm each, and the distance between holes was 0.10 m. The dimensions (L x W x D) of the units were: 2.00 m × 1.00 m × 1.05 m (CEvaT) and 2.00 m × 1.00 m × 0.60 m (HSSF-CW). The layers in the CEvaT, from bottom to top were: Gravel nº 4 (porosity: 0.50; particle size: 32 to 150 mm; layer height: 0.60 m), Gravel nº 2 (porosity: 0.48; particle size: $d_{10}$ = 20 mm, $d_{30}$ = 17 mm $d_{60}$ = 12 mm; layer height: 0.15 m) and (on top of a geotextile blanket) 0.30 m of soil. The HSSF-CW was filled with fine gravel (porosity: 0.44; particle size: d10 = 13 mm, d30 = 11 mm, d60 = 10 mm; and height: 0.60 m). The 0.2 m inlet and outlet portions were filled with Gravel nº 2. A detailed description of the system is presented elsewhere (da Silva et al., 2017). In this study, the EvaTAC was monitored for

51 days and was operated with approximately 0.160 ± 0.08 $m^3.day^{-1}$ and 0.151± 0.06 $m^3.day^{-1}$ of raw-light greywater for the CEvaT and HSSF-CW respectively, with a corresponding organic load of 25.08±14.29 $g.COD.m^{-2}.day^{-1}$ for the CEvaT and 19.62±8.34 $g.COD.m^{-2}.day^{-1}$ for the HSSF-CW. A schematic view of the system and sampling points is shown in Figure 1.

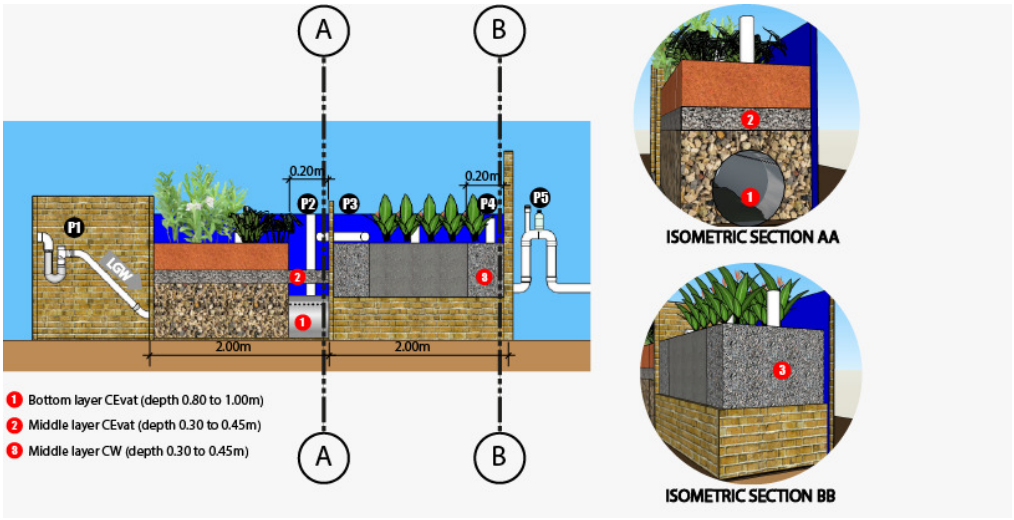


*Figure 1. Schematic view of the EvaTAC system, including effluent sampling points: P1(system inlet); P2 (piezometer, inside at the final of CEvaT); P3 (CEvaT outlet and HSSF-CW inlet); P4 (piezometer, inside at the end of the HSSF-CW) and P5 (system outlet). The numbers in red show the three biofilms sampling points: the bottom layer CEvaT (AnC sludge); the middle layer CEvaT (Gravel nº2), and the middle layer CW (Gravel nº2).*

### *2.2.2 Water and Biofilm Sampling*

The performance of the EvaTAC system was monitored over 51 days. Samples were taken twice a week from the piezometers (P2 and P4 – Figure 1) for pH, temperature, redox potential. Once a week the liquid samples were taken for chemical oxygen demand (COD) from P1, P3 and P5 (Figure 1). In order to characterise the environmental conditions of the mesocosm system, pH, temperature and redox potential were investigated in the same zone from which the biofilms were sampled. The environmental conditions analyses were performed using portable meters (Hanna Instruments, HI 9829, USA) in P2 and P4. In P2 a depth of 0.80 to 1.00 m was chosen to characterise the bottom layer of the CEvaT (BL.CEvaT), a depth of 0.30 to 0.45 m for the middle layer of the CEvaT (ML.CEvaT) and in P4 a depth of 0.30 to 0.45 m to characterise the middle layer of the HSSF-CW (ML.CW). All physicochemical analyses were performed according to standard methods for the examination of water and wastewater (APHA, 2012).

After 51 days of system monitoring, biofilm samples were collected from the final section of the subsystems (0.20 $m^2$ before the outlet) in two depth zones: the bottom layer (0.80 to 1.00 m) and the middle layer (0.30 to 0.45 m). Three samples were extracted from the same zones where the environmental conditions were investigated (Figure 1). For each sample, three subsamples (250 g each) were collected with a horizontal distance of about 0.30 m between them, and numbered. To avoid sample contamination the subsamples were collected in ziplock plastic bags, stored on ice after sampling and immediately transported to the laboratory. In the laboratory, the subsamples were homogenized manually to obtain a representative sample, and visible root or plant materials were removed. The samples were stored at -4 ºC.

### *2.2.3 DNA extraction, PCR amplification, and high-throughput sequencing*

A 400 g portion of homogenized sample was transferred to settle in mili-Q water for 3 hours. The supernatants collected were centrifuged at 11000 rpm for 15 minutes. Afterwards, the DNA pelleted by centrifugation was performed as described by (YU et al., 2005) with modifications. After centrifugation, 1.0 ml of lysis buffer (500mM NaCl, 50mM Tris-HCl, pH 8.0, 50mM EDTA, and 4% SDS) and 0.4g sterile zirconia beads ( 0.3 g of 0.1 mm and 0.1 g of 0.5 mm) was mixed into the samples and the mixture was incubated for 20 min at 70 °C with gentle agitation every 5 min. The mixture was then centrifuged at room temperature for 10 min at 13.600 rpm (Eppendorf 5804). The supernatant was transferred to a new tube, an equal volume of isopropanol was added, and after 20 min at room temperature the mixture was spun at maximum speed in a microcentrifuge for 10 min. The pellet was resuspended in 750 µl TE. Potassium acetate was added to a final concentration of 0.5 M, the mixture was incubated on ice for 5 min and after a centrifugation for 10 min at 13600 rpm, the supernatant was transferred to a new tube. This solution was then extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1). The final aqueous supernatant was transferred to a new tube. An equal volume of isopropanol was added to the recovered supernatant and after 1 h at room temperature total DNA was recovered by centrifugation at top speed for 15 min. The pellet obtained was washed with ethanol 70%, dried and resuspended in 30 µl milli-Q water.

For bacterial 16S rDNA, a 466 bp fragment was amplified using the primers: 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) flanking the V3 and V4 regions with the barcode (YU et al., 2005). For Archaeal 16S rDNA, a 288 bp fragment was amplified using the primers U519F (CAGYMGCCRCGGKAAHACC) and 806R (GGACTACNNGGGTATCTAAT) flanking the V4 region (PORAT et al., 2010). Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations, and index codes were added. Finally, the library was sequenced on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated by Genone company. This targeted locus study project has been deposited at DDBJ/EMBL/GenBank under the accession KCJY00000000. The version described in this paper is the first version, KCJY01000000.

### *2.2.4 Data Analysis*

Microbial sequences analyses were performed using Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/). Sequences above 97% of similarity were assigned to the same OTUs. A representative sequence for each OTU was screened for further annotation. The Green Gene Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) was used for each representative sequence, based on the RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) algorithm to annotate taxonomic information. In order to study the phylogenetic relationships of different OTUs, and the differences in the dominant species in different samples (groups), multiple sequence alignment was conducted using the PyNAST software (Version 1.2) against the "Core Set" dataset in the Green Gene database. OTU abundance information was normalized using a standard of sequence numbers corresponding to the sample with the fewest sequences. Statistical tests on the taxonomic differences between the samples were run on STAMP software using the Fisher's exact test with multiple Bonferroni correction ($p < 0.01$) (nominal coverage of 99%) (PARKS; BEIKO, 2010).

Subsequent analyses of alpha and beta diversity were all performed based on this output of normalized data. Alpha diversity was applied to analyse the complexity of species diversity for a sample through four indices: Chao1 to identify community richness, Shannon and Simpson to identify community diversity and Coverage to

characterised sequencing depth. Unweighted UniFrac using the Quantitative Insights into Microbial Ecology (QIIME) software was applied to compare microbial communities between samples, using the unweighted pair group method with arithmetic mean (UPGMA) clustering. All the indices and the beta diversity analysis in our samples were calculated using QIIME software (Version 1.7.0) and displayed via R software (Version 2.15.3).

In the analyses of physicochemical parameters, we tested the dispersion of dependent variables using the Shapiro–Wilk test, after which a one-way ANOVA test was applied. After one of these tests indicated that there was an effect of the factor on the dependent variable measured among the sampled averages, the Tukey's post-test was used to make pairwise comparisons between the results of each zone. Pearson's correlation analysis was carried out to test the strength of relationships between microbial community metrics (richness and evenness) and the different water metrics (redox potential, pH and temperature). With the significant results, the correlation coefficient r was interpreted as a strong correlation, when $r \geq |0.7|$ and a moderate correlation when $|0.5| \leq r < |0.7|$) (MILTON; BULL; BAUMAN, 2011; ZHANG et al., 2018). The Canonical Correlation (CCA) was performed for the two strongest correlations between microorganism abundance at the genera level, and environmental conditions. All statistical tests involving physicochemical parameters were employed using the R software and $p<0.05$ was considered significant.

## 2.3 Results

### *2.3.1 Microbial community richness and diversity*

The 206,727 filtered bacterial high-quality sequences were classified into 40 phyla, 103 classes, 156 orders, 105 families, 251 genera and 1657 OTUs, and the 137,059 sequences for archaea were classified into 5 phyla, 7 classes, 7 orders, 7 families, 9 genera, and 896 OTUs. Good's coverage estimator ≥ 99.5% suggested that most of bacterial and archaeal OTUs in each sample had been captured (Table 1). The results of the Venn diagram indicated that for bacteria 1448 OTUs (87.3%) were present in the HSSF-CW with 645 OTUs (38.9%) being specific to this subsystem (Figure 2). The bacterial community was mostly shared between the middle layer CW and middle layer CEvaT. Similarly, a higher number of total Archaea and specific OTUs were found in the

CW. The archaeal communities in the ML.CW and the ML.CEvaT shared most of the OTUs. The Chao1 richness estimator illustrated a remarkable variation in the studied samples, ranging from 519.1 to 1472.5 for bacteria and 320.1 to 850.2 for archaea (Table 1). The Shannon and Simpson diversity index also demonstrated a considerable variation, ranging, for bacteria, from 5.34 to 7.90 and 0.92 to 0.99, and for archaea from 3.61 to 5.92 and 0.74 to 0.93 respectively.

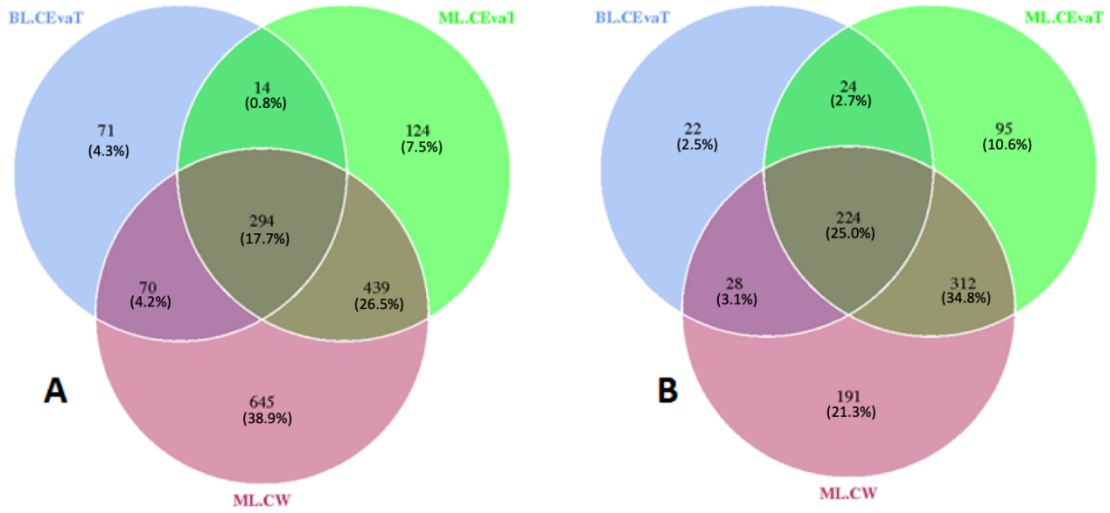


*Figure 2. Venn diagrams to show the unique and shared OTUs. A - for bacterial community and B - for archaeal community.*

### *2.3.2 UPGMA clustering analysis of microbial communities*

UPGMA was used to cluster the microbial communities and identify variability. The UPGMA tree indicated that, for both bacteria and archaea, the communities could be divided into two distinct groups with the ML.CW and ML.CEvaT samples clustered together. The results indicated that BL.CEvaT may form relatively independent ecosystems.

### *2.3.3 Microbial community structure*

Among the 40 bacterial phyla identified, Proteobacteria was predominant (66.2-77.9% of total sequence reads) in all the samples. The second largest bacterial group varied according to the sample: Synergistetes (15.1%) for BL.CEvaT, Firmicutes (13.2%) for ML.CEvaT and Acidobacteria (7.1%) for ML.CW. The others abundant phyla (>1%)

were Fusobacteria, Bacteroidetes and Cyanobacteria. A remarkable spatial difference in the samples was demonstrated, with Synergistetes decreasing and Acidobateria increasing along the water flow. In the archaeal group, Euryarchaeota was the dominant phyla (3.0-36.9%) and MCG appeared abundant (3.5%) only in ML.CW.

At the bacterial class level, Gammaproteobacteria (37.3%), Deltaproteobacteria (26.5%), Synergistia (15.1%) and Alphaproteobacteria (9.4%) were the most abundant in the BL.CEvaT, conversely Bacilli (1.0%) reached a lower relative abundance. In the ML.CEvaT Deltaproteobacteria were the most abundant (45.1%), followed by Bacilli (11.85%), Alphaproteobacteria (11.3%) and Gammaproteobacteria (9.8%) with Synergistia at only 5.9% of relative abundance. The most abundant classes in the ML.CW were Deltaproteobacteria (27.3%), Alphaproteobacteria (20.9%) and Gammaproteobacteria (12.5%), with Synergistia (3.6%) and Bacilli (1.6%) reaching lower abundance. A remarkable spatial difference in the proportion of these bacterial classes was also found, with Synergistia and Gammaproteobacteria abundance decreasing and Methanomicrobia and Alphaproteobacteria, with dominance of the *Rhizobiales* order, increasing along the greywater flow. A high abundance of Bacilli was encountered only in the ML.CEvaT and the Deltaproteobacteria expanded from BL.CEvaT to ML.CEvaT, then decreased again in the ML.CW final part of the system. Figure 3 demonstrates the average ratio of sequences, the difference in the average proportions of each pair of samples and the p-value, indicating whether the average proportion is equal for a given pair.

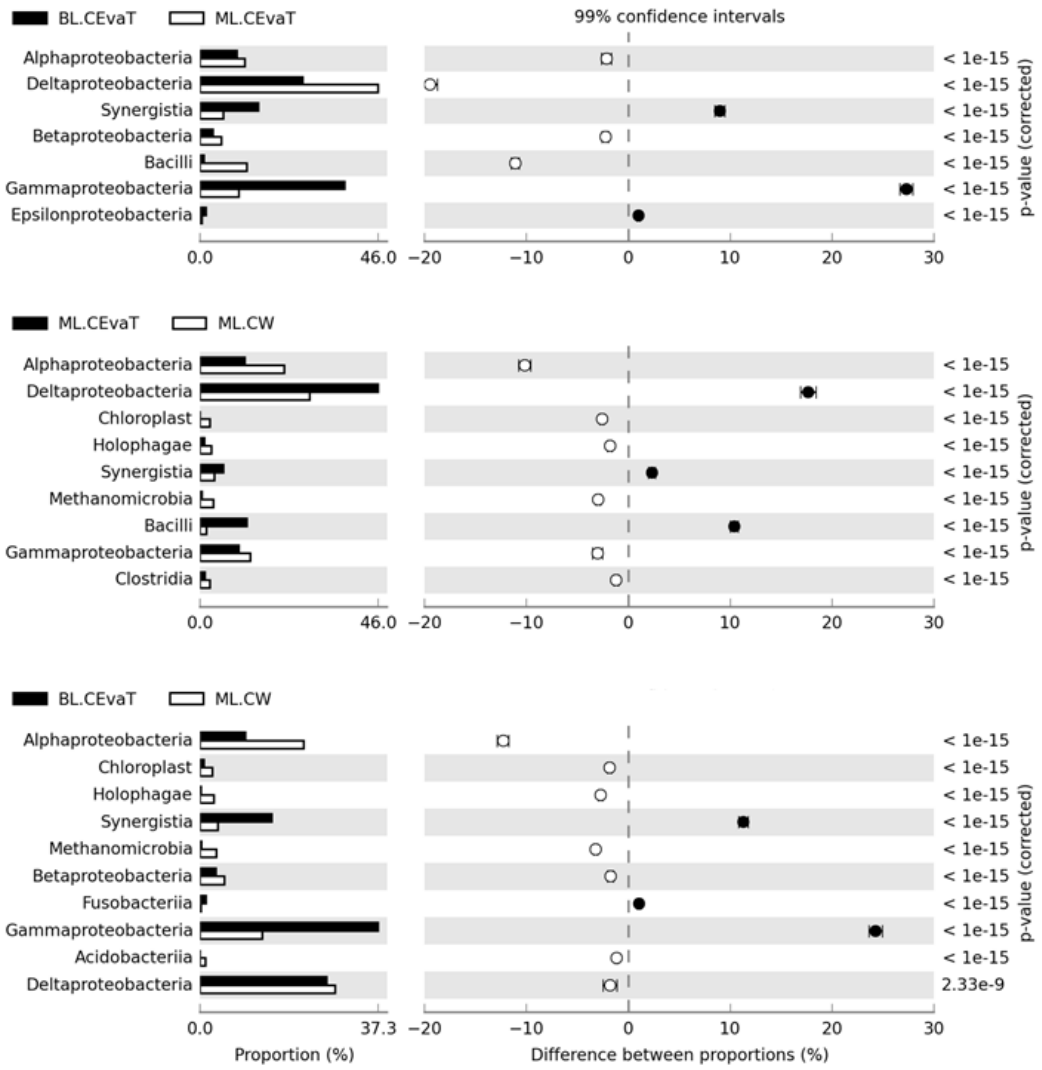


*Figure 3. Reference category for the distributed bacteria classes domain in EvaTAC that showed difference significance statistic with effect size > 1,00 (difference between proportion).*

At the archaeal class level, *Methanobacteria* (2.2 – 30.1%) was dominant in all samples, followed by *unidentified MCG* (0-3.5%), which appeared increasingly along the flow (Figure 4). The Methanomicrobia archaea was identified with abundance between 0.2 and 3.3%, where the highest abundance was found in the ML.CW. Similar to the unidentified MCG, its abundance increased along the flow (Figure 3).

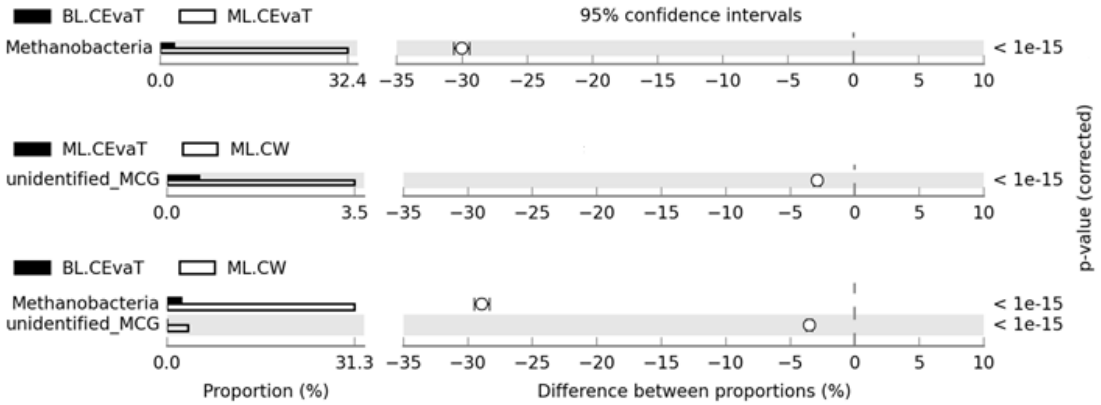


*Figure 4. Reference category for the distributed archaea classes domain in the EvaTAC that showed a statistically significant difference for effect size > 1.00 (difference between proportion).*

At the genus level, the relative abundance in the BL.CEvaT was dominated by *Acinetobacter* (31.6%), *Desulfovibrio* (13.2%) and *VadinCA02* (8.5%). The genus *Desulfovibrio* also appeared in high abundance in the ML.CEvaT (10.8%). The genus *Brevibacillus* (11.7%) only appeared in the ML.CEvaT and was the most abundant bacterial genus in this sample, others of relevance were *Pseudomonas (*3.7%*), Propioniovibrio (3.7%), Aminiphilus* (3.6%), *Syntrophobacter* (3.5%), *Pleomorphomonas* (2.4%) and *PD-UASB-13* (1.9%). *Methanobacterium* (29.3%) was also dominant in the archaeal domain. In the ML.CW, as the richness index demonstrated, many bacterial genera with high relative abundance (> 1.0%) were represented by: *Desulfovibrio* (4.6%), *Parvibaculum* (3.8%), *Bradyrhizobium* (2.7%), *Methanosaeta* (2.7%), *Methylosinus* (2.5%), *Syntrophobacte*r (1.8%), *Pseudomonas* (1.7%), *Rhodoplanes* (1.7%), *Desulfomonile* (1.4%) and *VadinCA02* (1.4%). In these genera it is important to highlight the dominance of those that belonged to the Rhizobiales order (*Bradyrhizobium, Methylosinus, Rhodoplanes*). Concerning Archaea, *Methanobacterium* (29.8%) and *unidentified MCG* (3.3%) were the most abundant.

### *2.3.4 Effects of environmental conditions on microbial community*

The physicochemical properties of LGW along the EvaTAC system are summarised in Table 1. A primary anaerobic condition was verified for all samples, the BL.CEvaT being the most anaerobic area, with a significant difference from the others zones ($p<0.05$). There was no significant difference in COD between the inlet and outlet of the CEvaT (P3) in the monitored period. The significant difference in the COD is only

noted in the outlet of HSSF-CW (P5) compared to both P1 and P3 ($p<0.05$). The pH was different only in the BL.CEvaT, where the neutrality was slightly higher compared to the other sampling points. The temperature was decreasing along the greywater flow, showing significant difference between inlet LGW and BL.CEvaT to ML.CW and LGW-effluent, with the ML.CEvaT functioning like a transition zone.

*Table 1. Physicochemical parameters measured (mean ± standard deviation) and bacterial richness and diversity of EvaTAC samples. For Simpson, Shannon, Chao1 and Good Coverage: upper numbers were bacterial Indices values and lower numbers (with parentheses) were archaeal Indices values.*

| **LGW flow** | **EvaTAC** | | **Environmental conditions** 14 samples | | | **Efficiency analyses** 8 samples | **Community diversity** | | **Community richness** | **Sequencing depth** |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | ORP (Eh) | pH | Temp. (ºC) | COD (mg.L$^{-1}$) | Simpson | Shannon | Chao1 | Good Coverage |
| | **LGW-Affluent (P1)** | | 76.7 ± 94.5$^{a}$ | 6.9 ± 0,1$^{a}$ | 26.2± 1.0$^{a}$ | 313.6 ± 178.7$^{a}$ | - | - | - | - |
| | CEvaT (P2) | BL.CEvaT | -364.1± 8.5$^{ab}$ | 7.2 ± 0.4$^{ab}$ | 25.5 ± 0.4$^{b}$ | | 0.92 (0.74) | 5.34 (3.61) | 519.1 (320.1) | 0.998 (0.998) |
| | | ML.CEvaT | -277.7± 37.6$^{abc}$ | 6.7 ± 0.2$^{b}$ | 25.6 ± 0.5$^{c}$ | | 0.96 (0.92) | 6.03 (5.39) | 913.3 (728.3) | 0.998 (0.995) |
| | **LGW-middle (P3)** | | - | - | - | 259.9 ± 106.7$^{b}$ | | - | - | - |
| | CW (P4) | ML.CW | -240.4± 21.1$^{ab}$ | 6.9 ± 0.1$^{b}$ | 24.8 ± 0.5$^{ab}$ | | 0.99 (0.93) | 7.90 (5.92) | 1472.4 (850.2) | 0.999 (0.995) |
| | **LGW-Effluent (P5)** | | -212.2 ± 26.2$^{abc}$ | 6.8 ± 0.1$^{b}$ | 24.3 ± 1.2$^{abc}$ | 151.1 ± 53.2$^{ab}$ | - | - | - | - |

*Notes: Same letters within the same column indicate significant difference ($p<0.05$). P1, P2, P3, P4 and P5 are the sampling points (see Figure 1).*

The overall community dissimilarities in the samples were mainly attributed to the Eh environmental variable, showing a positive correlation (Figure 5). The temperature and pH environmental variables showed a negative correlation with microbial community diversity and richness, which had a strong and moderate impact, respectively, in both microorganism groups.

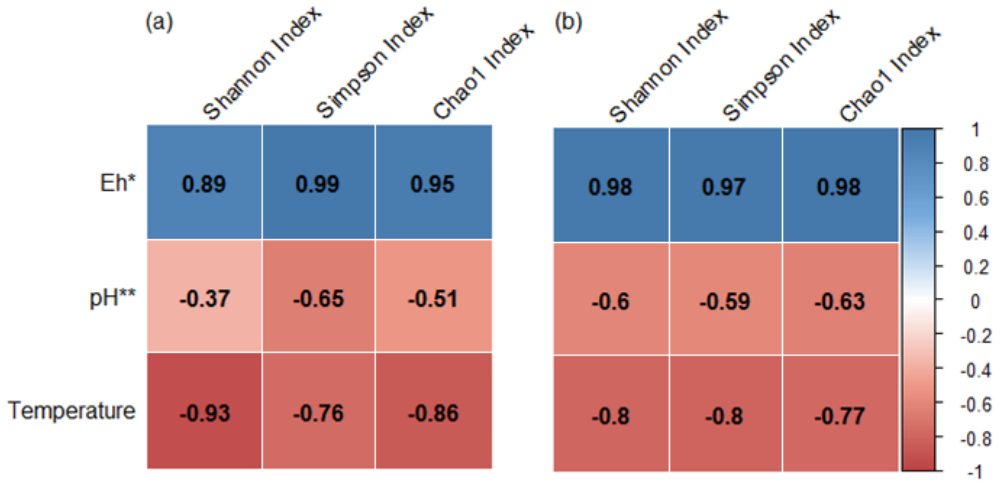


*Figure 5. Pearson correlation coefficient between physicochemical parameters and metrics Indices. (a) Bacterial metrics Indices (b) Archaeal metrics Indices. *Eh – redox potential. ** Potential of hydrogen.*

A canonical correlation analysis (CCA) was applied using the environmental conditions that demonstrated the strongest correlations in order to investigate the impact of these physicochemical variables on the spatial microorganisms at the genus level (Figure 6).

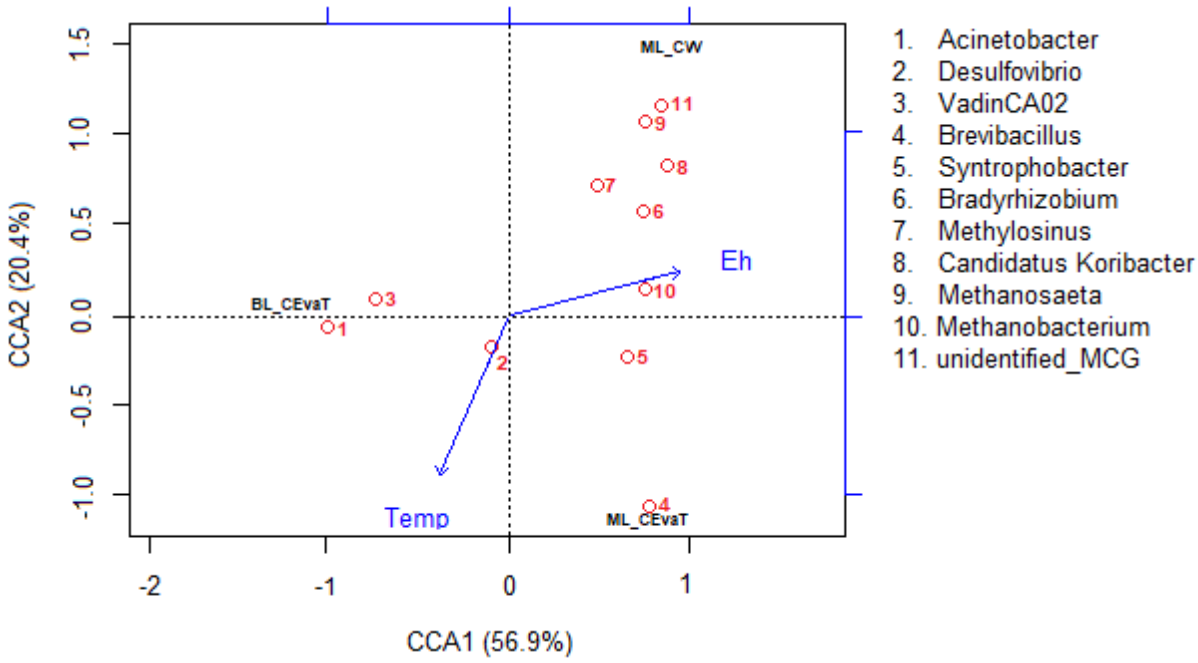


*Figure 6. Canonical correlation analysis (CAA) for environmental conditions and microbial genera level.*

First of all, it is important to identify the sites that have a particularity of certain genera and then the effects of environmental conditions. *Acinetobacter* and *VadinCA02* species were found mainly in the BL.CEvaT, showing a negative correlation with Eh and positive correlation with temperature. *Brevibacillus* and *Syntrophobacter* were most commonly found in ML.CEvaT, where the influence of Eh and temperature was low for both genera, whereas, *Methylosinus*, *Bradyrhizobium*, *Methanosaeta, Candidatus Koribacter* and *unidentified MCG* were more commonly found in ML.CW. These microbial genera were negatively affected by the temperature, once it is observed that the lower the temperature measured in the system, the higher their relative abundance. Conversely, the Eh showed a positive correlation with both archaea and bacteria genera. *Desulfovibrio* appeared closer to BL.CEvaT zone, but not far from ML.CEvaT, indicating the higher relative abundance of this genus in both zones. *Methanobacterium* appeared in the middle between *ML.CEvaT* and *ML.CW,* indicating a similar abundance in both zones, influenced by the increase in Eh and the decrease in temperature. We can infer that the microbial communities could be divided into three distinct groups for these selected genera, one for each sampled zone ($p<0.05$) as shown in Figure 6.

## 2.4 Discussion

The predominant respiration process, and therefore pollution removal, depends on the oxidation–reduction (redox) conditions prevailing in the wetland environment (ANSOLA; ARROYO; SÁENZ DE MIERA, 2014; FAULWETTER et al., 2009). In the present study, we verified that redox potential had a strongest correlation with the diversity and richness of the microbial community (Figure 5). The measured environmental conditions along the EvaTAC characterised anaerobic environment (FAULWETTER et al., 2009; STEIN et al., 2007; XU et al., 2018). The pH was the only environmental condition that did not show a strong correlation with the diversity and richness of the microbial community, corroborating other studies such as Lauber et al. (2009) and Yan et al. (2018). The most anaerobic zone was found in the deeper layer of the EvaTAC, where a greater decrease in the redox condition of the greywater flow was detected, considering that the Eh changed from anoxic conditions in the affluent light greywater to extremely anaerobic conditions in the BL.CEvaT (Table 1). This decrease could be characterised by the supply of more complex substrates (LIU et al., 2012) and by an accumulation of organic matter (ALLEN et al., 2002; BERNARDES et al., 2016), since this zone has the function of supporting and mixing the organic and hydraulic loads (DA SILVA et al., 2017).

The most abundant genera in the BL.CEvaT were *Acinetobacter, Desulfovibrio* and *VadinCA02* which belong to the *Pseudomonadales, Desulfovibrionales and Synergistales* orders, respectively. *Acinetobacter* is a group of microorganisms mostly adapted to lipid degradation (characterised as fats and long-chain fatty acids) contained in wastewater (CHIPASA; MĘDRZYCKA, 2006; MA et al., 2015), the phylum Synergistetes has been identified as responsible for the degradation of protein (MILITON et al., 2015) and genus *Desulfovibrio* is constituted by well-known sulphate reducers bacteria (SRB), typically using $H_2$, lactate and alcohols as electron donors for sulphate reduction (KUEVER, 2014; PLUGGE et al., 2011). The presence of these groups suggests the achievement of the first steps of anaerobic digestion, mainly hydrolysis, in this zone. Similar results were found by Wang et al. (2018), who studied the microbial characteristics in anaerobic processes. The relative abundance of archaea in BL.CEvaT was low, which could be explained by the high relative abundance of SRB, which can outcompete methanogenic archaea, for many reasons: SRB are less sensitive to organic load variation (DAMIANOVIC; FORESTI, 2009; HANSEN, 1994), less sensitive to free sulphide (HAO et al., 2014; MIZUNO; LI; NOIKE,

1998; STEIN et al., 2007) and present greater affinity to a wide variety of substrates (KRISTJANSSON; SCHÖNHEIT, 1983; LOVLEY; KLUG, 1983; THAUER et al., 2008). Additionally, by the low presence of Bacillus and Clostridia classes belonged to Firmicutes and *Synthrophotobacter*, which are well-known syntrophic bacteria partners of methanogens (HE et al., 2015; NOBU et al., 2015; ZHANG; JIAO; LU, 2018), and are responsible for decomposing volatile fatty acids such as butyrate and propionate into hydrogen, acetate and carbon dioxide, which are primordial substrates in the methanogenesis process (LIU; CONRAD, 2017; RIGGIO; COMINO; ROSSO, 2015; YAN et al., 2018; ZHANG et al., 2015a).

There was an average increase of 24.0% in the potential redox values in the ML.CEvaT compared to BL.CEvaT. The factors of influence could be: i) the design of the system, characterised by the presence of plants and a shallower depth (FAULWETTER et al., 2009; LV et al., 2017) and ii) microbial activity, considering the AnC, where hydrolysis might play a role in the conversion of complex to simpler substrates (LIU et al., 2012; XU et al., 2017b). This also explains the increase of the phylum Firmicutes and the Deltaproteobacteria class, mainly represented by the increase of *Synthrophobacter* genus, since *Desulfovibrio* suffered a slight decrease from BL.CEvaT (13.2%) to ML.CEvaT (10.8%). As the CCA analysis (Figure 6) showed the minimal influence of Eh in the abundance of these microorganisms in ML.CEvaT, the higher abundance of Firmicutes and *Synthrophobacter* may be as a result of the type of media filter used and the microbial activity in the BL.CEvaT. The microorganisms found in the BL.CEvaT could be responsible for the increase in the abundance of *Methanobacterium,* well-known hydrogenotrophic methanogens (WANG et al., 2018), that could have played an important role in the maintenance of the redox condition in the media, since the production of $H_2$ may cause a drop of redox potential (LIU et al., 2012). On the other hand, the continuing high relative abundance of *Desulfovibrio* indicates that it outcompeted archaea of the *Methanosaeta* genus, well-known acetoclastic methanogens (HE et al., 2015), that were only found in high abundance in the ML.CW zone. A syntrophic relationship could be hypothesised between Bacillus and Clostridia classes belonged to Firmicutes and *Syntrophobacter* with *Desulfovibrio* and *Methanobacterium* (KRUMHOLZ et al., 2015; LIU; CONRAD, 2017; NOBU et al., 2015). The greatest microbial diversity and richness in the EvaTAC was verified in the ML.CW, even with the lowest COD (Table 1).

The greater diversity could be a result of the increased redox condition (XU et al.,

2017a), which almost reached the anoxic zone, aligned with a considerable decrease in the relative abundance of *Desulfovibrio* (4,6%), the genus that was dominant in the other zones. With a lower abundance of SRB in the mesocosm, the acetoclastic Archaea *Methanosaeta* genus and the archaeal MCG phylum were found in greater abundance. The MCG phylum is formed by anaerobic heterotrophs and either access a wide variety of fermentative substrates or is linked to the degradation of refractory organic matter, potentially using electron acceptors other than sulphate and supposed to exist in the presence of methanotrophs, where the highest methane oxidising activity is presumed to occur (KUBO et al., 2012). An increase in Alphaproteobacteria was thus observed in this zone, which included the methanotrophic genera, such as *Methylosinus*, a type II methanotroph, and *Bradyrhizobium* (BAO et al., 2014; HE et al., 2015; MÄKIPÄÄ et al., 2018). Another group found together with methanotrophs was the Acidobacteria phylum (KIELAK et al., 2016; KOCH et al., 2008), with the highest relative abundance in ML.CW and mainly represented by the *Candidatus Koribacter* genus.

Usually, in horizontal subsurface flow constructed wetlands, the potential redox increases from the inlet to the outlet zone, which means the conversion of complex to simpler substrates and degradation of pollutants (HEADLEY; HERITY; DAVISON, 2005). This is in accordance with the behaviour of the EvaTAC system, which demonstrates a significant increase in redox conditions starting right after the AnC zone to final effluent, and a decrease in chemical oxygen demand (COD) from inlet to outlet (Table 1). Another similar finding is the ratio between the relative abundance of Proteobacteria and Acidobacteria (P/A), which may provide insight into the general carbon source in the environment, where the smaller P/A ratio may be indicative of the lower availability of carbon sources (SMIT et al., 2001). A negative correlation between the abundance of Acidobacteria and the concentration of organic carbon was also reported by Kielak et al. (2016). The P/A ratios of BL.CEvaT, ML.CEvaT and ML.CW were 343.3, 22.4 and 9.4 respectively, following the redox condition and the principles of pollutants degradation, indicating that the availability of carbon sources decreases along the pathway flow.

The analyses of UPGMA clustering showed that only the microbial community of BL.CEvaT was different from the other zones. This indicates that the related depth and the difference of structure where the biofilm is attached could be important factors to define the microbial community, which is also supported in other studies (He, Guan, Luan & Xie, 2016; Long et al., 2016; He et al., 2015). The number of OTU, Chao1, Shannon and Simpson

Indices increase along the greywater flow for both archaea and bacteria (Table 1). Ansola et al. (2014), treating municipal wastewater in natural and constructed wetlands, inferred that the lower diversity could be a result of the more controlled and stable conditions observed. This assumption, allied with the results, is interesting for the operation of the EvaTAC, since the insertion of an inbuilt AnC was intended to create more stable zones to decrease the impact of high loads of pollutants in the filter media zones.

The alpha diversity Indices found for the EvaTAC samples (Table 1) were similar to other CW studies treating different types of effluents: greywater (HYLANDER et al., 2014), surface water (HE et al., 2016; YUN et al., 2014), domestic wastewater (ANSOLA; ARROYO; SÁENZ DE MIERA, 2014; BOUALI et al., 2013; LONG et al., 2016) and municipal and agricultural wastewater (AN et al., 2019; ZHANG; JIAO; LU, 2018). In these natural and constructed *wetlands* the Shannon, Chao1 and Simpson Indices varied respectively on average in ranges of 5.11 to 7.57; 619 to 2347 and 0.91 to 0.99 for bacterial communities and 1.44 to 4.95; 248 to 703 and 0.73 to 0.96 for archaeal communities. Besides, our results support previous findings that archaeal communities are lower in richness and diversity than bacterial communities in constructed wetlands systems (BOUALI et al., 2013; DORADOR et al., 2013; HE et al., 2016; LONG et al., 2016).

Finally, an interesting finding in this study was the low abundance of phylum Chloroflexi in the EvaTAC. This phylum has been reported to exist in high abundance in CW (LONG et al., 2016; URAKAWA; BERNHARD, 2017; ZHANG; JIAO; LU, 2018). Bernardes et al. (2016), treating greywater in mesocosms simulating zero discharge CW, suggested that Chloroflexi*,* in addition to the degradation of organic material, could play a role in sulphide oxidation in anoxic environments. The combination of i) anaerobic conditions, ii) neutral pH, iii) odour released in the inlet portion of the CW and, iv) the absence of the genus *Chloroflexi* in this system could indicate that sulphide, the end product of the most abundant microorganism encountered in the EvaTAC (SRB) was released to the atmosphere.

## 2.5 Conclusions

This study explored the composition and diversity of bacteria and archaea communities, and is an initial step to understand microbial community structure in CW systems treating greywater. Microbial community showed variation depending on their

spatial position within the system, with the redox potential (Eh) being the environmental condition that most influenced microbial diversity and richness, and, as well as Eh, increased along the greywater flow. The anaerobic chamber, first zone of the EvaTAC system, presented the most dissimilar microbial community of the system, responsible for the conversion of complex to simpler substrate. As a result, this provided, in the middle layer of the CEvaT, the predominance of microorganisms involved in sulfidogenesis and methanogenesis processes, dominated by *Desulfovibrio* and *Methanobacterium* genera. The final part of the CW, which showed higher alpha diversity microbial Indices and lowest COD, was characterised by the presence of methanotrophs that were directly influenced by the increase of redox potential. The figure 7 show the overview of main processes occurring in each zone of EvaTAC.

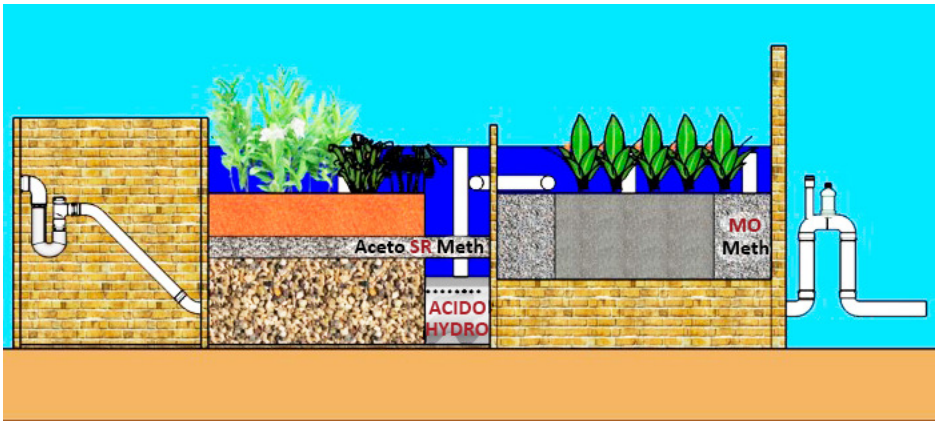


*Figure 7. Main processes occurring in each zone. The red colours indicate the main occurrence of the process was in that zone. The black colours indicating the secondaries processes occurring in the zones. HYDRO – Hydrolysis; ACIDO – Acidogenesis; ACETO – Acetogenesis; SR – Sulphate Reduction; METH – Methanogenesis; MO – Methane Oxidation.*

## 2.6 Acknowledgments

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# CAPÍTULO 3 - Spatial shift of microbial community and environmental condition in a constructed wetland system treating greywater*

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## Abstract

The global application of constructed wetlands (CWs) has suggested some environmental issues such as methane and sulphide release, mostly in anaerobic zones. Recent researches are showing that horizontal subsurface flow constructed wetland (HSSF-CW), beyond reduction, have also oxidation zones, which can contain microbial community to act in oxidation parts of biogeochemical cycles, and thus, avoiding these gases release. The objective of this work was to verify the relationship between environmental conditions and microbial processes along raw-light greywater flow in an improved constructed wetlands (CW) system, called EvaTAC. The EvaTAC system is composed of an upflow evapotranspiration and treatment tank (CEvaT) which has an inbuilt anaerobic chamber (AnC), followed by a HSSF-CW. Physicochemical analysis and high-throughput DNA sequencing were performed in the different zones to investigate the environmental conditions and microbial community. The results showed that the system operated predominantly in anaerobic conditions, with redox potential (Eh) increasing from the inlet (-342.9 mV) to the outlet (-316.4 mV) zone. Conversely, the chemical oxygen demand (COD) decreased along the greywater flow, suggesting negative correlation among these factors. The zones of CEvaT were characterized by lower community diversity and richness and by the presence of specific groups: Proteobacteria and Synergistetes related to hydrolysis and fermentation, in the sludge of bottom layer (AnC); methanogens (*Methanosaeta* and *Methanobacterium)* and sulphate reduction bacteria (*Desulfovibrio*, *Desulforhabdus* and *Desulfomonile*) in the gravel of middle layer and microorganism associated to nitrogen cycle and oxygen release area (*Acinetobacter, Novosphingobium, Candidatuas Nitrososphaera*) in the soil of top layer. On the other hand, the rise of ORP and decrease of organic matter were associated with higher community diversity and richness in the fine gravel of middle layer of CWs, that showed higher abundance of microorganisms involved in the methane (*Methylobacterium* and *Candidatus Koribacter*) and sulphur (*Rhodoblastus* and *Thiobacillus*) oxidation. Thus, this study suggests the influence of environmental conditions on microbial diversity and the occurrence of reduction and oxidation dynamics of C, S and N cycling in EvaTAC system.

**Keywords**: *High-throughput sequence, Microbial diversity, Anaerobic degradation, Methane oxidation*

**Esse artigo será submetido, como Bernardes et al., 2019, "Spatial shift of microbial community and environmental condition in a constructed wetland system treating greywater", para Water Research.*

## 3.1 Introduction

In most of the developing countries centralized sewer systems and wastewater treatment plants are provided mainly in larger cities, whereas the small and medium-sized towns generally discharge their waste water untreated into the environment (BRIX; ARIAS, 2005). With the pressure of local and global organizations for the public health, the resource-oriented sanitation that aims sustainable solution with cost effective, easy operation, low energy uses and mainly the health of user is gaining ground. In this context, separation at source is an important step for simplifying household treatment (OTTERPOHL, 2001). The light greywater (LGW), fraction that does not receive the contribution of the effluents from the toilet flush water, kitchen sink and dishwasher (ERIKSSON et al., 2002; OTTOSON; STENSTRÖM, 2003), beyond represent 70% of domestic sewage (HERNÁNDEZ LEAL et al., 2011), is the fraction of sewage with low organic material, nutrients and pathogens (PRÜSS et al., 2002).

Following the principles of resource-oriented sanitation, the constructed wetlands (CWs) systems are often used for the decentralized treatment of GW (ARDEN; MA, 2018). However, the global application of constructed wetlands has suggested the methane release (CHEN et al., 2013; CONRAD, 2009; ZHANG; JIAO; LU, 2018), sulphide production (CHEN et al., 2016a; WU et al., 2013) and gradual clogging in the filtering media (BARBAGALLO et al., 2016; NIVALA et al., 2013b). Methane is a potent greenhouse gas, with the warming effect per molar mass approximately 28–34 times of carbon dioxide over a 100- year period basis (MYRHE et al., 2014). Natural wetlands and paddy fields contribute about one third of the global methane emissions (CHEN et al., 2013). Sulphur is a popular target of bioremediation in constructed wetlands because it is highly reactive, redox-sensitive, harmful for aquatic life and cause aesthetic problems (taste, color and/or odor) when it is reduced form as sulphide (CHEN et al., 2016a; STURMAN et al., 2008).

The presence of methane and reduced forms of sulphides in constructed wetlands are performed by microbial processes called methanogenesis and sulphate reduction respectively. The methanogenesis is the process realized by methanogens that use $CO_2$ and $H_2$ and/or small organic molecules, such as acetate, formate, and methylamine and convert it to methane (ENZMANN et al., 2018). The sulphate reduction is the most

important biotic reactions influencing sulphur cycling in CWs and are catalysed by dissimilatory sulphate-reducing bacteria (SRB), which reduce sulphate to sulphide using the energy generated from the transfer of electrons from organic substrates (STURMAN et al., 2008). Both sulphate reduction and methanogenesis are regarded as the final step in the substrate degradation process in anaerobic environments (CHOU et al., 2008). In this context, the CW treating greywater is a conducive environment to the development of these processes, once greywater contains a wide range of complex organic macromolecules (carbohydrates, protein and lipids) (HYLANDER et al., 2014) and are also source of sulphur, which is frequently present in all organisms and occurs in organic compounds such as amino acids, proteins, enzymes, antibiotics and fats (POKORNA; ZABRANSKA, 2015).

On the other hand, constructed wetlands are well-known for showing different zones inside the system that could develop different environmental conditions, like rhizosphere zones (BAI et al., 2014; ZHANG et al., 2013), bottom and top layers (BOUALI et al., 2013; HUANG et al., 2013), inlet and outlet zones (BUTTON et al., 2015; ZHANG et al., 2018). These abiotic changes and well-known relevance of microbial interactions can shift microbial mediated processes (HO et al., 2016). As shifts in terminal acceptor electrons and products of microbial metabolism can change the dynamics of biological processes (ROYCHOWDHURY et al., 2018), the methane and sulphide produced can be also oxidized in this system (CHEN et al., 2016a; HE et al., 2015; ZHANG; JIAO; LU, 2018). Bacterial sulphur oxidation is mainly driven by S oxidizing bacteria (SOB), and sulphide is oxidized to sulphur (or sulphate) using oxygen or nitrate as electron acceptors in wetlands (FAULWETTER et al., 2009), while bacterial methane oxidation is driven by methanotrophs and methylotrophs, that convert $CH_4$ to $CO_2$, and have been serving as a biological filter significantly reducing $CH_4$ emissions (CHEN et al., 2013; CONRAD, 2009).

For this reason, it is important to carry out studies to understand the microbiological process inside the constructed wetlands. The microorganisms such as bacteria and archaea have been rarely used in wetland bio-assessment because of their complexity and a lack of understanding of their occurrence in wetland soils and water. The rapid development of molecular techniques during the past decade, however, made it possible to develop a new assessment tool using bacteria and archaea (SIMS et al., 2012). Still, the current understanding on microbial abundance and diversity in wetland

environments treating greywater is in its infancy, with just a few studies investigating the microbiological process (DING et al., 2014; HYLANDER et al., 2014; JONG et al., 2010; RODRÍGUEZ-MARTÍNEZ et al., 2016). Therefore, the goal of this research was to investigate the composition of the bacterial and archaeal communities present in the different zones of the improved constructed wetlands (EvaTAC) and verify the relation between environmental conditions and microbial processes along the greywater flow with special attention for the reduction and oxidation phases of carbon and sulphur cycle.

## 3.2 Material and Methods

### *3.2.1 System description*

The pilot scale EvaTAC system, located in Brazil (20°31' S e 54°39' W), was built in fiberglass to treat light greywater of an experimental bathroom (BanhEX) at the university campus. The BanhEX had a mean flow of 0.15 $m^3.day^{-1}$ generated by two showers, two sinks, two laundry sinks and one washing machine. The EvaTAC system is composed by two subsystems: an upflow evapotranspiration and treatment tank (CEvaT) with an inbuilt anaerobic chamber (AnC), followed by a horizontal subsurface flow constructed wetland (HSSF-CW). The dimensions (L x W x D) of the units were: CEvaT: 1.00 m × 1.00 m × 1.20 m and HSSF- CW: 1.00 m × 1.00 m × 0.70 m to the. For the CEvaT, the layers, starting from bottom to top were: gravel nº 4 (layer height: 0.40 m), gravel nº 2 (layer height: 0.30 m) and, on the top of a geotextile blanket a layer of 0.30 m of soil. The outlet layer (0.25 m x 1.00 m x 0.25 m) was filled with blast-furnace slag (particle size: retained in sieves with 19.1 mm mesh). The HSSF-CW was filled with fine gravel (layer height: 0.50 m). Sixteen seedlings of *Canna x Generalis* were planted in the CEvaT and 16 seedlings of *Equisetum Giganteum in the* HSSF-CW. The plants were distributed in 4 rows, 0.20 cm apart, with 4 seedlings in each. Before starting the presenting experiment, the system has been in operation for 2 years. In this study the EvaTAC was monitored for 104 days in the following conditions: hydraulic loading rate: 0.15 ± 0.08 $m^3.day^{-1}$ (CEvaT) and 0.14 ± 0.06 $m^3.day^{-1}$ (HSSF-CW). Organic loading rating of 48.00 ± 12.29 $g.COD.m^{-2}.day^{-1}$ (CEvaT) and 35.02 ± 10.34 $g.COD.m^{-2}.day^{-1}$ (HSSF-CW). A schematic view of the system and sampling points are shown in Figure 1.

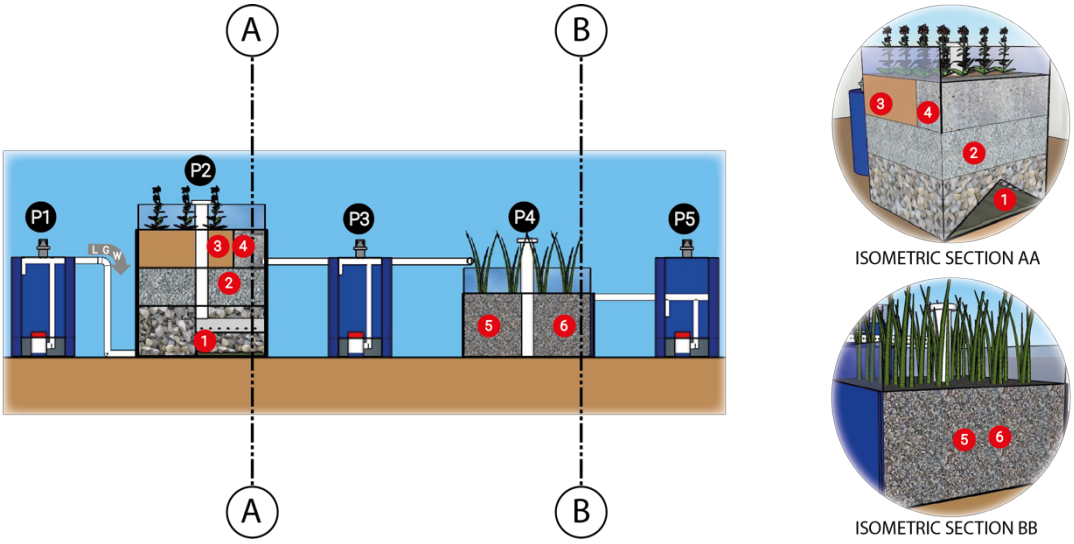


Figure 1. Schematic view of the EvaTAC system, including sampling points. Liquid samples: P1 (system inlet); P2 (piezometer, inside the CEvaT); P3 (CEvaT outlet and HSSF-CW inlet); P4 (piezometer, inside of HSSF-CW) and P5 (system outlet). The circles in red colours stand for the six biofilms sampling points: 1 - Bottom layer CEvaT – UF.AnC (AnC sludge); between 0.40 to 0.60 m of length and 0.80 to 1.00 m of depth; 2 - Middle layer CEvaT - G2.CET (Gravel nº2), between 0.50 m to 1.00 m of length and 0.30 to 0.45 m of depth; 3 - Top Layer CEvaT – SO.CET (Soil), between 0.5 m to 0.75 m of length and 0.10 to 0.15 m of depth; 4 - Top Layer CEvaT – SL.CET (Blast-furnace slag), between 0.75 m to 1.00 m of length and 0.10 to 0.15 m of depth; 5 - Initial Middle Layer CW – IFG.CW (Fine Gravel), between 0.00 to 0.50 m of length and 0.30 to 0.45 m of depth; and 6 - Final Middle Layer CW – FFG.CW (Fine Gravel), between 0.50 to 1.00 m of length and 0.30 to 0.45 m of depth.

### *3.2.2 Liquid and Biofilms Samplings*

Liquid samples were collected twice a week, from the indicated points in Figure 1 (P1 to P5). Portable meter (Hanna Instruments, HI 9829, USA) was used for pH, temperature and redox potential. Once a week the liquid samples were taken for chemical oxygen demand (COD), total carbon (TC), total organic carbon (TOC), total inorganic carbon (TIC), total nitrogen bound (TNb), sulphate and sulphide in the points: P1, bottom layer P2 (0.80 to 1.00 m depth), P3, middle layer P4 (0.30 to 0.45 m depth) and P5 (Figure 1). All physicochemical analyses were performed according to the standard methods for the examination of water and wastewater (APHA, 2012).

In the last day of system monitoring, biofilm samples were collected from the subsystems in different zones (Figure 1): bottom layer CEvaT (sludge), middle layer CEvaT (Gravel nº 2), top layer CEvaT (Soil and blast-furnace slag), initial middle layer CW (fine gravel) and final middle layer CW (fine gravel). In this sense, to form each

sample, three subsamples (250g each) were collected with horizontal distance about 0.30 m among them and numbered. The subsamples were stored in ziplock plastic bags and placed on ice immediately after sampling. In the laboratory, the subsamples were homogenized manually to obtain a representative sample and visible root or plant materials were removed. The samples were stored at -4ºC.

In order to characterize the environmental conditions of the zones, the potential redox, pH and temperature were measured inside the piezometers in the same depth where the biofilms were extracted. For the other parameters, the following correlations were assumed: having in mind that the CEvaT is an upflow system, the conditions correlated for the bottom layer biofilm (UF.AnC - sludge) was that measured in the bottom of P2, while for the middle layer (G2.CET - Gravel nº 2) was the average between that measured in P2 and P3 and, for the top layer (SO.CET - Soil and SL.CET -blast-furnace slag) it was considered that measured in P3. For the CW, which has a horizontal subsurface flow, the conditions correlated with the initial middle layer (IFG.CW) was the average between that measured in P3 and P4 and, for the final middle layer (FFG.CW) it was considered the average between measured in P4 and P5 (Figure 1).

### *3.2.3 DNA extraction, PCR amplification and high-throughput sequencing*

A 400 g portion of homogenized sample was transferred to settle in mili-Q water. The vessel was shaken by hand each 20 minutes during 3 hours. Afterwards, the cells were removed by centrifugation was performed as described by (YU et al., 2005) with modifications. After centrifugation, 1.0 ml of lysis buffer (500mM NaCl, 50mM Tris-HCl, pH 8.0, 50mM EDTA, and 4% SDS) and 0.4g sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm) was mixed into the samples and the mixture was incubated for 20 min at 70 °C with gentle agitation every 5 min. The mixture was then centrifuged at room temperature for 10 min at 13.600 rpm (Eppendorf 5804). The supernatant was transferred to a new tube, an equal volume of isopropanol was added, and after 20 min at room temperature the mixture was spun at maximum speed in a microcentrifuge for 10 min. The pellet was resuspended in 750 µl TE. Potassium acetate was added to a final concentration of 0.5 M, the mixture was incubated on ice for 5 min and after a centrifugation for 10 min at 13600 rpm, the supernatant was transferred to a new tube. This solution was then extracted twice with an equal volume of chloroform:isoamyl

alcohol (24:1). The final aqueous supernatant was transferred to a new tube. An equal volume of isopropanol was added to the recovered supernatant and after 1 h at room temperature total DNA was recovered by centrifugation at top speed for 15 min. The pellet obtained was washed with ethanol 70%, dried and resuspended in 30 µl milli-Q water.

For bacterial 16S rDNA, a 466 bp fragment was amplified using the primers: 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) flanking the V3 and V4 regions with the barcode (YU et al., 2005). For Archaeal 16S rDNA, a 288 bp fragment was amplified using the primers U519F (CAGYMGCCRCGGKAAHACC) and 806R (GGACTACNNGGGTATCTAAT) flanking the V4 region (PORAT et al., 2010). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. At last, the library was sequenced on an Illumina HiSeq 2500 platform by Genone company and 250 bp paired-end reads were generated.

### *3.2.4 Data Analyses*

Microbial sequences analyses were performed by Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/). Sequences above 97% of similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, the Green Gene Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) was used based on RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) algorithm to annotate taxonomic information. In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), multiple sequence alignment was conducted using the PyNAST software (Version 1.2) against the "Core Set" dataset in the Green Gene database. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences.

Subsequent analyses of alpha and beta diversity were all performed based on this output normalized data. Firstly, were analysed both the common and unique information for different samples (groups), and generate the Venn and Flower diagram. The unifrac

distance between pairwise samples are visualized as a heatmap to measure and view the dissimilarity extent. Alpha diversity was applied to analyse the complexity of species diversity for a sample through 3 indices: Chao1 to identify community richness, Shannon to identify community diversity and Coverage to characterized sequencing depth. To compare microbial communities among samples, unweight pair group method with arithmetic mean (UPGMA), nonmetric multi-dimensional scaling analysis (NMDS), analysis of similarity (Anosim) and linear discriminant analysis effect size (LfSe) were applied. All these alpha diversities and the beta diversity analysis in our samples were calculated with QIIME software (Version 1.7.0) and displayed with R software (Version 2.15.3).

In the analyses of physicochemical parameters, we tested the dispersion of dependent variables using the Shapiro–Wilk test, after which, the one-way ANOVA test was applied. After one of these tests indicated that there was an effect of the factor on the dependent variable measured among the sampled averages, the Tukey's post-test was used to make pairwise comparisons between the results of each zone. The Canonical Correlation (CCA) was performed to evaluate the influence of environmental condition on microorganism abundance. Pearson's correlation analysis was carried out to test the strength of relationships between and within microbial community metrics and the different water metrics. The correlation coefficient r was interpreted as a strong correlation, when $r \geq |0.75|$ and a moderate correlation when $|0.50| \leq r < |0.75|$) (MILTON; BULL; BAUMAN, 2011; ZHANG et al., 2018). All statistical tests involving physicochemical parameters were employed with the aid of the R software and $p<0.05$ was considered.

## 3.3 Results

### *3.3.1 Microbial community variability*

The 876,625 filtered bacterial high-quality sequences were classified into 59 phyla, 135 classes, 189 orders, 324 families, 663 genera, 283 species and 3532 OTUs, while the 969,622 sequences for archaea were classified into 5 phyla, 9 classes, 8 orders, 10 families, 11 genera, 4 species and 2663 OTUs. Good's coverage estimator ≥ 99.0% suggested that most of bacterial and archaea OTUs in each sample has been captured (Table S1).

The higher bacterial numbers of OTUs was encountered in the top layers of CEvaT (Figure S1A), with 57.5% and 53.7% of total OTUs were presented in SL.CET and SO.CET respectively (Figure S2A). These zones were also responsible for the highest specific number of OTUs (Fig. 2). Within CEvaT subsystem, the higher number of OTUs shared (59.8%) was between SL.CET and G2.CET and within HSSF-CW the IFG.CW and FFG.CW share 52% of the OTUs found in these zones, been 27.3% of OTU specific in the beginning and 20.7% of OTU in the outlet zone of this subsystem. The flowers diagram (Fig. 2) indicated that each zone had their specific microorganisms and 16.8% of total OTUs were shared for all zones of the system. The community diversity indices indicated that the higher diversity was found in the SL.CET, followed by FFG.CW and IFG.CW. On the other hand, the SO.CET, UF.AnC and G2.CET showed the lowest community diversity between the all zones (Table S1). The community richness index showed that the zones of CW had the higher evenness between found communities, the lower evenness followed the community diversity results, with the 2 first zones studied (UF.AnC and G2.CET) showing the lowest values (Table S1).

The highest archaeal number of OTUs was encountered in the FFG.CW, followed by IFG.CW and G2.CET (Fig. S1B), with more than a half of total OTU was presented in these zones (Fig. S2). On the other hand, regarding specifics OTUs, the highest number was encountered again in the top layers of CEvaT (SO.CET and SL.CET) (Fig. 2). The most shared numbers of OTUs (49.4%) in the CEvaT subsystem were between SL.CET and G2.CET and in the HSSF-CW the two zones studied shared (69.4%) of total reads found in both places. In the same way to bacteria target, the flowers diagram (Fig. 2) indicated that each zone had specific microorganisms and 18.4% of total OTUs were shared for all zones of the system. The alpha diversity indices indicated the higher archaeal community diversity and richness were found in the two zones of CW, followed by G2.CET, on the other hand, the UF.AnC showed lowest values for the diversity and richness Indices (Table S1).

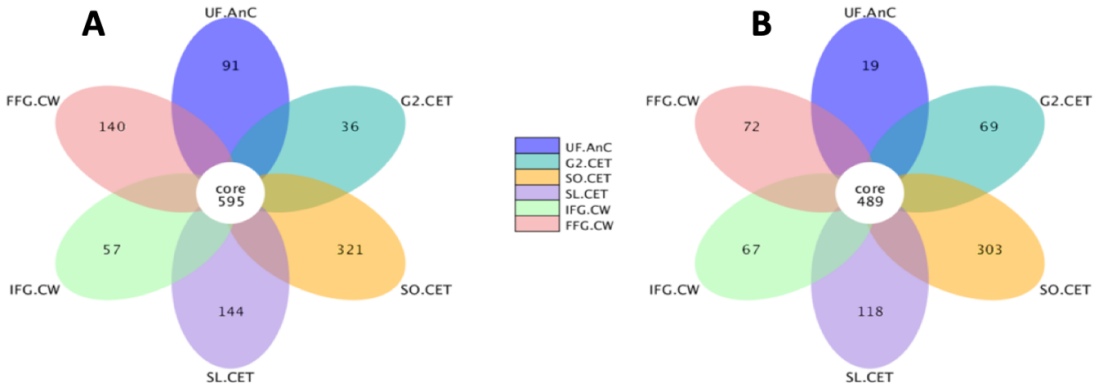


*Figure 2. Flower diagrams showing the unique and shared OTUs. A - for bacteria community and B - for archaeal community. UF.AnC (AnC sludge) - Bottom layer CEvaT; G2.CET (Gravel no2) - Middle layer CEvaT; SO.CET (Soil) - Top Layer CEvaT; SL.CET (Blast-furnace slag) - Top Layer CEvaT; IFG.CW (Fine Gravel) - Initial Middle Layer CW and FFG.CW (Fine Gravel) - Final Middle Layer CW.*

### *3.3.2 Clustering analysis of microbial communities*

To elucidate the variability of microbial communities within the system, we used Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Non-metric Multidimensional Scaling (NMDS) to cluster the microbial communities as a function of the zones investigated. The UPGMA tree indicated that the communities could be divided into four and five distinct groups for bacteria and archaea, respectively (Fig. 3). For bacteria, G2.CET and SL.CET and IFG.CW and FFG.CW showed similarity between their communities. The UF.AnC zone was more related with the CW than the CEvaT zones, indicating that the subsystem did not have great influence in clustering communities. For the archaea, only the CWs zones were clustering together (Fig. 3B), on the other hand these zones plus G2.CET and UF.AnC were clustered in the same branch. The top layers of CEvaT samples (SL.CET and SO.CET) were distantly separated from each other, suggesting that the media filter had influence on the clustering archaea communities.

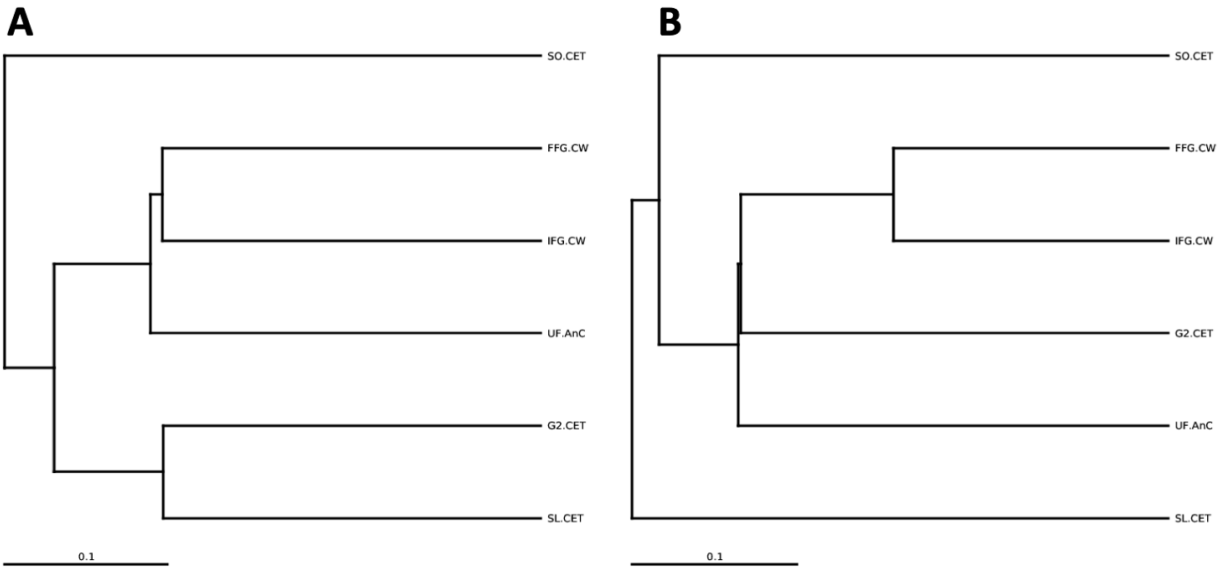


*Figure 3. UPGMA cluster tree based on Unweighted UniFrac distance. A - for bacteria community and B - for archaeal community.*

The NMDS analyses helped to further examine spatial variation among zones (Fig 4). The top layers of CEvaT (SL.CET and SO.CET) showed the greater variability within the replicas. The SO.CET was the zone that was ordinated furthers apart the other ones, for both analysed regions (V3V4 and V4). Anosim analyses confirmed that variation among groups was significantly larger than variation within replicas (Table S2).

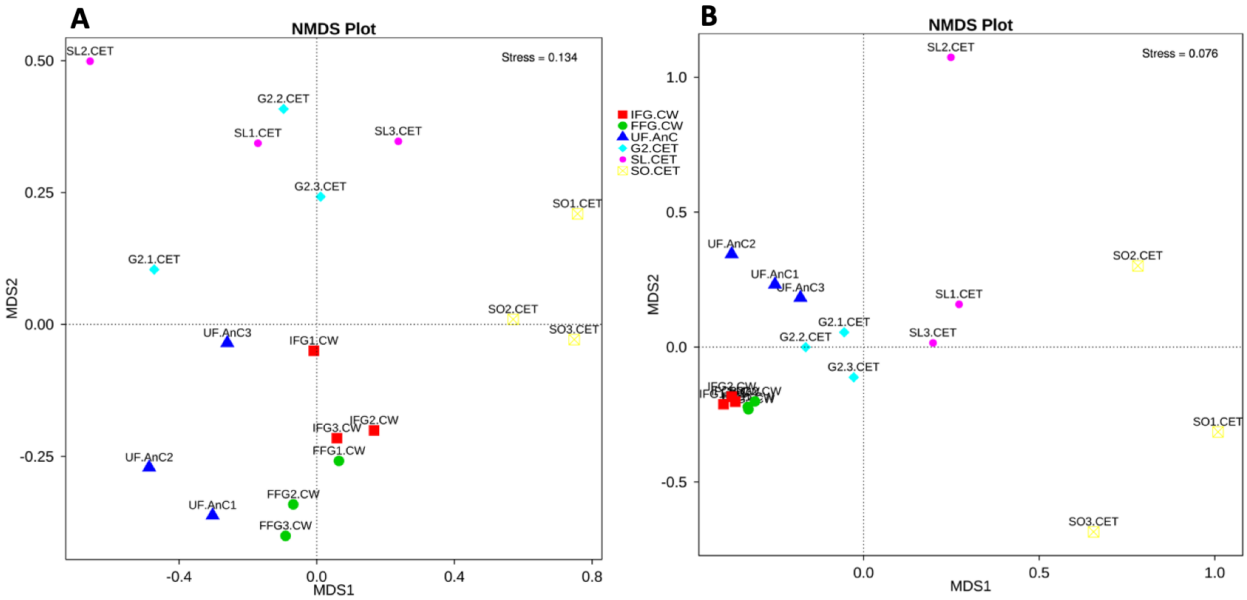


*Figure 4. Non-metric multi-dimensional scaling analysis (NMDS). A - for bacteria community and B - for archaea community. UF.AnC (AnC sludge) - Bottom layer CEvaT; G2.CET (Gravel no2) - Middle layer CEvaT; SO.CET (Soil) - Top Layer CEvaT; SL.CET (Blast-furnace slag) - Top Layer CEvaT; IFG.CW (Fine Gravel) - Initial Middle Layer CW and FFG.CW (Fine Gravel) - Final Middle Layer CW.*

### *3.3.3 Microbial community structure*

Of the 59 phyla identified using the V3-V4 region of 16SrRNA gene, only 8 were present at N>1% relative abundance in any of the sequencing libraries. Proteobacteria was the most abundant phylum, accounting for 58.2% of total reads. Euryarchaeota was the second most abundant phylum (13.7%), followed by Firmicutes (7.1%), Synergistetes (7.0%), Acidobacteria (3.8%), Actinobacteria (2.5%), Bacteriodetes (2.4%) and Chloroflexi (1.2%). Within the Proteobacteria, Alphaproteobacteria (32.5%), Betaproteobacteria (12.7%) and Deltaproteobacteria (9.0%) stand out, presenting the higher abundance, composes the top 5 of all classes. Others with high abundance were Methanomicrobia (10.9%), Synergistia (7.0%), Clostridia (5.9%) and Gammaproteobacteria (4.1%). The most abundant order of total reads was Rhizobiales (23.1%), belong the Alphaprotebacteria class, followed by Synergistales (10.2%) and Methanosarcinales (9.7%). For this region, the most abundant genus was *Methanosaeta* (13.0%), followed by *Dechloromonas* (8.9%), *Rhodopseudomonas* (8.4%), *Rhodoblastus* (7.8%) and *Novosphingobium* (5.9%). Figure 5A summarizes the distribution of the 35 most abundant genera across the zones of the system. For the archaeal community (identified using the V4 region of 16SrRNA gene), the most abundant phylum was Euryarchaeota (86.4%), mainly composed by Methanobacteria (85.6%) in the class level, Methanobacteriales (88.4%) in the order and *Methanobacterium* (87.3%) in the genus level. The second most abundant phylum was Thaumarchaeota (13.3%), mainly composed by SAGMCG-1(8.4%), Unidentified_SAGMCG-1 (8.6%) and *Candidatus Nitrosotalea* (8.7%) for class, order and genus level, respectively. Figure 5B summarizes the distribution of the 11 most abundant genera across the zones of the system.

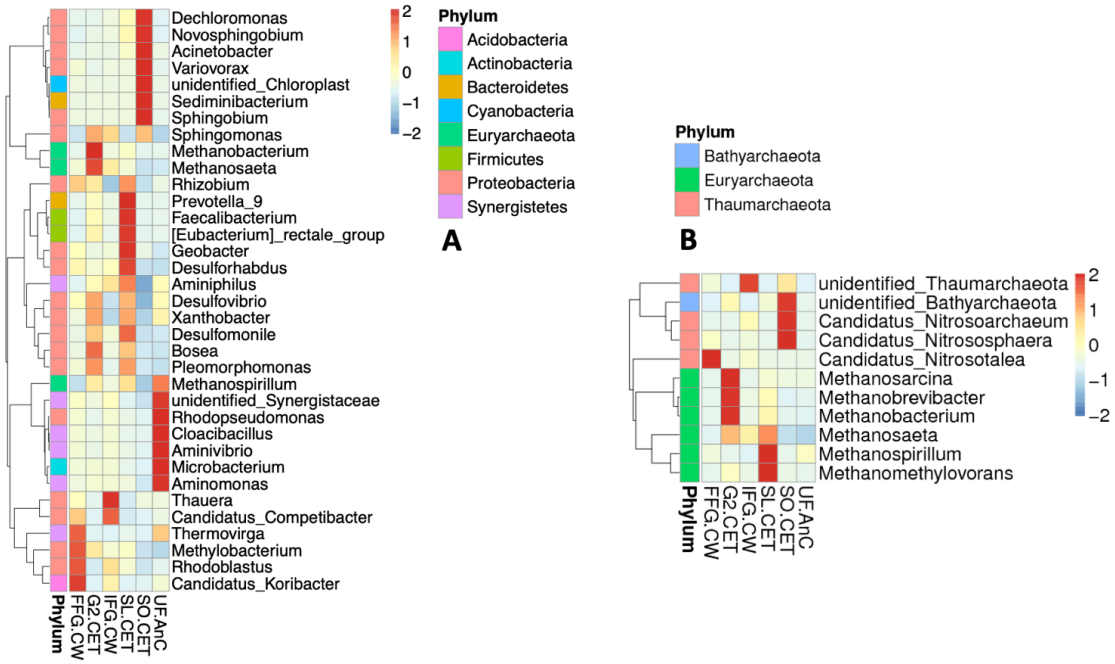


*Figure 5. Heatmap showing the distribution of top 35 most abundant OTUs for bacteria V3V4 region (A) and 11 most abundant OTUs for archaea V4 region (B). The absolute value represents the distance between the raw score and the mean of the standard deviation. It is negative when the raw score is below the mean, and vice versa. UF.AnC (AnC sludge) - Bottom layer CEvaT; G2.CET (Gravel no2) - Middle layer CEvaT; SO.CET (Soil) - Top Layer CEvaT; SL.CET (Blast-furnace slag) - Top Layer CEvaT; IFG.CW (Fine Gravel) - Initial Middle Layer CW and FFG.CW (Fine Gravel) - Final Middle Layer CW.*

The LEfSE was applied to elucidate the statistical significance, biological consistency, and effect relevance, allowing identifying features of abundance and related classes (Figure 6). The first zone, which receives the higher load of pollutants (UF.AnC), had the Synergistaceae and Rhizobiaceae as highlights. The second layer of the system (G2.CET) showed higher abundance of Euryarchaeota phylum, mainly dominated by Methanosaetacea family. The top layers of CEvaT showed the abundance of Firmicutes phylum in the SL.CET and Betaproteobacteria class in the SO.CET. In the second subsystem (HSSF-CW), the initial part (IFG.CW) had higher abundance of Deltaproteobacteria, Gammaproteobacteria and Betaproteobacteria class, mainly composed by Syntrophobacterales, Xanthomonadales and Rhodocyclales order respectively. The final part of the system was dominated by Acidobacteria. Finally, the greatest difference in the archaeal communities was encountered between the middle layer of CEvaT (G2.CET) and final middle layer of the CW (FFG.CW), the first one was dominated by methanogenic archaea of the Euryarchaeota phylum, mainly Methanobacteriaceae family and in the second one Thaumarchaeota phylum and SAGMCG 1 class appeared in higher abundance.

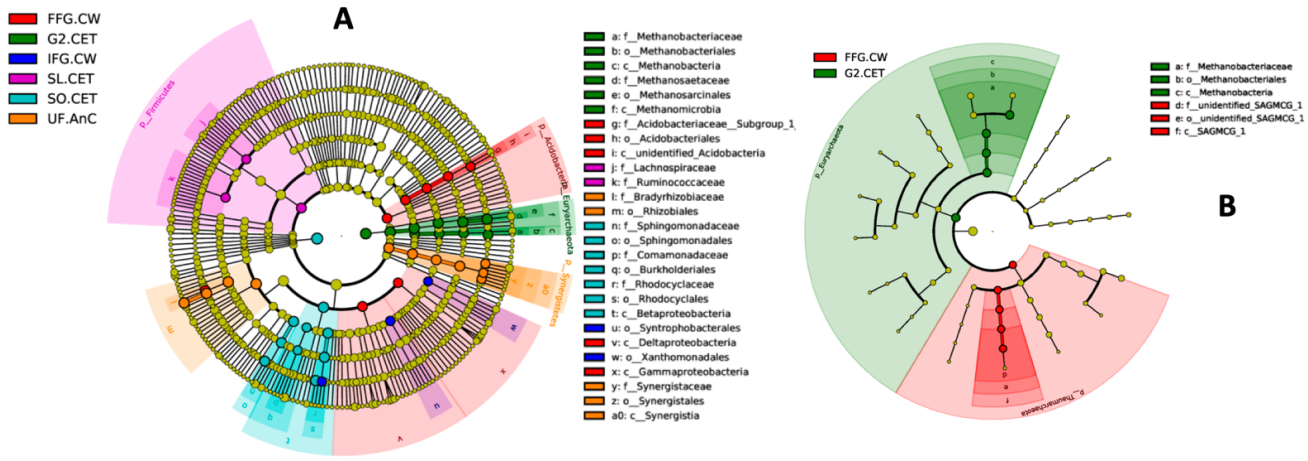


*Figure 6. Cladograms representing the results of LEfSe analysis for evaluation of biomarkers with statistically difference among group. A – for bacteria and B – for archaea.*

### *3.3.4 Relationship between environmental conditions and microbiota*

The physicochemical properties of LGW along the EvaTAC system are summarised in Table 1. The results showed a large variability of the physical chemical parameters measured in influent greywater, typical for GW. The focus of this research is the variation of physicochemical parameters between the first zones of the system (UF.AnC) to the subsequent zones along greywater flow (Figure 1). In this way, the majority of measured parameters had the trend to decrease along the greywater flow (Table 1). The TIC and ORP were the only parameters that showed the trend to increase along the flow. The sulphate and sulphide had different behaviours, with sulphate decreasing in the CEvaT subsystem, and increasing from P3 to P4 (from the inlet to the middle of the CW) and dropping again until leaving the EvaTAC system (CW outlet). Conversely, as expected, the sulphide increased from the inlet to the outlet of CEvaT and decreased along the flow from the inlet to the outlet of the CW subsystem. An anaerobic condition was verified for all zones.

The top 500 most abundant OTU and all physicochemical parameters were selected to construct the CCA model (Figure 7). The pH, temperature and COD/TOC ratio showed linear combination with other parameters and for this reason were automatically withdrawn of the model. Sulphide, Sulphate, COD and ORP were important environmental parameters impacting the bacteria communities and ORP, TN, COD, Sulphide and Sulphate for the archaea communities. As shown in Figure 7a and 7b, there is a negative correlation between ORP and TIC with other environmental

conditions, while ORP and TIC showed a trend to increase along the flow, with the others ones decreasing. Pearson Correlation analyses (Table S3) confirm that hypothesis, which showed negative correlation with ORP and TIC with others parameters. Only these two parameters also were that showing positive correlation with the alpha diversity indices (Fig. S3).

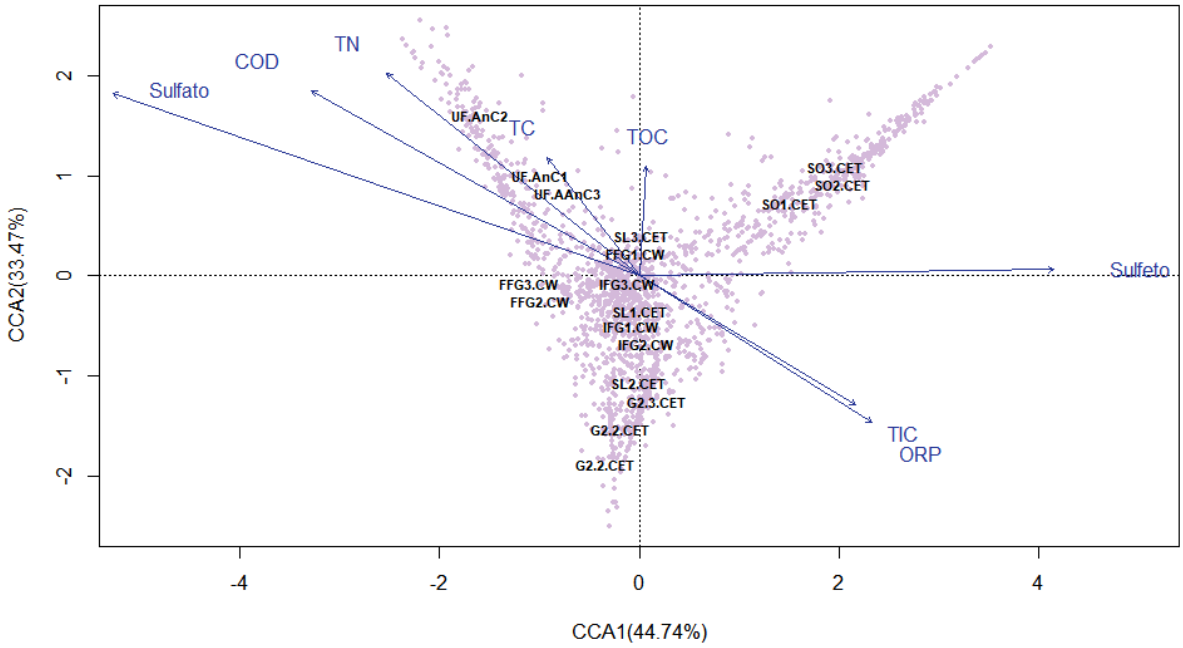


*Figure 7A. Canonical correlation analysis (CAA) for environmental conditions and species diversity diagram of 500 most abundant bacteria OTUs across the six zones studied.*

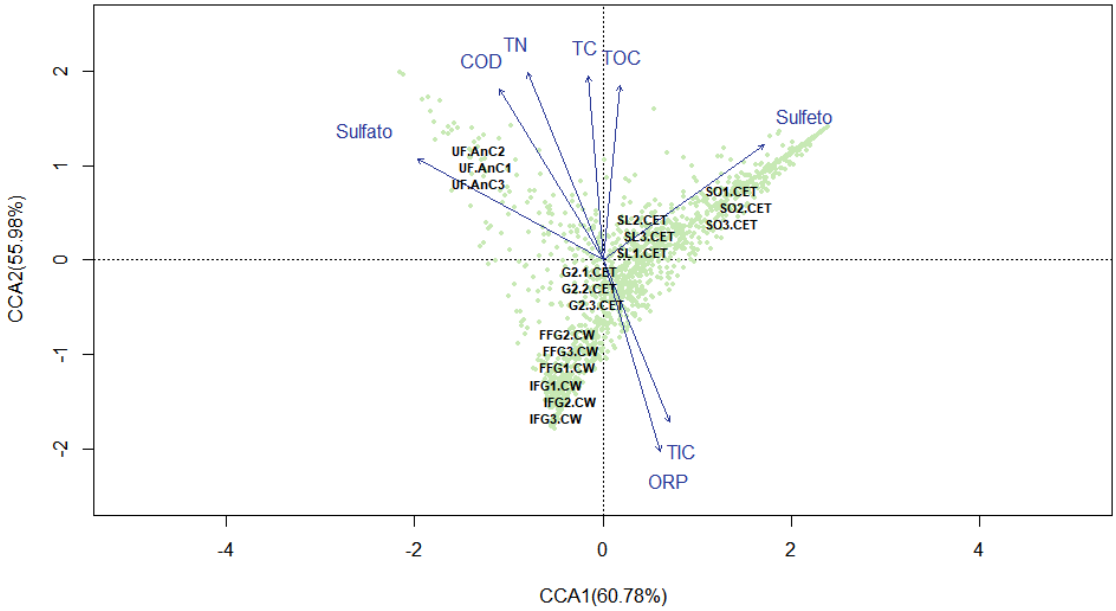


*Figure 7B. Canonical correlation analysis (CAA) for environmental conditions and species diversity diagram of 500 most abundant archaea OTUs across the six zones studied.*

*Table 1. Physicochemical parameters measured (mean ± standard deviation). Same letters within the same column indicate significant difference ($p<0.05$). P1, P2, P3, P4 and P5 are the sampling points (see Figure 1).*

| | EvaTAC | | Environmental conditions | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ORP$^{y}$ (Eh) | pH$^{y}$ | Temp$^{y}$. (ºC)* | COD$^{x}$ (mg.L$^{-1}$) | TC$^{x}$ (mg.L$^{-1}$) | TOC$^{x}$ (mg.L$^{-1}$) | TIC$^{x}$ (mg.L$^{-1}$) | COD/TOC$^{x}$ | TNb$^{x}$ (mg.L$^{-1}$) | Sulphate$^{x}$ (mg.L$^{-1}$) | Sulphide$^{x}$ (mg.L$^{-1}$) |
| ↓ | **LGW-Influent (P1)** | | 211.2 ± 111.1$^{a}$ | 7.9 ± 0.6$^{a}$ | 26.2 ± 3.1 | 319.8 ± 212.8$^{a}$ | 96.8 ± 32.4$^{a}$ | 81.0 ± 27.9 | 15.8 ± 6.3$^{a}$ | 3.9 ± 2.5$^{a}$ | 19.0 ± 7.3$^{a}$ | 46.6 ± 50.9 | 0.3 ± 0.4$^{a}$ |
| | CEvaT | BL.CEvaT (UF.AnC) **(P2)** | -342.9 ± 39.1$^{ab}$ | 6.8 ± 0.3$^{ab}$ | 26.7 ± 1.4 | 361.7 ± 102.0$^{b}$ | 136.6 ± 33.0$^{ab}$ | 104.7 ± 16.8$^{a}$ | 22.3 ± 6.4$^{ab}$ | 3.0 ± 0.4$^{b}$ | 43.8 ± 10.5$^{ab}$ | 82.7 ± 66.0$^{a}$ | 32.9 ± 10.0$^{ab}$ |
| | | ML.CEvaT (G2.CET) | -337.2 ± 41.4$^{a}$ | 6.9 ± 0.3$^{ac}$ | 26.9 ± 1.6 | 292.4 ± 66.6$^{bc}$ | 132.7 ± 31.9$^{ac}$ | 101.2 ± 19.5$^{b}$ | 24.4 ± 5.0$^{ac}$ | 2.6 ± 0.4$^{a}$ | 39.6 ± 8.7$^{a}$ | 65.3 ± 45.9 | 35.9 ± 11.6$^{a}$ |
| | | TL.CEvaT (SL.CET and SO.CET) | -328.0 ± 44.2$^{a}$ | 6.9 ± 0.3$^{ad}$ | 26.9 ± 2.1 | 231.5 ± 53.0$^{bcd}$ | 128.7 ± 33.0$^{a}$ | 100.6 ± 28.6$^{c}$ | 26.5 ± 4.3$^{ad}$ | 2.4 ± 0.5$^{a}$ | 37.1 ± 12.2$^{a}$ | 47.9 ± 37.0$^{a}$ | 42.3 ± 19.1$^{abc}$ |
| | **LGW-middle (P3)** | | - | - | - | | | | | | | | |
| | CW | IFG.CW | -316.4 ± 42.8$^{ab}$ | 6.8 ± 0.3$^{ae}$ | 26.1 ± 2.1 | 216.4 ± 44.1$^{bc}$ | 121.5 ± 31.9 | 93.0 ± 26.0$^{d}$ | 26.8 ± 4.4$^{ae}$ | 2.4 ± 0.5$^{a}$ | 34.9 ± 11.4 | 58.4 ± 30.9 | 33.2 ± 12.3$^{a}$ |
| | | ML.CW **(P4)** | | | | 208.6 ± 73.1$^{ab}$ | 114.3 ± 31.3 | 85.5 ± 24.0 | 27.0 ± 5.9$^{af}$ | 2.2 ± 0.7$^{a}$ | 32.6 ± 13.0 | 69.0 ± 49.6$^{b}$ | 26.1 ± 16.3$^{ac}$ |
| | | FFG.CW | | | | 153.7 ± 50.5$^{ab}$ | 109.9 ± 27.8 | 76.9 ± 20.1$^{abc}$ | 31.7 ± 7.3$^{abc}$ | 1.9 ± 0.4$^{a}$ | 30.9 ± 11.1$^{b}$ | 49.8 ± 28.6$^{a}$ | 26.2 ± 13.8$^{ac}$ |
| | **LGW-Effluent (P5)** | | -289.2 ± 31.2$^{ab}$ | 7.5 ± 0.2$^{bcde}$ | 26.5 ± 1.4 | 112.8 ± 51.6$^{abcd}$ | 105.4 ± 25.6$^{bc}$ | 68.2 ± 17.6$^{abcd}$ | 37.2 ± 10.1$^{abcdef}$ | 1.5 ± 0.4$^{ab}$ | 29.2 ± 11.9$^{b}$ | 30.6 ± 20.8$^{ab}$ | 27.7 ± 15.9$^{ac}$ |

*Note: * Indicates that the statistical tests showed no significant difference. $^{x}$ indicates 16 samples and $^{y}$ 28 samples.*

To further investigate the relationship between the environmental parameters and the dominant lineages across the zones, the CCA was applied to the 20 dominant genera (Figure 8) and for the groups classified within the known functional microbial genera, it was divided into 11 groups (Figure 9). The *Rhodopseudomonas, Aminivibrio, and Unidentified Synergistaceae,* all microorganisms performing the first steps of anaerobic degradation (hydrolysis, fermentation, acidogenesis and acetogenesis) were most abundant in the UF.AnC and were more associated with high COD and sulphate. The methane producing archaea (methanogens), mainly *Methanobacterium, Methanosaeta* and *Methanobrevibacter,* were most abundant in the G2.CET and showed low influence of environmental condition. In the SO.CET zone, microorganisms composed of denitrifiers, *Dechloromonas,* nitrifiers, *Candidatus Nitrososphaera* and aromatic compounds degraders group, *Novosphingobium,* were abundant. Another important group of microorganisms in this zone was the photosynthesizers, mainly represented by Cyanobacteria-*Unidentified Chloroplast* (Figure 9). In the SO.CET, the microbial structure showed positive correlation with sulphide, ORP and TIC. The *Desulfomonile* and *Desulforhabdus* were most abundant in the SL.CET zone and had negative correlation with sulphate and COD. The denitrifiers *Thauera* and *Candidatus Competibacter* were most abundant in the IFG.CW and the bacteria involved in methane oxidation, *Candidatus Koribacter,* nitrogen fixing, *Rhizobium,* nitrifier, *Candidatus Nitrosotaela* and sulphur oxidation, *Rhodoblastus* in the FFG.CW. The microorganisms of CW zones showed negative correlation with all parameters measured excepted with TIC and ORP. The last three zones of the system (SL.CET, IFG.CW and FFG.CW), as demonstrated by the alpha diversity index, had the highest diversity of microorganism, sharing different functional group mainly formed by SOB, Methanotrophs, Methylotrophs and SRB.

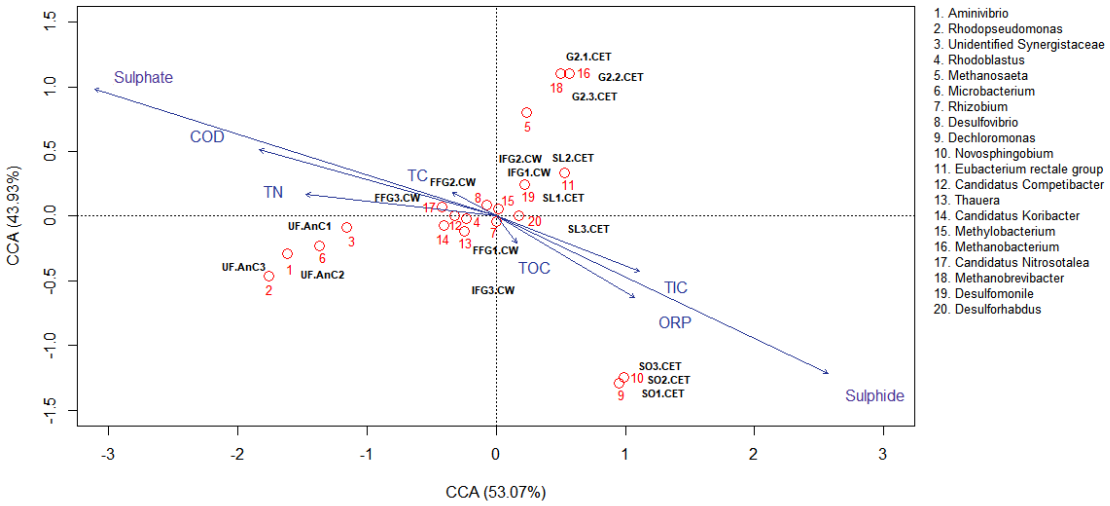


*Figure 8. Canonical correlation analysis (CAA) for environmental conditions and microbial genera level. The top 20 more abundant genera in this study were selected to build the model (17 for bacteria V3V4 region and 3 for archaea V4 region).*

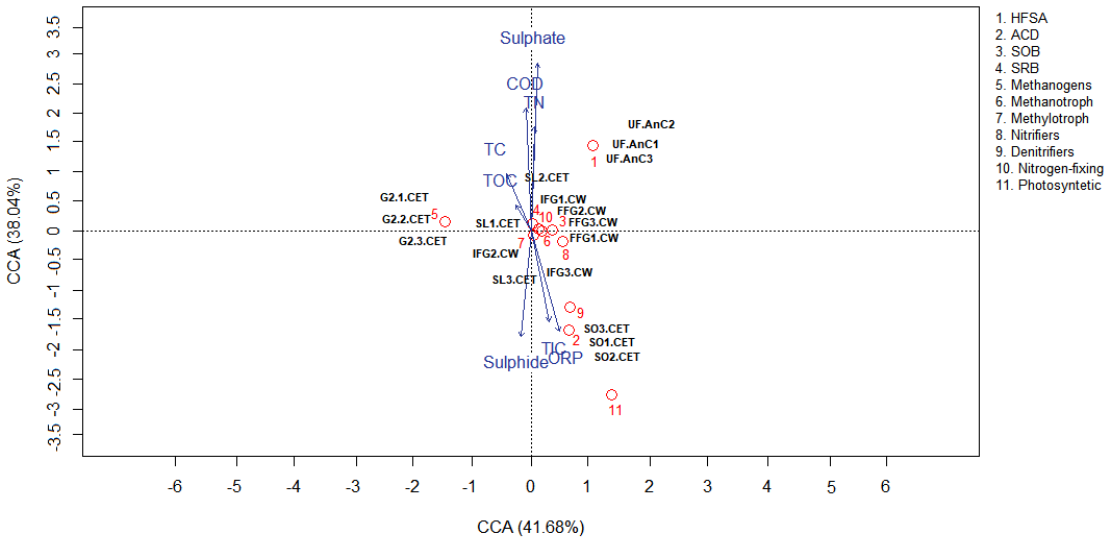


*Figure 9. Canonical correlation analysis (CAA) for environmental conditions and relative abundance of sequences classified within known functional bacteria and archaea genera. To build the model microbial genera were divided into 11 functional group (Table S3 – Supplementary Material).*

## 3.4 Discussion

### *3.4.1 Effect of zones on microbial communities*

The six studied zones of the EvaTAC system showed an anaerobic environment (from -342.9 to -316.4 mV) (FAULWETTER et al., 2009; STEIN et al., 2007; XU et al.,

2018), with the trend of ORP increasing along the greywater flow. Conversely, parameters representing carbon availability (TOC, TC and COD) trended to decrease (Table 1). The CCAs analyses (Figure 7 and 8) and Pearson Correlation (Fig. S3) confirmed this negative correlation between COD, TC and TOC with ORP. Usually in horizontal subsurface flow constructed wetlands, the potential redox increases from the inlet to the outlet zone, which means the conversion of complex substrates to simpler substrates and degradation of pollutants (HEADLEY et al., 2012). In agreement, the UF.AnC was the zone with the highest COD and lowest ORP value. It is interesting to note that within the CEvaT, the higher standard deviation of redox potential was in the top layers (SO.CET and SL.CET), suggesting that oxygen was released by the roots of the plants (ANGEL et al., 2016; BUTTON et al., 2015; LV et al., 2017; STEIN; HOOK, 2005; ZHANG; WU; HU, 2014) and atmospheric diffusion (NIVALA et al., 2013a; STEIN; HOOK, 2005) in these zones. This oxygen supply was not enough to differentiate ($p<0.05$) the redox condition permanently in the top layers to other ones in the CEvaT, but we presumed that it was responsible for the greater total and specific number of bacterial OTUs have been identified in the SO.CET and SL.CET (Figure S1), mainly for encountered in these zones high abundance of microorganism well-known for surviving in the area with oxygen release, like *Acinetobacter, Rhizobium,* Cyanobacteria-*Chloroplast* and *Novosphingobium* (Figure 5)(CHIPASA; MĘDRZYCKA, 2006; HYLANDER et al., 2014; KUSCHK et al., 2003; ZHONG et al., 2014). The micro-environment of alternating microaerobic–anaerobic condition in the plant roots zone supported the suitable habitat for aerobic, anaerobic, and facultative anaerobic microorganisms (LIU et al., 2016).

The higher numbers of archaeal OTUs, which are mostly anaerobic (FAULWETTER et al., 2009; THAUER et al., 2008), were encountered in the zones of the HSSF-CW and in the G2.CET. However, the highest numbers of specific OTUs, low diversity and richness of archaea communities and the higher abundance of *Candidatus Nitrosarchaeum* and *Candidatus Nitrososphaera (Fig. 5)*, which are related to the nitrification cycle and well known for be living in rhizosphere layers with release of oxygen (BOUALI et al., 2013; LIU et al., 2014; TOURNA et al., 2008; ZHANG et al., 2015a), were encountered in the top layers of CEvaT. Therefore, these evidences reinforce the possibility of oxygen release in these zones of CEvaT. Bouali et al. (2012), studying the structure and spatio-temporal distribution of the archaea in a horizontal

subsurface flow constructed wetland treating domestic sewage, found the dominance of *Candidatus Nitrososphaera* in the rhizosphere layer where the environment was similar to soil conditions (oxygenation, reed roots, gravel media, humus, etc.).

The results of UPGMA and NMDS confirm that the soil (SO.CET) was the most different zone among the others. However, this difference was not caused only by environmental condition, but also influenced by the media filter type, once the SL.CET and SO.CET were in the same layer, showing similar environmental conditions (Table 1). The higher microbial diversity found in the SL.CET than SO.CET could be an indicative of better adherence of microorganism to gravel media filters than soil. Hylander et al. (2014) testing filtering materials to treat greywater in pilot scale, verified that the performance of sand was less efficient in potential respiration rate and organic material degradation than bark and charcoal filters, mainly due to the small specific surface area, low porosity and high hydraulic conductivity for the sand, that reduce the contact of the greywater with the biofilm, thus, reducing its development.

Other interesting observation was that, as SO.CET, the UF.AnC and the G2.CET zones also showed lower microbial community diversity and richness, being zones with higher dominance of microbes belonging to specific functional groups, causing them to be ordinated distant from the others (Figure 8 and 9). This finding is really interesting regarding the system operation suggesting zones with specific activity. Monard et al. (2011), investigating the relationship between bacterial diversity and function under biotic control in soil, studying samples collected from top layer of an agricultural field, concluded that keystone phylotypes are important to maintain function more than the higher richness. In agreement, Deng, (2012), in a review study about the relationship of diversity-stability in soil microbial community, reported that degradation efficiency was not linearly related to the species richness but relied on keystone species, therefore eliminating keystone species may profoundly impair functional stability. On the other hand, the higher microbial diversity and richness found in the last zones of the system (SL.CET, IFG.CW and FFG.CW) could be an indication of different parts of biogeochemical cycles taking place, suggesting adaptation to the lower availability of carbon source and electrons acceptors. The rich bacterial community offered redundant actors with the capacity to degrade different types of organic matter (HYLANDER et al., 2014). This is supported by the CCA analyses of the most abundant genera and functional groups, which showed these zones always plotted in the centre. Therefore, indicating

higher evenness and diversity of microorganism that consequently suffered a similar influence of all environmental conditions.

The beta diversity microbial results (Figure 4 and Table S2), suggest that each zone have a kind of particularity and could be important for the development of biogeochemical cycle occurring inside the system. The impact of environmental parameters on the microbial assemblages was revealed by CCAs and the microbial community characterization (Figure 5 and 6) confirms that the dynamic of C, S, and N cycling occur in wetlands systems, with similar results found by (ROYCHOWDHURY et al., 2018; SIMS et al., 2012; URAKAWA; DETTMAR; THOMAS, 2017). The figure 10 show the overview of main processes occurring in each zone of EvaTAC.

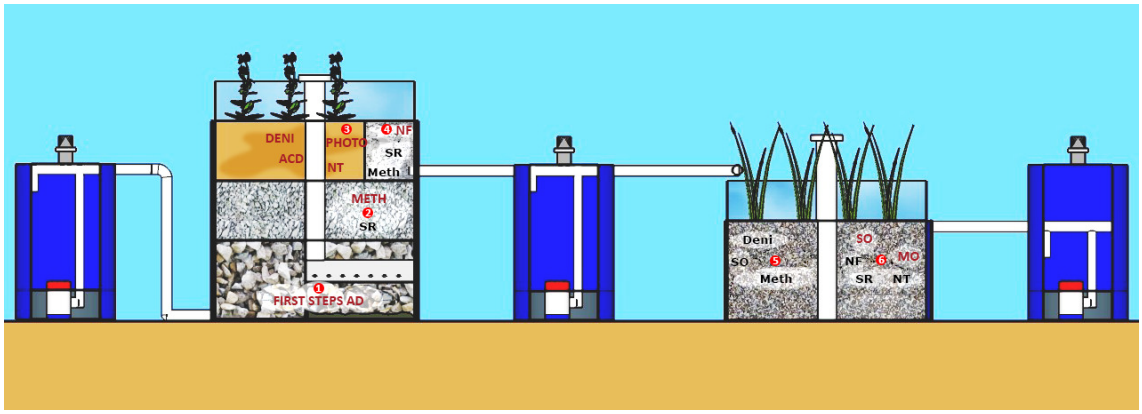


*Figure 10. Main processes occurring in each zone. The red colours indicate the main occurrence of the process was in that zone. The black colours indicating the secondaries processes occurring in the zones. FIRST STEPS ANAEROBIC DEGRADATION (AD) – Hydrolysis, Fermentation, Acidogenesis and Acetogenesis; SR – Sulphate Reduction; METH – Methanogenesis; DENI – Denitrification; ACD – Aromatic compounds degradation; PHOTO – Photosynthesis; NT – Nitrification; NF – Nitrogen Fixation; SO – Sulphur oxidation; MO – Methane Oxidation.*

### *3.4.2 Microbial community shift along the flow*

The predominance of anaerobic microorganisms found in the system is in accordance with the physicochemical parameters measured. With the LfSe analyse (Figure 6), it was possible to visualize the remarkable spatial difference in the microbiota in accordance with the greywater path, with emphasis to the Synergistetes phylum, which has the majority of their genus well known to be responsible for the initial step of anaerobic digestion process (WANG et al., 2018) in the UF.AnC. Following the flow, in the G2.CET, Euryarchaeota, the methanogens (THAUER et al., 2008), and lastly, the

Acidobacteria and Alphaproteobacteria, linked with methanotrophs (KIELAK et al., 2016) and sulphur cycle (ZHOU et al., 2015a) respectively, in the CW zones. However, microbial community analysis at lower taxonomic levels, such as genus and species levels, may provide better phylogenetic resolution than higher taxonomic groups such as phylum (HO et al., 2016; SUN et al., 2016, 2018).

In this way, there was the dominance of *Rhodopseudomonas (*29.7%), represented by *Rhodopseudomonas Palustris* species, followed by*, Aminivibrio (*5.2%)*, Unidentified Synergistaceae (5.2%) and Cloacibacillus (3.6%)* in the sludge of UF.AnC zone*. (*Figure 8*). Rhodopseudomonas palustris* obtain energy from carbon from organic compounds (mainly pyruvate, lactate, malate and succinate) to hydrogen producing (ADESSI et al., 2016; KOKU et al., 2002; MCKINLAY; HARWOOD, 2011; TANG; TANG; BLANKENSHIP, 2011), the others genera are members of Synergistaceae family, well know responsible for the degradation of proteins (MILITON et al., 2015; VEERAVALLI et al., 2016). The presence of these groups, mainly in the anaerobic chamber (UF.AnC) (Figure 9), suggests the achievement of the first steps of anaerobic digestion showing that, one of the functions expected to the AnC is to receive and convert the particles of higher molecular weight to simpler substrates. In the next layer (G2.CEvaT), methanogens were dominant, mainly represented by the *Methanosaet*a (29.7%) and *Methanobacterium* (12.2%) genera. This might be related to the substrate availability, because methanogenesis relied on substrates like hydrogen, formate, acetate and carbon dioxide (SCHINK, 1997), all of which could have been products of the organic matter degradation by the downstream former steps. This hypothesis can be supported by the fact that these microorganisms, in G2.CET zone, had no high influence of the environmental conditions (Figure 8 and Figure S3A). The presence of these groups of microorganisms in these zones achieved a reduction of COD in 35.9%.

In the SO.CET zone, there were the dominance of *Decloromonas* (25.2%) and *Novosphingobium* (16.8%) and the low abundance of *Methanosaeta* (0.6%). *Dechloromonas* are recognized like nitrate-reducing bacteria (SUN et al., 2018) and *Novosphingobium* are aromatic compounds degraders (GAN et al., 2013), both of them were identified in environments with the presence of oxygen release (RODRÍGUEZ-MARTÍNEZ et al., 2016; WU et al., 2016; ZHANG; JIAO; LU, 2018; ZHONG et al., 2014). Moreover *Methanosaeta* are strictly anaerobic and sensitive in the presence of some oxygen (ENZMANN et al., 2018; THAUER et al., 2008; WELTE;

DEPPENMEIER, 2014). In the SO.CET was yet noted the presence of Cyanobacteria-*Unidentified Chloroplast* (1.6%), belonging to the Cyanobacteria phylum, that are linked to the development of photosynthesis (KONG; RICHARDSON; HONG, 2017) in this zone. In the SL.CET, it was yet observed the high presence of *Decloromonas* (7.4%) and *Novosphingobium* (5.4%), but *Methanosaeta* (7.4%) also was one of the most abundant, confirming that the media filters also could drive the microbial communities. In this zone it is showed the beginning of the higher abundance of genus *Rhodoblastus* (4.9%), related as sulphur oxidizing bacteria (URAKAWA; DETTMAR; THOMAS, 2017) and highlighted (Figure 6) the presence of genera belong to Firmicutes phylum: *Eubacterium Rectale Group* (1.4%), *Faecalibacterium* (1.3%) and *Christensenellaceae R7 group* (0.6%). The Clostridia class are microorganisms found in anaerobic treatments and have been reported as being able to degrade a large number of organic compounds into hydrogen, acetate and carbon dioxide (WANG et al., 2018).

It is important to notice the reduction of sulphate in the CEvaT (Table 1), which could be explained by the presence of SRB, like *Desulfovibrio*, *Desulfomonile* and *Desulforhabdus* manly in the SL.CET and G2.CET zones of this subsystem. The relative low and similar abundance of these microorganisms in that zones, could be explained by the dominance of *Methanosaeta* genus, that in saturated and neutral pH condition, outcompete SRB on the consumption of acetate (ROYCHOWDHURY et al., 2018; YAN et al., 2018). In this case, the SRB could utilize the liable fermentation products (hydrogen, lactate and pyruvate) as electron donors, and thus reduce sulphate to sulphide (CHEN et al., 2016a; STEIN et al., 2007; STURMAN et al., 2008). The presence of *Desulfovibrio, Desulfomonile* and *Desulforhadus* suggests that the SRB could reduce sulphate using other fermentation products than acetate as electron donors. The rise (28.6%) of sulphide from bottom layer to top layer of CEvaT was inferred to be responsible for the appearance of SOB, mainly represented by *Rhodoblastus,* that increased 200% in abundance from SL.CET to FFG.CW. Consequently, the rise of SOB suggesting the decrease of sulphide (38.0%) from the effluent of CEvaT to the final zone of CW (Table 1 and Figure S3).

In the IFG.CW, the higher abundance of *Rhodoblastus* (9.3%), that have been reported as being able to oxidize sulphur under anaerobic conditions (PFENNIG, 1975; URAKAWA; BERNHARD, 2017; URAKAWA; DETTMAR; THOMAS, 2017), and the increase in the abundance of *Methanosaeta* (14.2%), that doubled in abundance from

SL.CET, could be the reason for this increase (44.1%) of sulphate from P3 to P4. Other interesting higher abundance in the IFG.CW were *Thauera* (5.3%), *Dechloromonas* (3.8%), *Candidatus Competibacter* (2.2%) and *Candidatus Koribacter* (2.1%). The *Thaurea, Dechloromonas* and *Candidatus Competibacter* have been reported as nitrate-reducing bacteria (MCILROY et al., 2014; SUN et al., 2018). The high abundance of Acidobacteria phylum in the IFG.CW, mainly represented by *Candidatus Koribacter*, also suggested the presence of methanotrophs (KIELAK et al., 2016; KOCH et al., 2008).

In the FFG.CW microorganisms belonging to the SOB and Methanotroph group were dominating (Figure 9). The relative abundance in this zone, identified using the V3-V4 region of 16SrRNA gene, were: *Rhodoblastus* (14.9%), *Methanosaeta* (7.2%), *Candidatus Koribacter* (5.0%), *Rhizobium* (2.5%), *Methylobacterium* (1.7%) *Desulfovibri*o (1.5%) and *Candidatus Competibacter* (1.5%), and for archaeal identified using the V4 region of 16SrRNA gene: *Candidatus Nitrosotalea* (4.3%), belong to the Thaumarchaeota phylum. The decrease by half in abundance of *Methanosaeta*, combined by the presence of SRB and the *Candidatus Koribacter* and *Methylobacterium*, that are involved in methane oxidation cycle (GILLIS et al., 2006; KIELAK et al., 2016), could be responsible to the observed sulphate decrease (55.6%) (Table 1). It has been proposed in the literature that one of anaerobic methane oxidation pathways is mediated by consortia of methane oxidizers and sulphate-reducing bacteria via "reverse methanogenesis" (CALLAGHAN, 2013). Sun et al. (2018) studying the microbial community in diverse paddy soils, environment similar a *wetlands*, found co-occurrence of sulphate reducing bacteria with methane oxidation microorganism. Moreover, other co-occurrence inferred the presence of methane oxidizing activity in our constructed wetlands, firstly Osborne and Haritos, (2018) investigating the phylogenetic analysis of Proteobacteria related to methanotrophs verified that *Rhodoblastus* could contain suitable membrane structures to support the metabolic process of methanotrophs, then Ho et al. (2016) studying biotic interactions in microbial communities in different sites (sediment from fresh water lake, rice paddy soil, grassland soil and others) reported the co-occurrence of methanol oxidizer, like *Methylobacterium,* and methanotroph in all sites, suggesting the methylotroph feed on the methanol derived from methane oxidation. In this way, we suggest that along the greywater flow the carbon source was converted from higher molecular weight to simpler substrate until methane and sulphide, supporting the shift on the dominance of archaea methanogens in the CEvaT for the methane and

sulphide oxidizing microorganisms in the CW that could be avoiding methane and sulphide release to the atmosphere.

Finally, it is important to mention some microorganism considered rare (<1%) in the EvaTAC system: *Geobacter (0.5%), Xhantobacter (0.7%), Thiobacillus (0.2%), Acidovorax (0.2%) and Variovorax (0.7%)*. The less abundant taxa could serve as a reservoir of genetic and functional diversity and/or buffer ecosystems against species loss or environmental change (BROWN et al., 2009). Thus, *Acidovorax*, *Variovorax* and *Xhantobacter,* which contain denitrifying and nitrogen fixation species (GILLIS et al., 2006; HYLANDER et al., 2014; ZHONG et al., 2014), could helped in the nitrogen cycle, *Geobacter* in the iron cycle (SUN et al., 2018) and *Thiobacillus*, well known sulphur oxidizing bacteria (CHEN et al., 2016a; POKORNA; ZABRANSKA, 2015), involved in the oxidation of sulphide.

## 3.5 Conclusion

The results suggest that each zone of EvaTAC is unique and could be important for the development of different biogeochemical cycles occurring inside the system. The impact of environmental condition on the microbial assemblages was revealed by CCAs and the microbial characterization confirms that the reduction and oxidation dynamics of C, S and N cycling occur in wetlands system. In the CEvaT subsystem, the lower ORP, the reduction of COD and sulphate and the increase of sulphide were associated to the methanogens (*Methanosaeta* and *Methanobacterium)* and SRB microorganism (*Desulfovibrio*, *Desulforhabdus* and *Desulfomonile*), confirming the complete methanogenesis and sulfidogenesis process. The occurrence of theses process also associated to a dominant specific group of bacteria, that convert complex to simpler substrate, identified in the downstream layer of the system (UF.AnC), suggesting that zones with specific microbiota are important for the system stability. In this way, the shifts in terminal electron acceptor with the products of anaerobic degradation and the rise of ORP, might have supported the higher abundance of genera involved in methane and sulphur oxidation (*Candidatus Koribacter, Methylobacterium, Rhodoblastus, Thiobacillus*) in CW subsystem. Therefore, the presence of these microorganisms can

avoid the methane and sulphide release to the atmosphere and improve the acceptance of onsite EvaTAC treatment system.

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## Supplementary material

# Spatial shift of microbial community and environmental condition in a constructed wetland system treating greywater

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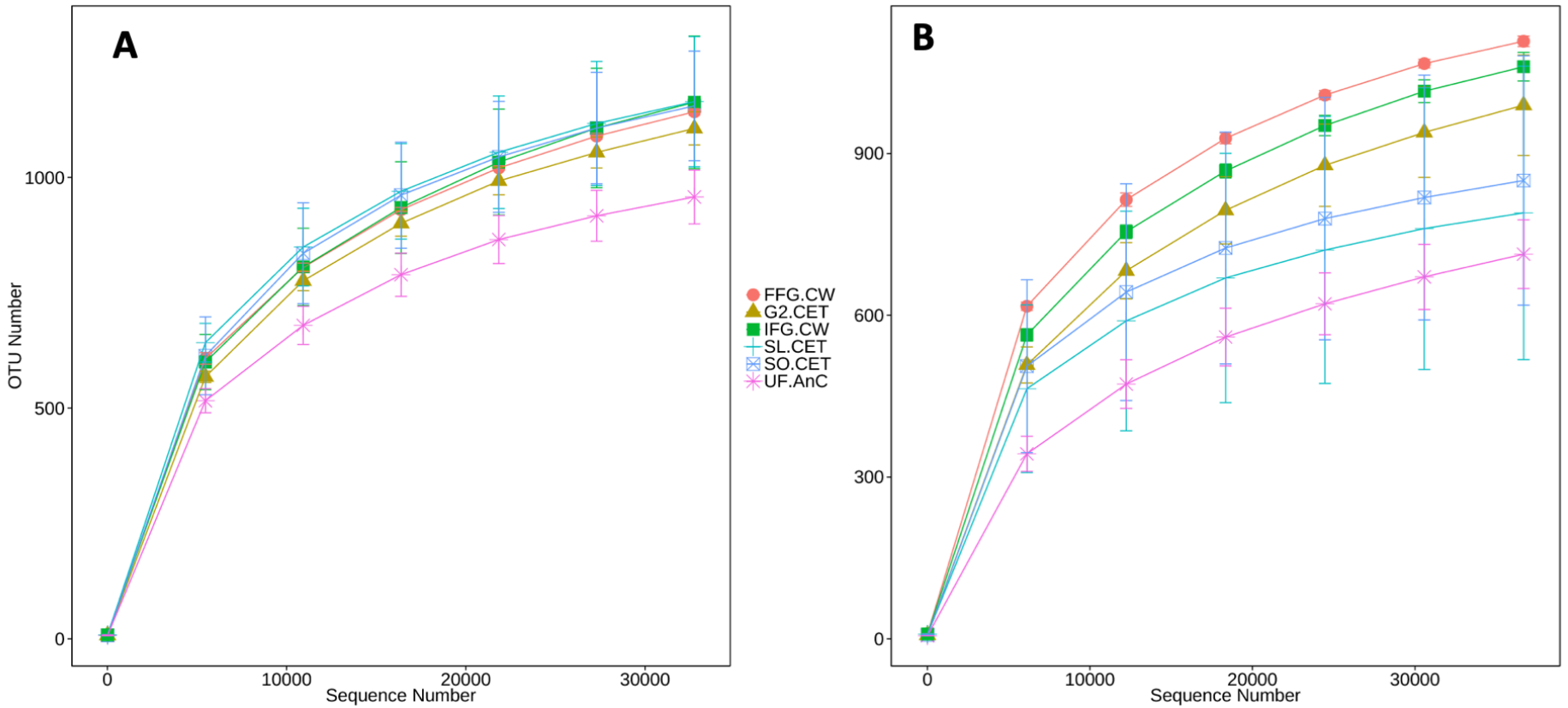


Figure S1. Rarefaction curves. Samples were composed of UF.AnC (bottom layer of CEvaT - sludge of AnC), G2.CET (middle layer of CEvaT – gravel), SO.CET (top layer of CEvaT – soil), SL.CET ( top layer of CEvaT – gravel), IFG.CW (middle layer of CW – gravel) and FFG.CW (middle layer of CW – gravel). For the constructed the model the 3 biological replicates were pooled. A – Bacteria and B - Archaea species diversity.

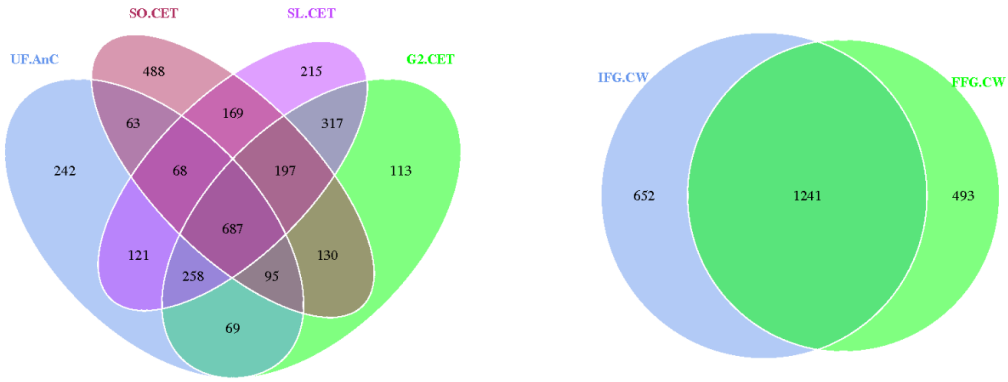


*Figure S2A. Venn diagrams to show the unique and shared OTUs for bacterial community.*

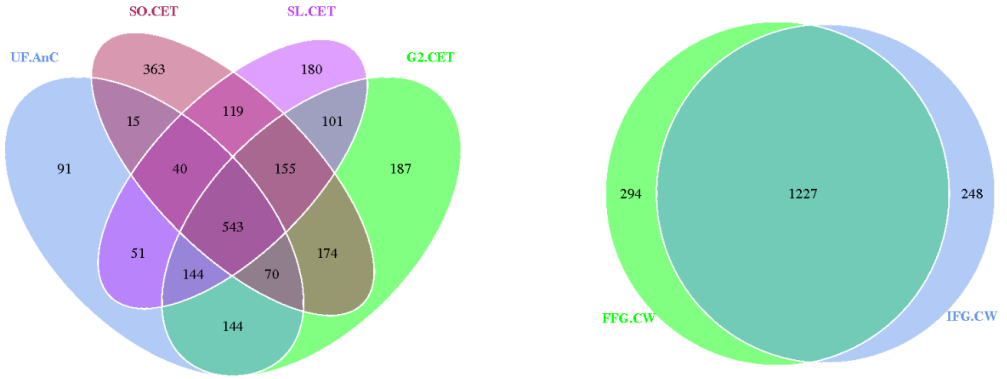


*Figure S2B. Venn diagrams to show the unique and shared OTUs for archaeal community.*

*Table S1. Alpha diversity indices of EvaTAC samples. Upper numbers were bacterial Indices values and lower numbers (with parentheses) were archaeal Indices values*

| Sample_name | **Community Diversity** | **Community Richness** | **Sequencing Depth** |
|---|---|---|---|
| | shannon | chao1 | goods_coverage |
| UF.AnC1 | 6.483 | 1.072.683 | 0.993 |
| | (3.809) | (1.040.276) | (0.993) |
| UF.AnC2 | 5.301 | 985.753 | 0.994 |
| | (3.839) | (822.000) | (0.995) |
| UF.AnC3 | 5.793 | 1.155.959 | 0.993 |
| | (3.893) | (916.669) | (0.994) |
| G2.1.CET | 5.283 | 1.200.521 | 0.993 |
| | (5.318) | (1.001.078) | (0.995) |
| G2.2.CET | 6.009 | 1.279.004 | 0.992 |
| | (5.517) | (1.177.531) | (0.993) |
| G2.3.CET | 5.902 | 1.313.530 | 0.991 |
| | (5.862) | (1.398.775) | (0.991) |
| SL1.CET | 7.342 | 1.346.890 | 0.993 |
| | (6.948) | (1.005.529) | (0.997) |
| SL2.CET | 7.370 | 1.083.917 | 0.994 |
| | (6.130) | (459.958) | (0.998) |
| SL3.CET | 6.546 | 1.469.459 | 0.991 |
| | (5.909) | (1.242.793) | (0.993) |
| SO1.CET | 6.126 | 1.382.199 | 0.993 |
| | (5.390) | (855.854) | (0.996) |
| SO2.CET | 5.017 | 1.140.048 | 0.992 |
| | (3.746) | (748.485) | (0.996) |
| SO3.CET | 6.434 | 1.336.000 | 0.993 |
| | (6.724) | (1.305.640) | (0.995) |
| IFG1.CW | 7.094 | 1.863.694 | 0.987 |
| | (6.864) | (1.186.544) | (0.994) |
| IFG2.CW | 6.031 | 1.256.197 | 0.992 |
| | (6.711) | (1.169.400) | (0.994) |
| IFG3.CW | 6.811 | 1.142.207 | 0.993 |
| | (6.849) | (1.399.019) | (0.992) |
| FFG1.CW | 6.926 | 1.330.091 | 0.992 |
| | (7.291) | (1.209.714) | (0.995) |
| FFG2.CW | 6.939 | 1.307.323 | 0.992 |
| | (7.301) | (1.244.122) | (0.994) |
| FFG3.CW | 7.053 | 1.322.832 | 0.991 |
| | (7.229) | (1.244.744) | (0.994) |

*Table S2 - The analysis of Similarity - ANOSIM*

| A - Bacteria | | |
|---|---|---|
| Group | R-value | P-value |
| FFG.CW-SL.CET | 0.6296 | 0.1 |
| SO.CET-SL.CET | 0.6667 | 0.1 |
| SO.CET-FFG.CW | 1 | 0.1 |
| IFG.CW-SL.CET | 0.5926 | 0.1 |
| IFG.CW-FFG.CW | 0.5185 | 0.1 |
| IFG.CW-SO.CET | 1 | 0.1 |
| G2.CET-SL.CET | 0.5556 | 0.1 |
| G2.CET-FFG.CW | 1 | 0.1 |
| G2.CET-SO.CET | 1 | 0.1 |
| G2.CET-IFG.CW | 1 | 0.1 |
| UF.AnC-SL.CET | 0.8148 | 0.1 |
| UF.AnC-FFG.CW | 1 | 0.1 |
| UF.AnC-SO.CET | 1 | 0.1 |
| UF.AnC-IFG.CW | 0.963 | 0.1 |
| UF.AnC-G2.CET | 1 | 0.1 |

| B - Archaea | | |
|---|---|---|
| Group | R-value | P-value |
| UF.AnC-IFG.CW | 1 | 0.1 |
| SO.CET-IFG.CW | 1 | 0.1 |
| SO.CET-UF.AnC | 1 | 0.1 |
| FFG.CW-IFG.CW | 1 | 0.1 |
| FFG.CW-UF.AnC | 1 | 0.1 |
| FFG.CW-SO.CET | 1 | 0.1 |
| SL.CET-IFG.CW | 1 | 0.1 |
| SL.CET-UF.AnC | 1 | 0.1 |
| SL.CET-SO.CET | 1 | 0.1 |
| SL.CET-FFG.CW | 1 | 0.1 |
| G2.CET-IFG.CW | 1 | 0.1 |
| G2.CET-UF.AnC | 1 | 0.1 |
| G2.CET-SO.CET | 1 | 0.1 |
| G2.CET-FFG.CW | 1 | 0.1 |
| G2.CET-SL.CET | 0.5556 | 0.1 |

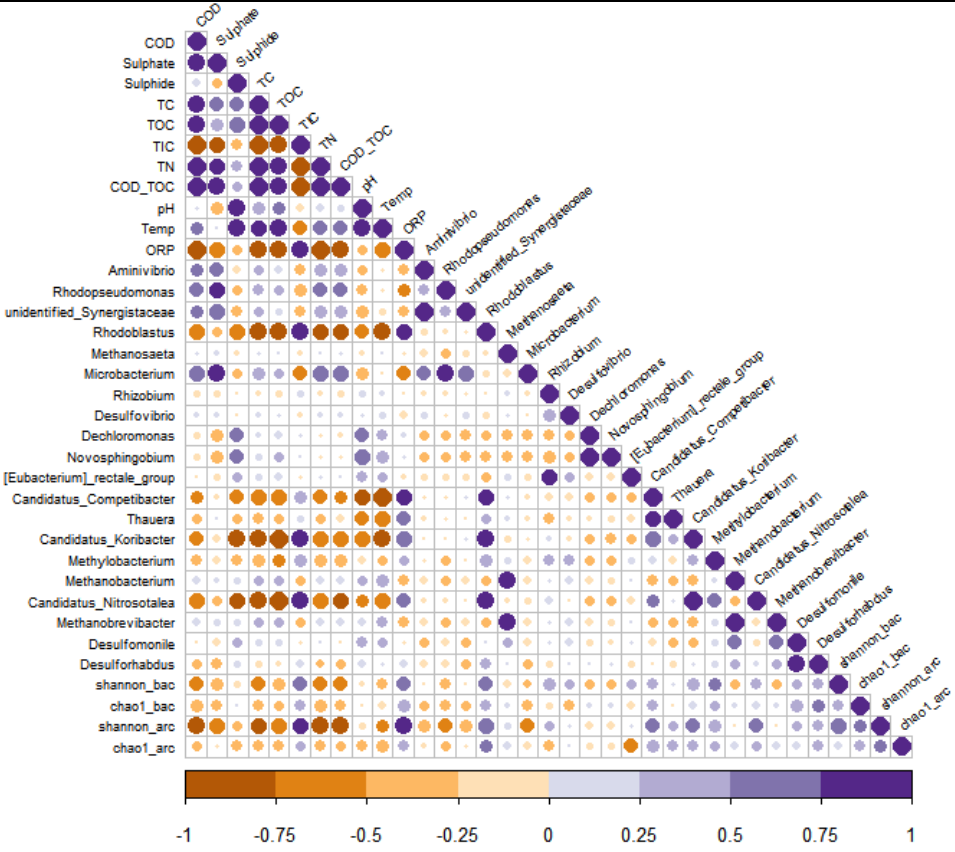


Figure S3A - Pearson Correlation between environmental parameters, alpha diversity Indices and relative abundance of top 20 microbial genera.

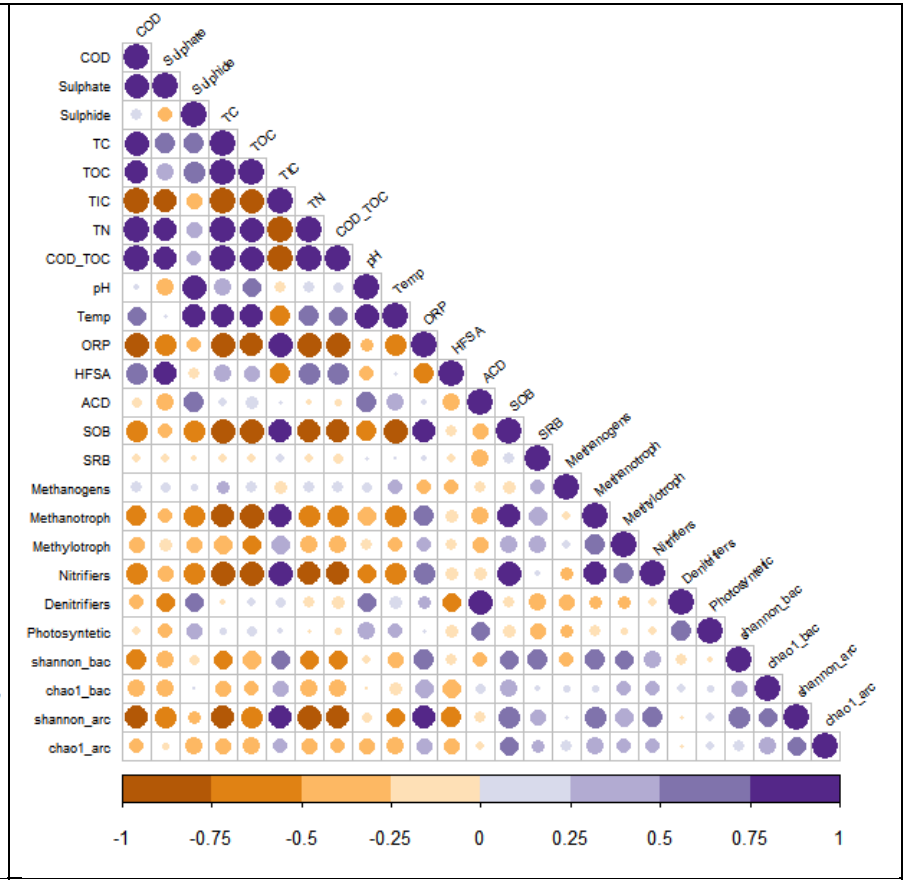


Figure S3A - Pearson Correlation between environmental parameters, alpha diversity indices and relative abundance of functional group

Table S3 - Genera of microorganism belonging in the specific functional group.

| Group | Genus | Reference | Group | Genus | Reference |
|---|---|---|---|---|---|
| Hydrolysis, Fermentative, Syntrophy and Acetogenesis (HFSA) | *Aminivibrio* | (Militon et al., 2015; Morató et al., 2014; Veeravalli et al., 2016; Wang et al., 2018) | Methanogens | *Methanosaeta* | (Chen et al., 2013; Enzmann et al., 2018; Sun et al., 2018; Zhang et al., 2018) |
| | *unidentified_Synergistaceae* | | | *Methanosarcina* | |
| | *Cloacibacillus* | | | *Methanomethylovorans* | |
| | *Aminomonas* | | | *Methanospirillum* | |
| | *Aminiphilus* | | | *Methanobrevibacter* | (Enzmann et al., 2018; Jing et al., 2013; Thauer et al., 2008) |
| | *Lactivibrio* | | | *Methanobacterium* | |
| | *Thermovirga* | | Methanotrophy | *Methylobacter* | (Osborne and Haritos, 2018; Prasse et al., 2015; Truu et al., 2009; Urakawa et al., 2017) |
| | *[Eubacterium]_rectale_group* | (Tang et al., 2011; Wang et al., 2018; Zeigler, 2016; Zhou et al., 2018) | | *Methylocaldum* | |
| | *Faecalibacterium* | | | *Methylococcus* | |
| | *Paenisporosarcina* | | | *Methylomicrobium* | |
| | *Christensenellaceae_R-7_group* | | | *Methylomonas* | |
| | *Ruminococcaceae_UCG-002* | | | *Candidatus_Koribacter* | (Kielak et al., 2016; Koch et al., 2008) |
| | *Roseburia* | | | *Candidatus_Methylomirabilis* | (Ettwig et al., 2010) |
| | *[Eubacterium]_coprostanoligenes_group* | | | *Pleomorphomonas* | (Bao et al., 2014; Dedysh et al., 2003) |
| | *Acinetobacter* | (Adrados et al., 2014; He et al., 2016; Hylander et al., 2014) | Methylotroph | *Methylobacterium* | (Knief et al., 2010; Tang et al., 2011; Urakawa et al., 2017) |
| | *Microbacterium* | (Anderson et al., 2011; Wang et al., 2018) | | *Methylophilus* | |
| | *vadinBC27_wastewater-sludge_group* | (Adrados et al., 2014; Dai et al., 2017; Yan et al., 2018) | | *Methyloversatilis* | |
| | *Prevotella_9* | | | *Candidatus_Methylospira* | (Ho et al., 2016) |
| | *Bacteroides* | | Nitrifiers | *Nitrosospira* | (Fan et al., 2016; Pelissari et al., 2017a) |
| | *Sediminibacterium* | | | *Nitrosomonas* | |
| | *Rhodopseudomonas* | (Adessi et al., 2016; Koku et al., 2002; Mckinlay and Harwood, 2011; Tang et al., 2011) | | *Nitrobacter* | (Zuma et al., 2009) |
| | *Syntrophus* | (Kersters et al., 2006; Zhang et al., 2018) | | *unidentified_Nitrospiraceae* | (Zhong et al., 2014) |
| Aromatic Compound Degraders (ACD) | *Brevibacterium* | (Kielak et al., 2016) | | *Candidatus_Nitrosotalea* | (Bouali et al., 2013; Zhang et al., 2018) |
| | *Arthrobacter* | | | *Candidatus_Nitrososphaera* | |
| | *Alcaligenes* | (He et al., 2016; Zhou et al., 2018) | | *Candidatus_Nitrosoarchaeum* | |
| | *Alicycliphilus* | | | *unidentified_Thaumarchaeota* | |
| | *Ideonella* | | Denitrifiers | *Opitutus* | (Pelissari et al., 2017b) |
| | *Ramlibacter* | | | *Dechloromonas* | (Adrados et al., 2014; Sun et al., 2018; Wu et al., 2016) |
| | *Massilia* | | | *Thauera* | |

| Group | Genus | Reference | Group | Genus | Reference |
|---|---|---|---|---|---|
| Aromatic Compound Degraders (ACD) | *Mycobacterium* | (Chipasa and Mędrzycka, 2006; Seo et al., 2009) | Denitrifiers | *Denitratisoma* | (Qian et al., 2015; Rodríguez-Martínez et al., 2016) |
| | *Rhodococcus* | | | *Zoogloea* | |
| | *Staphylococcus* | (Tommaso et al., 2002) | | *Propionivibrio* | |
| | *Novosphingobium* | (Gan et al., 2013; Kersters et al., 2006; Takeuchi et al., 2001) | | *Azospira* | |
| | *Sphingomonas* | | | *Variovorax* | (Zhong et al., 2014) |
| | *Sphingobium* | | | *Acidovorax* | |
| | *Sphingopyxis* | | | *Hydrogenophaga* | |
| | *Erythrobacter* | | | *Pseudomonas* | (Hylander et al., 2014) |
| | *Pseudoxanthomonas* | (He et al., 2016) | | *Bosea* | (Bao et al., 2014) |
| Sulphate Reducing Bacteria (SRB) | *Desulfomonile* | (Aida et al., 2014; Hao et al., 2014) | | *Xanthobacter* | (Kersters et al., 2006) |
| | *Smithella* | | | *Candidatus_Competibacter* | (McIlroy et al., 2014) |
| | *Desulfobacca* | | Nitrogen-fixing | *Rhizobium* | (Aya et al., 2015) |
| | *[Desulfobacterium]_catecholicum_group* | (Fawzy et al., 2015) | Sulphur Oxidizing Bacteria (SOB) | *Thiobacillus* | (Chen et al., 2016; Pokorna and Zabranska, 2015) |
| | *Desulfitobacterium* | (Villemur et al., 2006) | | *Roseobacter_clade_CHAB-I-5_lineage* | (Luo et al., 2013) |
| | *Desulforegula* | (Chen et al., 2016; Stein et al., 2007; Sturman et al., 2008) | | *Sulphuricurvum* | (Chen et al., 2016) |
| | *Desulfovibrio* | | | *Rhodoblastus* | (Pfennig, 1975; Urakawa et al., 2017;Urakawa and Bernhard, 2017) |
| | *Desulfomicrobium* | | | | |
| | *Desulforhabdus* | (Plugge et al., 2011; Urakawa et al., 2017) | Photosynthetic | *Cyanobacteria-unidentified_Chloroplast* | (Kong et al., 2017) |
| | *Syntrophobacter* | | | | |
| | *Desulfovirga* | | | *Prochlorococcus* | (Brown et al., 2009) |

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# CAPÍTULO 4 – CONCLUSÃO GERAL

Os dois EvaTACs estudados apresentaram condições anaeróbias com o potencial redox nas zonas do sistema variando entre -342,9 e -240,4 mV. Com essa condição predominaram os processos anaeróbios de metanogênese e sulfetogênese. No estudo em escala real (EvatAC 1, descrito no capítulo 2) as bactérias redutoras de sulfato (BRS) foram mais abundantes do que as arqueias metanogênicas (AM) em todas as zonas estudadas, por outro lado no estudo realizado em escala piloto (EvaTAC 2, descrito no capítulo 3) as AM foram mais abundantes que as BRS em todas as zonas do sistema. A competição entre esses dois grupos de microrganismos pode ter sido mediada pelas seguintes razões: diferença de potencial redox, relação DQO/$SO_2^{-4}$ e presença de sulfeto dissolvido nas águas cinza. No EvaTAC 2, dominado pelas arqueias metanogênicas, a condição se manteve mais anaeróbia, com o potencial redox sendo em media 20% mais baixo do que no EvaTAC 1. O sistema 2 apresentou a relação DQO/$SO_2^{-4}$ sempre acima de 3,0, condição em que as AM superam as BRS, além de ter sido encontradas maiores abundâncias de bactérias que oxidam o sulfeto nas zonas do sistema, o que pode ter evitado a toxicidade às arqueias metanogênicas, principalmente às *Methanosaetas*. No entanto, mesmo com um grupo sendo mais abundante que o outro em determinado sistema, os processos de metanogênese e sulfetogênese coexistiram nos EvaTACs e auxiliaram na degradação de matéria orgânica.

Os processos de metanogênese e sulfetogênese foram relacionados com a comunidade microbiana encontrada na câmara de digestão anaeróbia (Cdig). Nas duas Cdigs foram encontrados grupos de microrganismos específicos associados à conversão de substratos complexos para mais simples (primeiros passos da digestão anaeróbia). Essa abundância de microrganismos específicos, menor diversidade e riqueza da comunidade microbiana, foram interessantes para o desenvolvimento do sistema, uma vez que determinados grupos de microrganismos são imprescindíveis para manter a funcionalidade do sistema e a consequente degradação de poluentes. Dessa forma, sugere-se que a câmara de digestão anaeróbia e o grupo de microrganismos encontrado nela, foram responsáveis por assimilar as cargas orgânicas afluentes ao sistema e evitar grandes choques aos grupos de microrganismos das demais zonas.

O potencial redox, em ambos os sistemas estudados, apresentou forte influência na comunidade de microrganismos, sendo inversamente proporcional à presença de matéria orgânica (DQO, TOC, TC) e diretamente proporcional a diversidade e riqueza das comunidades microbianas. Nesse sentido, as zonas com maiores diversidades e riqueza de espécies foram

encontradas nos *wetlands* construídos, principalmente na zona final, próximo à saída do sistema. Nessas zonas, para ambos EvaTAC 1 e 2, foram encontradas maiores abundâncias de microrganismos que podem oxidar o metano e o sulfeto, evitando assim que esses gases se desprendam para a atmosfera, prevenindo a ocorrência de maus odores e consequentemente aumentando a aceitação do sistema e também prevenindo o aumento do aquecimento global. Com a identificação desses microrganismos pôde se confirmar a presença de zonas de redução e oxidação dentro dos EvaTACs.

Portanto, com essas constatações, algumas configurações podem ser sugeridas para um melhor funcionamento de *wetlands* construídos. Primeiramente compartimentos como a câmara de digestão anaeróbia são desejáveis, pois podem conter os impactos da carga orgânica e formar uma comunidade microbiana específica para degradação de substratos mais complexos para mais simples, possibilitando o desenvolvimento de outros tipos de microrganismos nas demais zonas, além de auxiliar em questões de entupimento. O sistema de fluxo em batelada também se mostra uma importante ferramenta para maior variação no potencial redox e consequentemente pode auxiliar em fatores como diversidade e riqueza das comunidades microbianas. No entanto zonas mais anaeróbias são necessárias na parte inicial do sistema para realização completa da digestão anaeróbia, principalmente via metanogênese. Dessa forma, subsistemas como CEvaTs, dimensionadas para ficar a maior parte do tempo saturadas podem auxiliar nesse ponto. Por outro lado, subsistemas como CW, podem ser dimensionados com maiores comprimentos, que podem ajudar no aumento do potencial redox, redução da matéria orgânica e com isso apresentar zonas com maior potencial de oxidação, que serão importantes para conversão de sulfetos, metano e realização dos processos de nitrificação e desnitrificação, evitando assim maus odores e aumentando a aceitação em escala domiciliar.