UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Microbiologia

Joyce da Cruz Ferraz Dutra

AMEAÇA BIOCORROSIVA: monitoramento da biocorrosão e diagnóstico metagenômico em campos de extração de petróleo

Belo Horizonte
2023

Joyce da Cruz Ferraz Dutra

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Federal de Minas Gerais como requisito parcial à obtenção do título de Doutora em Microbiologia.

Orientador: Vasco Ariston de Carvalho Azevedo

Coorientador: Aristóteles Góes-Neto.

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INSTITUTO DE CIÊNCIAS BIOLÓGICAS
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ATA DE DEFESA DE TESE

ATA DA DEFESA DE TESE DE **JOYCE DA CRUZ FERRAZ DUTRA**

Nº REGISTRO: **2019709435**

Às 08:00 horas do dia **28 de junho de 2023**, reuniu-se, por via remota, a Comissão Examinadora composta pelos Drs. Rodrigo Dias de Oliveira Carvalho (Universidade Federal da Bahia (UFBA), Lucymara Fassarella Agnez Lima (Universidade Federal do Rio Grande do Norte (UFRN), Ana Paula Trovatti Uetanabaro (Universidade Estadual de Santa Cruz - UESC-BA), Selma Soares de Oliveira (Universidade Federal do Rio de Janeiro (UFRJ), Prof. Dr. Vasco Ariston de Carvalho Azevedo (Orientador) e o Prof. Dr. Aristóteles Góes Neto (Coorientador), para julgar o trabalho final **"AMEAÇA BIOCORROSIVA: Monitoramento da biocorrosão e diagnóstico metagenômico em campos de extração de petróleo"** da aluna **Joyce da Cruz Ferraz Dutra**, requisito final para a obtenção do Grau de **DOUTORA EM CIÊNCIAS BIOLÓGICAS: MICROBIOLOGIA**. Abrindo a sessão, o Presidente da Comissão, Prof. Dr. Vasco Ariston de Carvalho Azevedo, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para a apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. A candidata foi considerada **APROVADA**. O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ata, que será assinada por todos os membros participantes da Comissão Examinadora. A candidata tem 60 (sessenta) dias, a partir desta data, para entregar a versão final da tese ao Programa de Pós-graduação em Microbiologia da UFMG e requerer seu diploma.

Membros da Banca:

Prof. Dr. Rodrigo Dias de Oliveira Carvalho

Profa. Dra. Lucymara Fassarella Agnez Lima

Profa. Dra. Ana Paula Trovatti Uetanabaro

Profa. Dra. Selma Soares de Oliveira

De acordo:

Prof. Dr. Vasco Ariston de Carvalho Azevedo

(Orientador)

Prof. Dr. Aristóteles Góes-Neto

(Coorientador)

Prof. Dr. Daniel de Assis Santos

(Coordenador do Programa de Pós-graduação

em Microbiologia)

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RESUMO

A corrosão influenciada por microrganismos (CIM) é um problema significativo nos campos de petróleo, representando de 10 a 20% das falhas ocorridas. Prever e monitorar esse processo é um desafio devido à variação dos biofilmes dentro dos sistemas industriais e à sua propensão à corrosão localizada, a qual que é mais difícil de monitorar em comparação à corrosão uniforme, apresentando uma série de desafios. Assim, este estudo propôs um pacote tecnológico metagenômico de monitoramento de campos de petróleo, prático e aplicável em processos de rotina. A revisão sistemática mostrou que a composição da comunidade microbiana das regiões petrolíferas avaliadas inclui grupos capazes de desempenhar diversos tipos de metabolismo, como o sintrófico, oxidativo e a produção de metabólitos corrosivos, dependendo das características físico-químicas do ambiente. Nos ensaios de padronização dos métodos de extração de DNA, o kit comercial específico para a em extração de DNA para de solos mostrou-se superior em termos de rendimento, pureza e integridade do DNA em comparação com outros kits comerciais avaliados em amostras de água produzida e óleo. A abordagem de amplicon 16S rRNA foi mais eficiente na detecção de organismos com abundância acima de 1%, comparada ao shotgun, e revelou vias metabólicas coerentes com o ambiente da água produzida em campos de petróleo, tornando-a a melhor opção para o monitoramento nesse contexto. O protocolo adaptado de Yu & Morrison (2004), de baixo custo foi capaz de obter DNA de qualidade satisfatória, resultando em sequenciamentos de alta qualidade Phred, sendo adequado para o monitoramento na indústria petrolífera. Observamos também que o sequenciamento de amplicon 16S rRNA aplicado em DNA de amostras extraídas diretamente do ambiente (amostras metagenômicas), combinada com a caracterização físico-química, oferece uma representação mais precisa no monitoramento dos grupos microbianos em instalações da indústria do petróleo, em comparação com a aplicação em comunidades enriquecidas com meios de cultivos específicos para os grupos da CIM. Por fim, o monitoramento de dutos utilizados no transporte de produtos e resíduos de petróleo indicou que a forma predominante de corrosão observada foi a corrosão por pites, possivelmente causada por bactérias produtoras de ácidos (BPAs) que utilizam predominantemente o metabolismo de degradação de 1,2 propanediol. Concluímos que os métodos de extração de DNA desenvolvidos na presente tese são aplicáveis e representam avanços significativos no monitoramento de rotina da corrosão microbiologicamente influenciada (CIM) na indústria petrolífera.

Palavras-chave: campos de petróleo. CIM; corrosão por pites; biocorrosão; bactérias produtoras de ácidos; monitoramento; metagenômica; shotgun; amplicon 16S rRNA.

ABSTRACT

A corrosion influenced by microorganisms (CIM) is a significant issue in oil fields, accounting for 10 to 20% of failures that occur. Predicting and monitoring this process is challenging due to the variability of biofilms within industrial systems and their susceptibility to localized corrosion, which is harder to monitor compared to uniform corrosion, presenting several challenges. Thus, this study proposed a metagenomic technological package for oil field monitoring that is practical and applicable in routine processes. The systematic review showed that the microbial community composition in evaluated oil regions includes groups capable of various types of metabolism, such as syntrophic, oxidative, and production of corrosive metabolites, depending on the physicochemical characteristics of the environment. In the standardization tests of DNA extraction methods, the specific commercial kit for soil DNA extraction proved superior in terms of yield, purity, and DNA integrity compared to other commercial kits evaluated in produced water and oil samples. The 16S rRNA amplicon approach was more efficient in detecting organisms with abundances above 1%, compared to shotgun sequencing, and revealed coherent metabolic pathways with the produced water environment in oil fields, making it the best option for monitoring in this context. The adapted low-cost protocol from Yu & Morrison (2004) was capable of obtaining satisfactory quality DNA, resulting in high-quality Phred sequencing, suitable for monitoring in the oil industry. We also observed that 16S rRNA amplicon sequencing applied to DNA directly extracted from the environment (metagenomic samples), combined with physicochemical characterization, provides a more accurate representation in monitoring microbial groups in oil industry facilities, compared to application in communities enriched with specific culture media for CIM groups. Finally, the monitoring of pipelines used for transporting petroleum products and waste indicated that the predominant form of corrosion observed was pitting corrosion, possibly caused by acid-producing bacteria (APBs) that predominantly utilize the 1,2-propanediol degradation metabolism. We conclude that the DNA extraction methods developed in this thesis are applicable and represent significant advances in routine monitoring of microbiologically influenced corrosion (CIM) in the oil industry.

Keywords: oil fields; CIM. pitting corrosion; biocorrosion; acid-producing bacteria. monitoring; metagenomics; shotgun; 16S rRNA amplicon.

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ORGANIZAÇÃO DO TRABALHO

Essa tese abrange a introdução, objetivos, justificativa e relevância, referencial teórico e quatro capítulos dedicados a temas específicos. As conclusões finais e perspectivas futuras encerram o trabalho de forma abrangente. As seções a serem abordadas nesta tese serão explicadas em detalhes a seguir:

I - Introdução Geral- A introdução fornece uma visão geral da tese, estabelecendo a contextualização e o problema de pesquisa relacionado à dificuldade de monitorar a biocorrosão em indústrias de petróleo.

II - Objetivos da Tese – Será destacado o objetivo principal da tese, (objetivo geral) e os objetivos específicos, que serão delineados e seguidos para atingir o objetivo principal proposto.

III - Justificativa e Relevância – Neste tópico serão abordadas as motivações e a importância do desenvolvimento desta tese. Será destacada a significância da parceria entre a Universidade Federal de Minas Gerais e a Petrobras, no âmbito de viabilizar o intercâmbio de conhecimentos e impulsionar avanços significativos no monitoramento da corrosão microbiologicamente influenciada (CIM) na indústria petrolífera.

IV – Referencial Teórico – Essa revisão teórica analisa criticamente teorias, conceitos e estudos anteriores relevantes para o tema da biocorrosão em indústrias de petróleo. Foram abordados tópicos importantes, como composição e densidade do petróleo, consumo global e produção crescente, produtos gerados em campos de petróleo, corrosão microbiologicamente influenciada (CIM) e seus mecanismos, técnicas de monitoramento da biocorrosão em oleodutos, técnicas de amostragem e metagenômica.

V - Capítulo I:- Este estudo, intitulado "Corrosion-influencing microorganisms in systematic review, analysis, and scientific petroliferous regions on a global scale: synthesis of 16S rRNA amplicon metagenomic studies", publicado na revista PeerJ em janeiro de 2023, teve como objetivo compilar informações sobre estudos de biologia molecular em plataformas NGS (*next-generation sequencing*) em campos de petróleo e extrair informações sobre os perfis microbianos das regiões petrolíferas do mundo e as funções metabólicas principais do microbioma.

VI - Capítulo II: Aborda a padronização dos experimentos em três etapas. A primeira etapa consiste em um experimento piloto que testa diferentes kits comerciais para extração de DNA de água produzida e petróleo bruto. A segunda etapa envolve a verificação da correlação entre duas abordagens (shotgun e a partir do 16S rRNA) em relação à taxonomia e às funções

metabólicas de microorganismos encontrados em amostras de água produzida em campos de petróleo. A terceira etapa propõe testar um protocolo adaptado de baixo custo para o monitoramento da CIM em campos de petróleo, usando a abordagem 16S rRNA. O estudo correspondente a essa etapa foi intitulado "CostSaver DNA: a simpler and cost-effective DNA extraction protocol for multiple sample sources for metagenome study" e foi submetido à revista "Memórias do Instituto Oswaldo Cruz".

VII- Capítulo III: Realiza análise de amplicom de 16S rRNA para investigar a variação espacial na estrutura das comunidades bacterianas e arqueas em água produzida e sua correlação com as características físico-químicas do ambiente e com cultivos de bactérias redutoras de sulfato (BRS), bactérias produtoras de ácidos (BPA) e bactérias anaeróbias totais (BANT). O estudo, intitulado "Effective Biocorrosive Control in Oil Industry Facilities: 16S rRNA Gene Metabarcoding for Monitoring Microbial Communities in Produced Water", foi publicado na revista "Microorganisms".

VIII - Capítulo IV – Monitoramento de dutos de transporte de compostos petrolíferos através da implantação de cupons metálicos nas paredes internas dos dutos. Assim, avaliar o tipo de corrosão gerada nesses dutos e comparar a taxonomia e as funções metabólicas da comunidade de biofilme residente no duto de maior taxa de corrosão por pites com a comunidade planctônica de um tanque de armazenamento que contém o mesmo tipo de resíduo circulante. Este estudo foi intitulado “Exploring the cause of biocorrosion in petroleum pipelines through taxonomic and functional study of the 16S rRNA gene in biofilms”, com pretensão de submissão para a revista “Fuel”.

X - Conclusões Gerais: sintetiza as principais descobertas e tecnologia desenvolvida nessa tese.

XI - Perpectivas: são apresentados os passos seguintes a essa tese.

XII - Anexos: material complementar à tese.

XIII - Apêndice: produção científica entre (2019 e 2023) que, apesar de não terem sido temas da tese, foram importantes para o desenvolvimento e maturidade acadêmica.

1 INTRODUÇÃO GERAL

O petróleo bruto é originado a partir da deposição de matéria orgânica de animais e vegetais, abrigada à margem marítima há milhões de anos. É o recurso energético mais utilizado nos dias atuais, com crescente aumento na produção mundial (Lisboa, 2021). No entanto, a produção do petróleo com menores prejuízos ambientais e financeiros, ainda é um desafio, o que pode gerar impactos ambientais e tornar o produto mais caro para o consumidor final. Assim, é importante analisar cada etapa de produção, visando ao melhor desempenho produtivo e à prevenção dos possíveis problemas operacionais, existindo diversificados processos que podem desencadear falhas na infra-estrutura e transporte desse produto. Um processo importante é a corrosão influenciada por microrganimos (CIM).

Os microrganismos estão presentes em praticamente todos os ambientes relacionados à indústria de petróleo e gás que contêm ou interagem com água livre. Esses ambientes variam desde a rede de poros nas rochas dos reservatórios de petróleo, até as instalações e equipamentos de superfície e subsuperfície que constituem a infraestrutura da indústria de petróleo e gás, tais como poços injetores, poços produtores, dutos, válvulas, membranas, tanques, bombas, separadores, unidades de tratamentos de água e de efluentes e torres de resfriamento (Ollivier & Magot, 2005; Waldow, 2018). As atividades microbianas que ocorrem nos ambientes da indústria de petróleo e gás podem gerar benefícios (i.e., biorremediação) ou prejuízos (i.e., biocorrosão), dependendo das espécies de microrganismos e das condições ambientais predominantes (Augustinovic et al., 2012).

A Corrosão Influenciada por Microrganismos (CIM) se refere à corrosão que é afetada pela presença e atividade de microrganismos, geralmente envolve o estabelecimento de biofilmes que iniciam e/ou aceleram processos corrosivos de natureza eletroquímica (Videla, 1996). A CIM é um processo difícil de prever e monitorar devido à heterogeneidade da distribuição dos biofilmes no interior dos sistemas industriais e por levar à corrosão localizada, cujo monitoramento é bem mais desafiador do que a corrosão uniforme (Little & Lee, 2007; Roche, 2007).

Os custos anuais globais com a corrosão de dutos e outros equipamentos industriais para a produção e o transporte de petróleo e gás foram calculados em 2,5 trilhões de dólares por ano, segundo o estudo *"NACE International's impact breaks new ground in the study of corrosion management"*, e se estima que entre 10 e 20% das ocorrências de corrosão envolvem CIM (Machuca & Salgar-Chaparro, 2019). Dentre as despesas operacionais para mitigação da CIM, incluem-se custos operacionais com limpeza mecânica, passagem de *pigs* (*pipeline inspection gauge*), injeção de produtos químicos (biocidas, inibidores da corrosão, sequestrantes de H_2S),

inspeção de linhas, e realização de análises físico-químicas e microbiológicas (Skovhus et al., 2017).

O método de detecção e quantificação de microrganismos mais empregados pela indústria de petróleo e gás é o método do Número Mais Provável (NMP), o qual consiste em um método dependente de cultivo que se baseia no princípio da diluição até extinção (Bhagobaty, 2014; Waldow, 2018). No entanto, o NMP possui diversas limitações, tais como a demora em obtenção de resultados e o grande volume de trabalho necessário para a preparação e a inoculação dos kits de quantificação (Eckert & Skovhus, 2018). Além disso, estima-se que os microrganismos "cultiváveis" em amostras ambientais não ultrapassam 1% do total das linhagens microbianas realmente presentes (Pedrós-Alió, 2011).

Os Métodos Microbiológicos Moleculares (MMM) são métodos independentes de cultivo que têm atraído o interesse da indústria de petróleo e gás para o monitoramento das comunidades microbianas. Esses métodos se baseiam em biomarcadores, tais como a adenosina trifosfato (ATP), o ácido ribonucléico (RNA) e o ácido desoxiribonucléico (DNA), proteínas e metabólitos (Skovhus et al., 2017). O advento dos MMM na indústria de petróleo e gás tornou possível a obtenção de um retrato mais abrangente e preciso das comunidades microbianas presentes nos diferentes ambientes que compõem essa indústria (Larsen et al., 2009).

O termo "metagenômica", utilizado pela primeira vez em 1998, se refere ao sequenciamento de moléculas de DNA extraídas diretamente de amostras ambientais, permitindo uma investigação genômica das comunidades microbianas, sem a necessidade de cultivo prévio em laboratório (Handelsman et al., 1998). Por muitos anos, desde a sua introdução, a análise metagenômica baseava-se na extração de DNA comunitário de uma amostra, utilização de enzimas de restrição para clivar as moléculas de DNA, seguida de clonagem de segmentos de DNA em vetores e varredura dos insertos nos milhares de clones das bibliotecas metagenômicas para explorar novos produtos e atividades biológicas (Handelsman et al., 1998).

O avanço das tecnologias de Sequenciamento de Nova Geração (do inglês *next-generation sequencing*, ou NGS), juntamente com o aprimoramento dos programas de análise de bioinformática, tem possibilitado um aumento significativo no número de genomas e metagenomas sequenciados a baixo custo, contribuindo assim para a ampla adoção e o aperfeiçoamento da metagenômica (Scholz et al., 2012; Stark et al., 2014). No contexto do sequenciamento de DNA de amostras ambientais, duas abordagens comumente utilizadas são o shotgun e o 16S rRNA.

A abordagem de metagenômica shotgun consiste no sequenciamento de todo o DNA

presente em uma amostra ambiental, sem amplificação prévia de uma região taxonômica específica. Em seguida, os reads sequenciados são analisados utilizando bancos de dados de metagenômica como referência, visando atribuir cada read a um táxon específico (Laudadio et al., 2018; Quince et al., 2017; Truong et al., 2015). Por outro lado, a técnica de metagenômica de amplicon 16S rRNA envolve a amplificação prévia de regiões genômicas específicas, como o gene 16S rRNA para bactérias e archaeas, seguido do sequenciamento em larga escala (Klindworth et al., 2013; Laudadio et al., 2018; Quince et al., 2017).

No entanto, a implantação de tecnologias moleculares continuam sendo um desafio nos campos de petróleo, devido à carência de um pacote tecnológico específico, economicamente viável e de aplicação prática na rotina de monitoramento da biocorrosão. Diante desse cenário, o objetivo principal do presente estudo é desenvolver um pacote tecnológico de monitoramento da CIM, baseados em metagenômica, para serem aplicados em indústrias petrolíferas com histórico de corrosão.

1.1 OBJETIVO GERAL

Desenvolver um pacote tecnológico metagenômico padronizado para monitoramento da CIM, em campos de petróleo com histórico de corrosão.

1.1.1 Objetivos específicos

I Sintetizar o conhecimento disponível na literatura sobre o uso da biologia molecular em campos de petróleo, com foco na análise dos perfis microbianos característicos de diversas regiões petrolíferas globais e nas principais funções metabólicas do microbioma associado, por meio de uma revisão sistemática;

II Definir diretrizes para padronização dos processos de extração de DNA dos materiais resultantes da atividade petrolífera;

III Analisar e contrastar as classificações taxonômicas e as funções metabólicas presentes nas abordagens de shotgun e amplicon 16S rRNA em amostras de DNA coletadas de campos petrolíferos, buscando identificar correlações entre essas metodologias;

IV Avaliar a eficácia de um protocolo de baixo custo em amostras coletadas de campos de petróleo, visando obter um melhor entendimento de suas vantagens e benefícios.

V Utilizar o sequenciamento de amplicon 16S rRNA para explorar a variação espacial na composição das comunidades bacterianas e archaeas em amostras de água produzida, investigando sua interação com as propriedades físico-químicas do ambiente. Além disso, estabelecer correlações entre essas comunidades e cultivos de bactérias redutoras de sulfato (BRS), bactérias produtoras de ácidos (BPA) e bactérias nitrificantes anaeróbias (BANT).

VI Analisar o padrão de corrosão em dutos de transporte de compostos e resíduos de petróleo, e contrastar a classificação taxonômica e as funções metabólicas da comunidade de biofilme presente no duto com maior incidência de corrosão por pites, em relação à comunidade planctônica em um tanque de armazenamento que contém o mesmo tipo de resíduo circulante.

2 JUSTIFICATIVA E RELEVÂNCIA

Em razão de frequentes acidentes de falhas em dutos e estruturas metálicas na indústria de petróleo e, aproximadamente entre 10 e 20% ser em razão da corrosão influenciada por microrganismos (CIM), o presente estudo tem como objetivo principal desenvolver métodos moleculares padronizados para monitoramento da CIM, baseados em metagenômica, a serem aplicados a indústrias petrolíferas com histórico de corrosão.

Esta pesquisa possui parceria com a empresa Petrobras S.A., é uma estatal de economia mista de capital aberto. Essa empresa possui uma produção diária de mais de 2 milhões de barris de petróleo (320 mil metros cúbicos), sendo importante para o cenário petrolífero internacional. Essa parceria é uma oportunidade para a universidade em gerar conhecimento público com os incentivos financeiros da empresa e esta se beneficiará com a aplicação do conhecimento fornecido e possíveis tecnologias desenvolvidas para potencializar e reduzir os custos no processo de monitoramento da CIM.

Esse estudo é parte de um projeto intitulado “Estudo integrado de metagenômicas e cultivo de microrganismos causadores de biocorrosão em dutos”, o qual tem o objetivo de coletar o máximo de informações (identificação, quantificação, produtos metabólicos, expressão gênica e proteínas em comunidades microbianas) sobre os microrganismos presentes em indústria petrolífera. Nos campos de petróleo, os métodos de detecção e quantificação de microrganismos mais empregados são ainda dependentes de cultivo, por exemplo, número mais provável (NMP), os quais detectam, aproximadamente, em torno de 1% do total de microrganismos que provavelmente ocorrem nas amostras e são altamente seletivos, o que o torna ineficiente.

O êxito no desenvolvimento desse estudo viabilizará a aplicação de métodos moleculares, baseados em metagenômica, para monitoramento de dutos e estruturas metálicas em campos petrolíferos e isso poderá reduzir os acidentes de vazamentos de petróleo, os quais podem causar desastres ambientais como a morte de organismos aquáticos entre outros, causados pela CIM. Dessa forma, os dados obtidos terão importância em âmbito global, visto que o petróleo é o produto mais utilizado na matriz energética mundial e brasileira e sua obtenção gerando menores danos ambientais ainda é um desafio.

Na literatura, já foram realizados estudos de caracterização metagenômica de produtos gerados em campos petrolíferos. No entanto, nesta pesquisa, propomos uma abordagem inovadora que vai além da caracterização. Nosso objetivo é desenvolver um "pacote tecnológico metagenômico de monitoramento de rotina em campos de petróleo". Esse pacote tecnológico terá a

capacidade de prever cenários de biocorrosão em qualquer campo de petróleo, promovendo um nível avançado de monitoramento e permitindo a implementação de métodos profiláticos personalizados e eficazes contra a biocorrosão.

3 REFERENCIAL TEÓRICO

3.1 PETRÓLEO

O petróleo bruto é originado a partir da deposição e transformação de matéria orgânica na subsuperfície ao longo de milhões de anos, consistindo em um líquido de cor escura, pegajoso e viscoso (Vieira et al., 2007).

O petróleo bruto compreende uma complexa combinação de hidrocarbonetos, com quantidade variáveis de nitrogênio, oxigênio e outros elementos químicos. Além disso, o petróleo bruto não-diluído possui frações de parafinas livres de metais, mistura de parafina-naftaleno, aromáticos e uma porção asfáltica (Huc, 2015). Os componentes do petróleo podem ser divididos em duas classes: hidrocarbonetos e não-hidrocarbonetos (Figura 1).

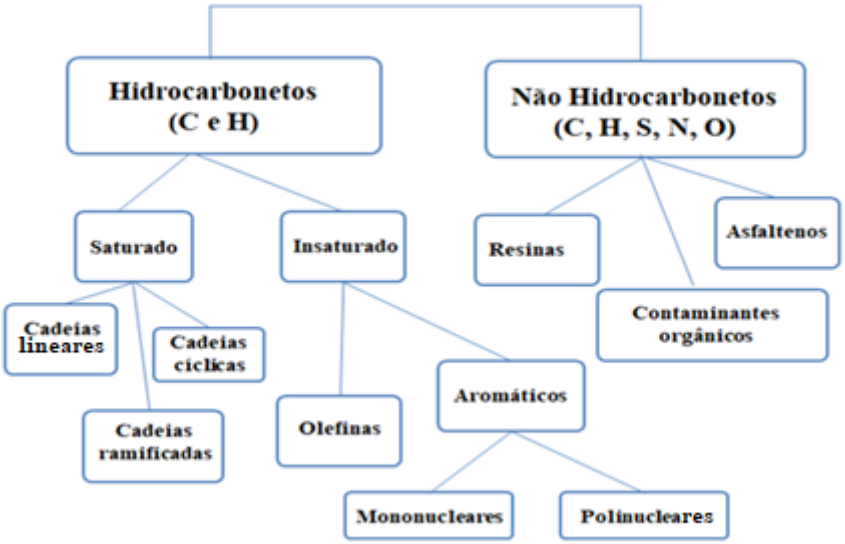

Figura 1 Composição do petróleo. Fonte: Ferreira (2018), adaptado.

Os hidrocarbonetos constituem a maior fração do petróleo, e o teor dessa fração pode estar intimamente associado às condições paleoestatigráficas e estilo estrutural durante a sedimentação dos organismos decompostos (Affouri et al., 2013). Esses compostos podem ser categorizados como alifáticos ou aromáticos. Os hidrocarbonetos alifáticos são arranjos saturados ou insaturados, de cadeia aberta podendo ser lineares ou ramificados, como *n*-alcanos, isoalcanos, cicloalcanos, terpenos e esteranos. Os hidrocarbonetos saturados possuem apenas ligações simples e correspondem ao maior percentual dos elementos do petróleo bruto (Varjani, 2017).

Em contrapartida, os hidrocarbonetos aromáticos possuem um ou mais anéis aromáticos - cadeias cíclicas com ligações duplas (uma ligação pi e outra sigma) e ligações simples de maneira intercalada e podem ser divididos principalmente em hidrocarbonetos aromáticos monocíclicos (BTEX - benzeno, tolueno, etilbenzeno e xilenos) e aromáticos policíclicos (PAHs) (Chandra et al., 2013; Costa et al., 2012). Os PAHs possuem mais de um anel benzênico, e os que apresentam até três anéis benzênicos (naftaleno, fenantrepeno e antraceno) são denominados como compostos de baixa massa molecular ou PAHs leves. Os PAHs que apresentam acima de três anéis benzênicos (pireno, crisenos, fluoranteno e benzopireno) são identificados com PHAs de alta massa molecular ou pesados (Chandra et al., 2013) (Figura 2).

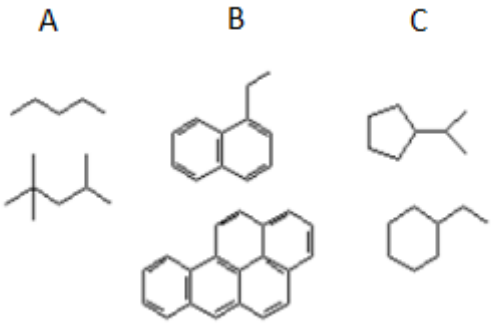

Figura 2 Exemplos de hidrocarbonetos do petróleo. (A) Hidrocarbonetos alifáticos; (B) Hidrocarbonetos aromáticos; e (C) Hidrocarbonetos naftênicos. Fonte: Micha (2015), adaptado.

Os compostos que não são hidrocarbonetos são as resinas e asfaltenos. As resinas são sólidos amorfos e verdadeiramente dissolvidos em óleo (Balba, 1998), e são moléculas policíclicas aromáticas normalmente contendo cadeias longas alifáticas, as quais se atribui a responsabilidade por essas moléculas serem solúveis em solventes parafínicos. Possuem vários grupos funcionais polares formados com N, S, O e vestígios de metais (Ni, V, Fe) (Chandra et al., 2013). Os asfaltenos, que são moléculas similares às resinas, mas com uma massa molecular maior, são constituídos por aglomerados policíclicos, variavelmente substituídos por grupos alquil e um núcleo de anéis aromáticos mais condensados ou fundidos, o que contribui para sua resistência à biodegradação. Eles são solúveis em hidrocarbonetos aromáticos leves, como benzeno e tolueno (Chandra et al., 2013).

3.1.1 Densidade do petróleo

O petróleo bruto pode ser categorizado como leve, médio ou pesado, baseado na quantidade proporcional dos componentes de alta massa molecular (Varjani, 2014, 2017). Sua constituição pode diferir conforme o local, tempo de existência de um campo petrolífero e de acordo com a profundidade do poço (Varjani, 2017). Existem diferentes métodos desenvolvidos ao longo dos anos por indústrias petroquímicas para classificar os tipos de petróleo através de métodos matemáticos, utilizando propriedades físicas de fácil mensuração como a temperatura de ebulição, densidade e viscosidade para classificação do petróleo (Menezes et al., 2007). O Grau API (°API), é uma grandeza arbitrária que mede a densidade relativa dos líquidos advindos do petróleo em relação à água. Quanto maior for a densidade, menor será o grau API (Ferreira, 2018) e assim, menos valor agregado. Na Tabela 1, é mostrado os diferentes graus de APIs.

Tabela 1 Definição de classes considerando a medida de gravidade específica.

Classe	**Mínimo API**	**Máximo API**
Leve	31,1	
Médio	22,3	31,1
Pesado	10	22,3
Extra-pesado		10

Fonte: Ferreira (2018).

3.2 PETRÓLEO NO BRASIL E NO MUNDO

O consumo global de energia pelos países da OCDE (Organização para Cooperação e Desenvolvimento Econômico) e não-pertencentes à OCDE entre os anos de 2006 e 2030 mostram um aumento (estimado) de 15,5 % para 73 % do petróleo com presença de recursos de energia, de acordo com a previsão da *Energy Information Administration (EIA, 2009)* (Bachmann et al., 2014). Na Figura 3, é mostrada a matriz energética mundial e do Brasil.

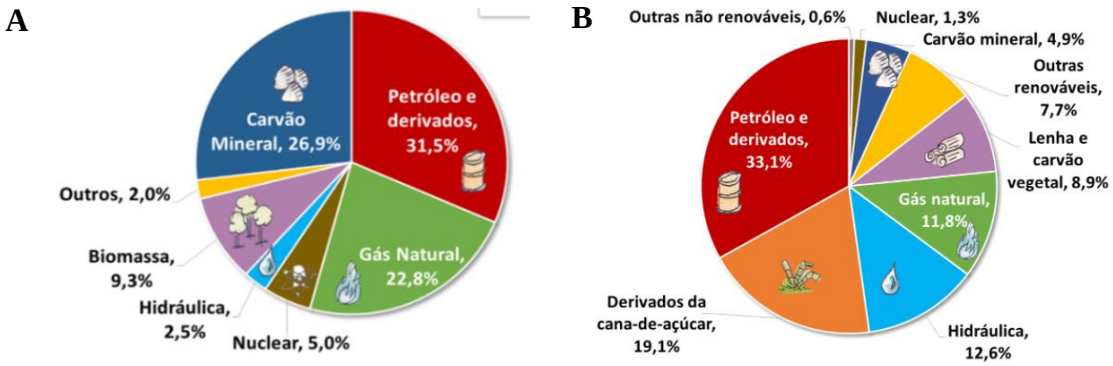

Figura 3 Matriz energética do Brasil e do mundo. (A) Matriz energética no mundo; (B) Matriz energética do Brasil. Fonte: EPE (2018).

Desde a construção do primeiro poço de petróleo em 1859, na Pensilvânia, nos EUA, vários campos de petróleo foram descobertos. No Brasil, o primeiro poço perfurado foi no Recôncavo Baiano, no final da década de 1940. Mais de 40 mil campos de petróleo estão espalhados ao redor do mundo, *onshore* e *offshore*, no entanto, a maioria não possui significância em seu impacto na produção mundial (Wikipédia, 2017). As duas maiores classes de campos são os supergigantes, campos com 1 bilhão ou mais de barris de petróleo recuperável, e gigantes, campos com 500 milhões de barris de óleo recuperável (Britannica, 2018; J. O. Silva et al., 2007). Menos de 40 campos de petróleo supergigantes foram encontrados em todo o mundo, mas esses campos originalmente continham cerca de metade de todo o petróleo descoberto até agora (Britannica, 2018).

A bacia sedimentar árabe-iraniana na região do Golfo Pérsico contém dois terços desses campos supergigantes. As demais supergigantes estão distribuídas entre Estados Unidos, Rússia, México, Líbia, Argélia, Venezuela, China e Brasil (Britannica, 2018). Na Figura 4, são mostrados os maiores países produtores de petróleo no mundo.

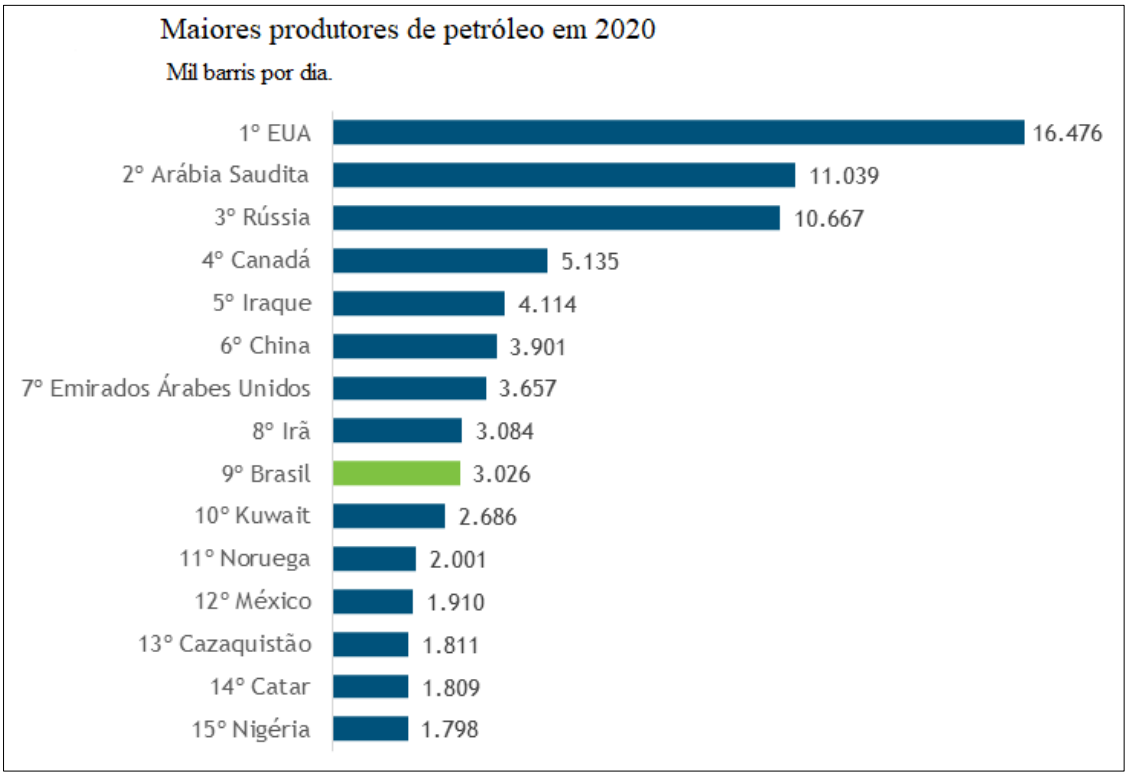

Figura 4 Maiores países produtores de petróleo no mundo. Fonte: "Oil: The Next Revolution"(Instituto Brasileio de Petróleo e Gás_ IBP, 2022).

O Brasil é o 9º maior produtor de petróleo no mundo (Instituto Brasileio de Petróleo e Gás_ IBP, 2022). A produção do Pré-Sal em águas ultraprofundas e os recentes leilões têm potencial para colocar o país ainda mais à frente deste ranking (IBP, 2022).

3.3 EXTRAÇÃO DE ÓLEO EM CAMPOS DE PETRÓLEO

A indústria petrolífera é dividida em três segmentos: *upstream*, *midstream* e *downstream* (Sahebi et al., 2014). *Upstream* se associa à exploração e produção de petróleo (descoberta de novas reservas, produção de petróleo e gás natural e processamento primário), *midstream* refere-se ao transporte de petróleo (oleodutos, gasodutos, navios transportadores e terminais), e o *downstream* abrange o refino, a petroquímica e a distribuição dos derivados de petróleo (Hassani et al., 2020; Sahebi et al., 2014). No presente estudo, os segmentos *upstream* e *midstream* são o foco, visto que sofrem mais com problemas de biocorrosão.

3.3.1 Produtos gerados no campo de petróleo

3.3.1.1 Água de injeção

A injeção de água é um método utilizado para aumentar a pressão interna de reservatórios de petróleo e assim estimular a produção, constituindo o que se denomina de recuperação secundária de petróleo. Em plataformas *onshore*, a água de injeção (AI) é

composta por água doce de origem superficial ou subterrânea, em contraste com as plataformas *offshore*, onde a água do mar compõe a base da AI. Além disso, a água produzida, que é extraída associada ao petróleo, pode ser tratada para reinjeção (Bedoya et al., 2021). Alguns estudos têm demostrado a presença de diversos grupos de microrganismos nas amostras de AI (Li et al., 2017; Nazira et al., 2017). Assim, o processo de recuperação secundária do petróleo pode contribuir para a introdução de microrganismos exógenos nos reservatórios (Magot et al., 2000; Shestakova et al., 2011; Nazira et al., 2017).

Para controlar os microrganismos desfavoráveis, são usados biocidas nessa fase de recuperação do petróleo (Vikram et al., 2014). Os biocidas podem ser classificados em dois grandes grupos: biocidas oxidantes (tais como cloro, bromo, ozônio e peróxido de hidrogênio) e biocidas não-oxidantes (tais como compostos de amônio quaternário, compostos alcoólicos e fenólicos, aldeídos, compostos contendo halogênio, aminas, e diaminas) (Kahrilas et al., 2015).

3.3.1.2 Água produzida

As amostras de água produzida (AP) consistem na água associada ao petróleo e ao gás que chega à superfície pelos poços produtores proveniente dos reservatórios, e que acaba compondo a fase aquosa que sai dos processos de separação trifásica (óleo/água/gás). A AP possui grandes quantidades de sólidos suspensos e dissolvidos, os quais podem ser de natureza orgânica ou inorgânica. Dentre os compostos orgânicos, os alifáticos mais leves (< C_5) são os mais solúveis em água. Já os sólidos inorgânicos da AP são similares aos encontrados na água do mar, no entanto, a salinidade pode variar até quatro vezes mais (Thomas, 2001).

A AP pode tanto ser tratada como efluente, para ser descartada no mar, quanto ser tratada como AI, para ser reinjetada no reservatório. A amostragem de AP a fonte mais comum na indústria de petróleo para se inferir os microrganismos presentes no interior dos reservatórios (Cluff et al., 2014; Rachel & Gieg, 2020).

3.1.1.3 Óleo e água

As amostras compostas por uma mistura de óleo e água constituem os fluidos produzidos que ainda não passaram pelo processamento primário, no qual é realizada a separação das fases. A quantidade de AP aumenta ao longo da vida produtiva dos poços, devido à injeção de água durante as etapas de recuperação secundária e terciária de petróleo. Contudo, mesmo no início da produção, AP já pode estar presente nos fluidos produzidos, derivada da água de formação orginalmente presente no reservatório (Gomes, 2009; Tales et

al., 2010; Zhu et al., 2021)

3.1.1.4 Óleo

No presente estudo, foi considerada amostra de óleo aquela obtida a partir do petróleo após o processamento primário, quando o óleo já está enquadrado para exportação. Mesmo na fase oleosa, há microgotículas de água, de modo que é possível haver microrganismos mesmo no petróleo já enquadrado (Lele et al., 2018).

3.1.1.5 Depósitos sólidos

Os depósitos sólidos compreendem o material que se acumula nas paredes dos dutos e fundo de tanques, e os biofilmes podem fazer parte da composição desses depósitos. Esse biofilme pode alojar microrganismos com metabolismos que podem acelerar ou mesmo desencadear a corroção localizada (Santos et al., 2022). A formação de um biofilme tem início quando determinadas células pioneiras empregam mecanismos químicos especializados para aderir a uma superfície, desempenhando um papel crucial ao tornar a superfície-alvo mais atrativa para as células subsequentes, que, ao longo do tempo, amadurecem e se organizam em um filme complexo e estruturado (Monroe, 2007). A Figura 5 mostra as fases da estruturação de um biofilme.

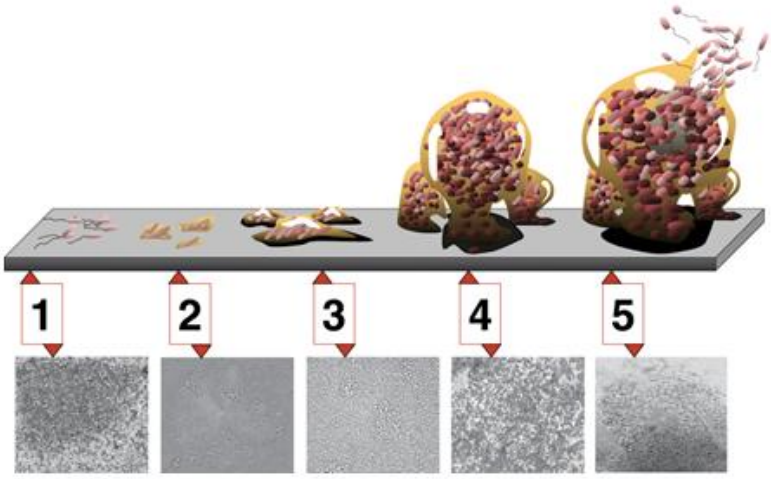

Figura 5 Fases da estruturação do Biofilme. Fonte: Monroe (2007).
Exemplo de estágios do desenvolvimento do biofilme. Fotomicrografias de P. aeruginosa em diferentes estágios para ilustração: (1) envolve a fixação inicial; (2), caracterizado pela fixação irreversível; (3), conhecido como maturação I; (4), correspondente à maturação II; (5), que envolve a dispersão.

3.4 BIOCORROSÃO

Microrganismos estão presentes em praticamente todos os ambientes da indústria de petróleo e gás que contêm ou interagem com água livre, e o monitoramento dessa microbiota é essencial para se reduzir os transtornos que afetam as operações industriais, com destaque para a bioincrustação, a acidulação biogênica e a biocorrosão.

Uma das principais causas de problemas na indústria de petróleo e gás relacionados à presença de microrganismos é a biocorrosão, também conhecida como *microbiologically influenced corrosion* (MIC) ou corrosão influenciada por microrganismos (CIM) (Little & Lee, 2007; Machuca & Salgar-Chaparro, 2019; Skovhus et al., 2017; Vigneron et al., 2018). A biocorrosão corresponde à deterioração de superfícies metálicas desencadeada ou potencializada pela presença e/ou pelas atividades metabólicas de microrganismos, podendo chegar ao ponto de provocar falhas como rompimentos, vazamentos, colapso de estruturas, mau funcionamento de equipamentos, entre outros transtornos (Hashemi et al., 2018)

Entre os equipamentos mais suscetíveis à biocorrosão na indústria de petróleo e gás estão os oleodutos dos segmentos *upstream* e *midstream*, os quais transportam petróleo entre poços produtores, plataformas e terminais (Skovhus et al., 2017; Vigneron et al., 2018). Os fluidos extraídos a partir dos reservatórios são multifásicos, uma vez que o petróleo (fase oleosa) apresenta-se associado à água (fase aquosa) e ao gás natural (fase gasosa). Esse fluido multifásico quando chega à plataforma é submetido ao processamento primário, no qual o objetivo central é separar as três fases e enquadrar o petróleo e o gás natural para exportação. O petróleo deve atender às especificações de qualidade antes que possa ser exportado da plataforma, e um dos parâmetros que precisam ser enquadrados é apresentar um teor menor do que 0,5 ou 1,0 % de água e sedimentos básicos (BSW, de *basic sediment and water*) (ASTM, 2017). Contudo, é preciso considerar que a água pode acabar ingressando em pontos a jusante dos sistemas de transporte e armazenamento de petróleo.

Os microrganismos ocorrem basicamente em qualquer ambiente onde haja água no estado líquido, o que é um requisito absoluto para eles exercerem atividades do metabolismo e reprodução (Fenchel, 2011). Dessa forma, quanto mais abundante for a fase aquosa num fluido, maior a possibilidade de ocorrer processos de biocorrosão nas estruturas em contato com esse fluido. Como a fase aquosa é mais densa que as fases oleosa e gasosa, ela tende a se acumular nos pontos mais baixos dos sistemas, como fundos de tanques e a geratriz inferior de oleodutos (Figura 6), as quais, por essa razão, acabam sendo as regiões mais propensas a eventos de biocorrosão (NACE International, 2018). A altura da interface água/óleo é uma região especialmente propícia à proliferação de micróbios, uma vez que os compostos orgânicos da fase oleosa servem como nutrientes para microrganismos presentes na fase aquosa.

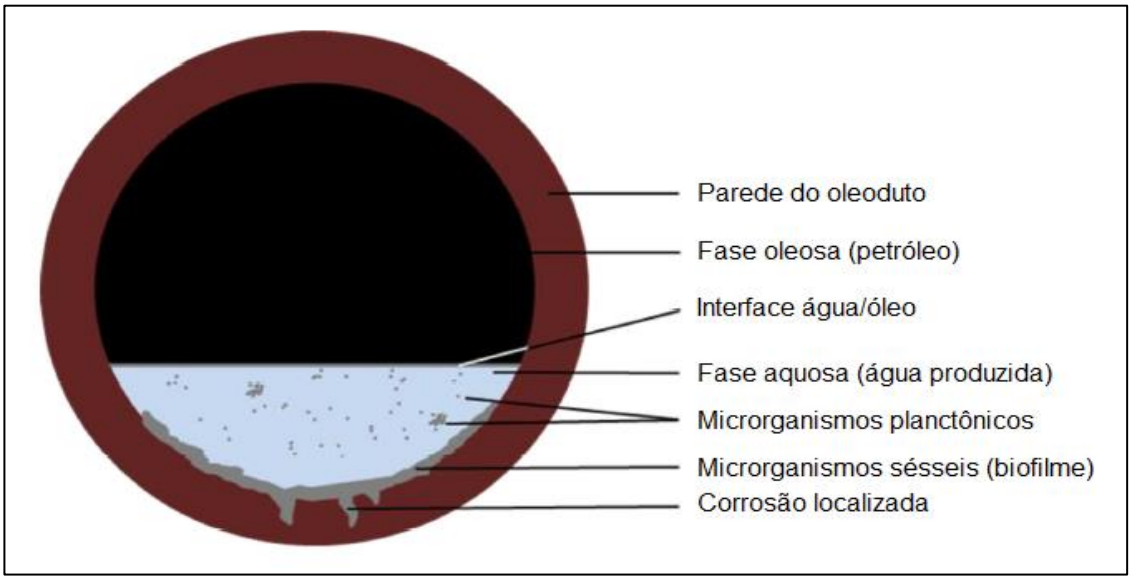

Figura 6 Corte transversal de um oleoduto ilustrando a heterogeneidade de hábitats para microrganismos. Fonte: Lomans et al. (2016).

Os oleodutos são geralmente compostos por algum tipo de aço, e acredita-se que os mecanismos de corrosão de superfícies metálicas são de natureza eletroquímica. Esse processo envolve: (1) a oxidação do ferro metálico como reação anódica ($Fe^0 \rightarrow Fe^{2+} + e^-$); (2) a redução de algum composto químico como reação catódica; e (3) a água (H_2O) como eletrólito. O oxigênio molecular (O_2) se difunde do ar para a água exposta à atmosfera, e quando dissolvido na água ele participa de uma reação catódica relacionada a altas taxas de corrosão do aço ($O_2 + 4\ e^- \rightarrow 2\ H_2O + 4\ OH^-$) (Waldow, 2018). Diante disso, as empresas de petróleo realizam a desaeração dos fluidos e adição de sequestrantes de O_2, como medida de controle da corrosão (Andrade, 2017). Em razão das condições predominantemente anóxicas no interior dos oleodutos, os microrganismos anaeróbicos se tornam os agentes principais no processo de biocorrosão. Considera-se que há dois mecanismos principais de biocorrosão em ambientes anóxicos (Enning & Garrelfs, 2014; Kato, 2016) (Figura 7).

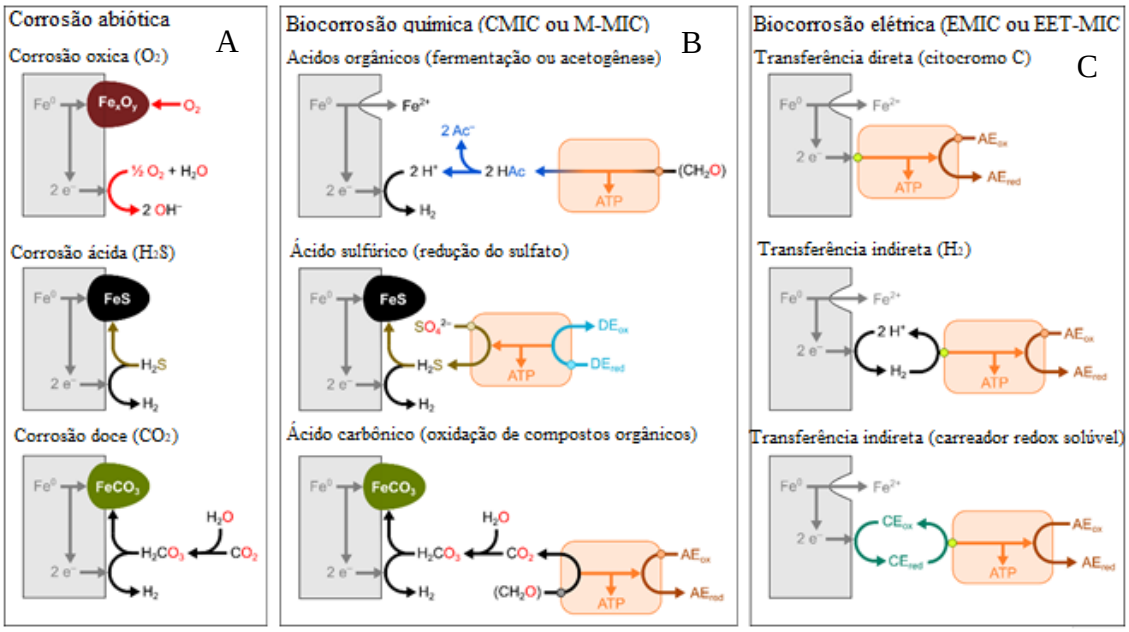

Figura 7 Principais tipos de corrosão abiótica e de biocorrosão. (A) Corrosão abiótica; (B) Biocorrosão química; (C) Biocorrosão elétrica. Ac = ácido orgânico; AE = aceptor de elétrons; ATP = adenosine trophosphate; CE = carreador de elétrons; CMIC = chemical mic = *chemical microbially influenced corrosion*; DE = doador de elétrons; EET-MIC = *extracellular electron transfer microbially influenced corrosion*; EMIC = *electrical microbially influenced corrosion*; M-MIC = *metabolite microbially influenced corrosion*. Fonte: Enning & Garrelfs, (2014) e Kato (2016), adaptado por Waldow (2018).

O primeiro mecanismo de biocorrosão (Figura 7B) é mais comumente conhecido e pode ser denominado de biocorrosão química, pois se refere à ação corrosiva de compostos químicos de caráter ácido produzido pelo metabolismo de certos grupos microbianos (Kryachko & Hemmingsen, 2017). Dentre esses compostos químicos, o sulfeto de hidrogênio (H_2S) é aquele mais frequentemente relacionado à biocorrosão na indústria de petróleo e gás (Souza et al., 2019). Essa associação é devido à água do mar injetada em reservatórios ser abundante em sulfato (SO_4) (B. Liang et al., 2018), que é o aceptor final de elétrons na respiração anaeróbica de microrganismos redutores de sulfato, os quais geram H_2S como produto da sua respiração (Fenchel, 2011). Os ácidos orgânicos de cadeia curta (C_1 a C_5) têm sido cada vez mais relacionados à biocorrosão química, uma vez que esses ácidos orgânicos podem ser produzidos por microrganismos fermentadores e por bactérias acetogênicas, alcançando elevadas concentrações na interface entre os biofilmes e as superfícies metálicas (Kryachko & Hemmingsen, 2017).

O segundo mecanismo de biocorrosão (Figura 6C) foi descoberto mais recentemente, e ele pode ser denominado de biocorrosão elétrica, pois consiste na transferência extracelular de elétrons (ou EET, de *extracellular electron transfer*) realizada por microrganismos quimiolitotróficos que utilizam, por exemplo, o ferro metálico (Fe^0) como doador de elétrons,

oxidando-o a Fe^{2+} (Kato, 2016; Kato et al., 2014). Essa extração de elétrons pode se dar por contato direto com a superfície metálica – através de enzimas citocromo multi-heme ligadas à membrana celular ou a nanofios – ou de maneira indireta – pela difusão no meio extracelular do H_2 ou de carreadores redox solúveis, tais como flavinas, fenanzinas e quinonas (Figura 6C – transferencia indireta de H_2) (Kato, 2016). Microrganismos pertencentes a diversos grupos fisiológicos têm sido descritos como causadores de biocorrosão elétrica, incluindo procariotos redutores de nitrato, procariotos redutores de sulfato, arqueias metanogênicas e bactérias acetogênicas.

A biocorrosão do aço é um processo complexo, que é desencadeado por uma sequência de eventos e interações entre diversos fatores ambientais, agentes químicos e agentes biológicos. Processos de biocorrosão podem ocorrer simultaneamente e contribuírem para processos de corrosão abiótica. Dessa forma, um programa de monitoramento considerando variáveis operacionais, parâmetros físico-químicos e avaliações microbiológicas são necessários para o gerenciamento apropriado da biocorrosão em oleodutos (NACE International, 2018).

3.5 MONITORAMENTO DA BIOCORROSÃO EM OLEODUTOS

A biocorrosão é um tipo de corrosão localizada e, como todas as corrosões desse tipo, ela é mais difícil de prever, monitorar e gerenciar do que a corrosão uniforme. Essas características da biocorrosão decorrem do fato de que biofilmes aderidos às superfícies metálicas são os principais responsáveis pela biocorrosão (Eckert, 2015; Vigneron et al., 2018). Os biofilmes se estabelecem de maneira heterogênea ao longo das superfícies internas dos oleodutos e outros equipamentos industriais, gerando microambientes na interface com as superfícies metálicas, os quais causam e/ou aceleram a corrosão em pontos específicos (Souza et al., 2019).

A grande dificuldade de monitorar a presença microbiana no interior dos oleodutos reside na inacessibilidade e na grande extensão desses ambientes. Além de serem exíguos, com um diâmetro nominal entre 4 e 48 polegadas (10 e 122 cm), os oleodutos também são ambientes remotos, pois se estendem por vários quilômetros no interior da crosta terrestre, no leito marinho, debaixo da terra, em florestas fechadas, etc. Portanto, o monitoramento da comunidade microbiana em oleodutos acaba se dando principalmente pela coleta e análise de amostras provenientes de trechos próximos ao início e ao final da extensão desses.

3.5.1 Tipos de amostragem

Os três tipos de amostragens comumente realizadas em oleodutos são discutidos a seguir:

1) Amostragem de fluidos por meio de torneiras. Nas plataformas e terminais dos quais partem ou nos quais chegam oleodutos e possuem tanques de armazenamento, geralmente há pontos com torneiras que permitem a amostragem dos fluidos circulantes nos oleodutos e dos fluidos armazenados nos tanques. Esse é o tipo mais fácil e mais comum de amostragem, pois pode ser realizada a qualquer momento. A desvantagem é que fornecem as amostras menos interessantes para fins de monitoramento da biocorrosão, pois só permite a avaliação dos microrganismos planctônicos suspensos nos fluidos circulantes (e não dos sésseis, presentes nos biofilmes aderidos a superfícies). Primeiramente, deixa-se escorrer o fluido da torneira aberta por um tempo suficiente para descartar o volume morto (i.e., o fluido retido na linha da torneira) a fim de que seja obtido, de fato, o fluido do interior do oleoduto ou tanque.

2) Amostragem de biofilme em cupons. O monitoramento da taxa de corrosão uniforme em certos oleodutos inclui a avaliação da taxa de perda de massa em cupons metálicos (também chamados de corpos de prova). Tomadores de cupons são equipamentos presentes em determinados pontos de alguns oleodutos, e neles se instalam os cupons metálicos para ficarem expostos às condições internas do oleoduto. Após um determinado período de tempo, geralmente algo na ordem de 1 a 6 meses, os cupons são retirados e substituídos por novos cupons. Quando há biofilme depositado sobre os cupons retirados, é possível coletar esse biofilme com um *swab* ou espátula previamente esterilizados antes que eles sejam encaminhados para a pesagem; esse *swab* ou espátula é então imerso(a) num frasco contendo alguma solução preservante. (Em alguns casos, é possível amostrar adicionalmente o fluido presente no interior do tomador de cupom.). A desvantagem é ser um tipo de amostragem nem sempre disponível, pois demanda que existam tomadores de cupom no oleoduto a ser analisado. E mesmo nesses casos, a amostragem precisa ser alinhada ao cronograma de troca dos cupons. Outra desvantagem é a possibilidade de não haver biofilme suficiente depositado sobre o cupom retirado para que análises microbiológicas possam ser realizadas.

3) Amostragem de resíduo de pig. Os únicos equipamentos que conseguem acessar toda a extensão de um oleoduto são os pigs, e isso é possível somente para os dutos 'pigáveis' (que contam com a presença de lançadores e recebedores de pig). Os pigs são equipamentos impulsionados pelo fluxo dos próprios fluidos circulantes, percorrendo o interior do oleoduto

para fins de limpeza das superfícies internas. Além de efetuarem essa limpeza, os pigs instrumentados realizam simultaneamente uma inspeção, obtendo informações quanto à espessura da parede do duto, as quais servem para o monitoramento da corrosão uniforme (e somente alguns tipos específicos de pig permitem também a detecção de corrosão localizada).

Quando o pig termina seu percurso e chega ao recebedor do pig, ele apresenta um acúmulo de material aderido ao seu corpo (chamado de resíduo de pig), o qual é parte do material removido das superfícies internas do oleoduto, incluindo porções de biofilmes. Esse material pode então ser coletado com uma espátula e colocado num frasco contendo alguma solução preservante; essa amostra pode ser submetida a análises microbiológicas. (Frequentemente, também é possível amostrar o fluido presente no interior do recebedor de pig antes que ele seja aberto.) A vantagem desse tipo de amostragem é ser mais garantida do que os cupons quanto à obtenção de material biológico em quantidade suficiente. A desvantagem desse tipo de coleta é que ela depende do cronograma de passagem de pigs, com a dificuldade adicional de que a chegada do pig pode se dar a qualquer hora dentro de uma janela de 2 a 3 dias, exigindo que o pessoal responsável pela coleta fique de sobreaviso nesse período.

3.5.2 Método dependente de cultivo

A finalidade mais comum na indústria de petróleo e gás para amostragens microbiológicas é a quantificação de grupos microbianos de interesse pelo método do Número Mais Provável (NMP), o qual consiste em um método dependente de cultivo que se baseia no princípio de diluição até extinção (Bhagobaty, 2014; Waldow, 2018). A inoculação pode ser feita em campo, logo após o momento da coleta, ou em laboratório, num prazo de até 48 h após o momento da coleta. Cada meio de cultivo utilizado é voltado para se quantificar um certo grupo fisiológico, tais como “redutores de sulfato”, “produtores de ácido” e “anaeróbios heterotróficos totais”. O método do NMP possui baixa acurácia, tais como a demora na obtenção de resultados e o grande volume de trabalho necessário para a preparação e a inoculação dos kits de quantificação (Eckert & Skovhus, 2018). Além disso, estima-se que os microrganismos “cultiváveis” fique ao redor de 1,0 % do total de linhagens microbianas presentes em amostras ambientais (Pedrós-Alió, 2011).

3.5.3 Obtenção de culturas

Esse método consiste em realizar o cultivo de microrganismos em laboratório, também utilizando as amostras de campo como inóculos em meios de cultura específicos para o

enriquecimento de um determinado grupo fisiológico. No contexto da biocorrosão, esses cultivos costumam visar à obtenção de culturas mistas para serem utilizadas em testes de eficiência biocida, os quais subsidiam o processo de escolha de qual produto biocida aplicar no sistema a ser tratado.

3.5.4 Biologia Molecular

Dadas as limitações do método do NMP, há uma forte tendência na direção de se incluir métodos de biologia molecular nos programas de monitoramento da biocorrosão (Eckert & Skovhus, 2018). Um método proeminente nessa tendência é a quantificação da adenosina trifosfato (ATP) por fotometria, a qual pode ser considerada como um indicador do nível global de atividade metabólica da comunidade microbiana presente na amostra (Keasler et al., 2013). A quantificação de ATP é realizada por meio de kits comerciais e deve ser executada imediatamente após a coleta da amostra.

Outros métodos que vêm sendo considerados analisam uma determinada classe de biomoléculas, tais como: os ácidos desoxirribonucleicos (DNA) nos métodos de qPCR e metagenômica; os ácidos ribonucleicos (RNA) nos métodos de RT-qPCR e metatranscriptômica; as proteínas nos métodos de metaproteômica; e metabólitos nos métodos de metabolômica (Beale et al., 2015). Percebe-se um certo compromisso entre os diferentes níveis de análise nas metaômicas, pois, quanto mais fácil é de se abordar metodologicamente uma das classes de biomoléculas, menos informativa ela é em termos da atividade microbiana existente no momento da coleta da amostra.

Independentemente da finalidade para quais as amostras estão sendo coletadas, certas precauções gerais devem ser tomadas. Em primeiro lugar, é preciso se tomar medidas a fim de minimizar a degradação das amostras por DNAses e RNAses (Rachel & Gieg, 2020). Logo, é imprescindível a utilização de materiais e soluções previamente esterilizados (por autoclave ou filtração em membrana de malha igual a 0,22 μm), a utilização de luvas esterilizadas durante todo o procedimento de coleta, e o fechamento dos frascos imediatamente após o material amostrado ter sido neles inseridos.

Quando a finalidade envolve avaliações por biologia molecular, é preciso minimizar as alterações na composição da amostra coletada (Rachel & Gieg, 2020). Dentre os processos que podem alterar a composição da amostra coletada estão a degradação diferencial das biomoléculas, a alteração nos padrões de expressão gênica (principalmente no caso do RNA), e a proliferação diferencial de linhagens microbianas.

Um dos grandes desafios para a implantação de métodos baseados em biologia

molecular no monitoramento em oleodutos é a padronização dos protocolos de preservação e extração de biomoléculas a partir das amostras coletadas. Em primeiro lugar, porque os pontos de amostragem se localizam em plataformas e terminais que podem ser de difícil acesso, limitando a quantidade de material para a coleta que pode ser levado a campo. Em segundo lugar, porque a logística de transporte das amostras coletadas costuma apresentar obstáculos, tais como limitação quanto ao volume de amostras que podem ser transportadas e uma demora de dias, semanas ou até meses até as amostras chegarem ao laboratório onde serão analisadas (Rachel & Gieg, 2020). Por fim, as amostras coletadas podem apresentar grande variação entre si quanto à sua composição físico-química (e.g., teor de sólidos e de óleo), o que exige protocolos robustos capazes de lidar com essas variações.

3.5.5 Metagenômica

O termo "metagenômica", utilizado pela primeira vez em 1998, se refere ao sequenciamento de moléculas de DNA extraídas diretamente de amostras ambientais, permitindo uma investigação genômica das comunidades microbianas, sem a necessidade de cultivo prévio em laboratório (Handelsman et al., 1998). Anteriormente, a análise metagenômica fundamentava-se na obtenção de DNA comunitário de uma amostra, aplicação de enzimas de restrição para clivar as moléculas de DNA, seguida de clonagem de segmentos de DNA em vetores e varredura dos insertos nos milhares de clones das bibliotecas metagenômicas para explorar novos produtos e atividades biológicas (Handelsman et al., 1998).

O progresso das tecnologias de Sequenciamento de Nova Geração (ou NGS, do inglês *next-generation sequencing*), associado ao desenvolvimento dos programas de análise de bioinformática, promoveu um aumento no número de genomas e metagenomas sequenciados com baixo custo, colaborando para a disseminação e o aperfeiçoamento da metagenômica (Scholz et al., 2012; Stark et al., 2014).

A metagenômica pode usar a amplificação prévia seguida do sequenciamento em larga escala de regiões genômicas biomarcadoras (por ex.: o gene 16S rRNA para Bacteria e Archaea) para viabilizar o reconhecimento dos gêneros em uma amostra retirada diretamente do ambiente, essa técnica é denominada metagenômica de amplicon 16S rRNA. Na outra abordagem de metagenômica, denominada de *shotgun*, todo o DNA ambiental é sequenciado (sem amplificação prévia de uma região biomarcadora taxonómica), no entanto, essa abordagem necessita de um DNA metagenômico em grande quantidade e de alta qualidade, o que não é comum em amostras ambientais (Truong et al., 2015).

CAPÍTULO 1

CAPÍTULO 1 - CORROSION-INFLUENCING MICROORGANISMS IN SYSTEMATIC REVIEW, ANALYSIS, AND SCIENTIFIC PETROLIFEROUS REGIONS ON A GLOBAL SCALE: SYNTHESIS OF 16S AMPLICON METAGENOMIC STUDIES

Microrganismos influenciadores da corrosão em revisão sistemática e análise científica de regiões petrolíferas em escala global: síntese de estudos metagenômicos de amplicon 16S.

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Os estudos metagenômicos realizados em campos de petróleo apresentam divergências em relação à taxonomia e ao metabolismo predominante associado ao processo de corrosão microbiologicamente influenciada (CIM). Essa falta de consenso dificulta o entendimento completo das interações envolvidas na CIM, prejudicando o monitoramento e o diagnóstico preciso da biocorrosão. Como resultado, podem ser implementadas medidas de intervenção inadequadas para combater o processo de biocorrosão.

Diante dessa problemática, é crucial estabelecer uma metodologia de estudo padronizada que compile e analise informações consolidadas na literatura relacionadas ao uso da metagenômica em campos de petróleo. Ainda há muitas lacunas de conhecimento sobre a estrutura e dinâmica das comunidades microbianas na infraestrutura de petróleo e gás. Nesse contexto, a presente revisão sistemática teve como objetivo avaliar a composição e a abundância relativa de bactérias e arqueas associadas à corrosão microbiologicamente influenciada (CIM), além de predizer as funções metabólicas presentes em diferentes amostras de sistemas de produção e transporte de petróleo em todas as regiões petrolíferas do mundo.

Após a análise das regiões petrolíferas, verificou-se que a maioria dos microrganismos identificados está potencialmente envolvida nos processos relacionados à corrosão microbiologicamente influenciada (CIM). Esses microrganismos abrangem grupos como os sulfidogênicos, formadores de biofilme, acidogênicos, acetogênicos e metanogênicos. Nas amostras de água produzida, foi observada uma maior abundância dos grupos sulfidogênicos, apesar da presença de alta diversidade microbiana. Já nas amostras de depósitos sólidos (biofilmes), foi identificado um perfil microbiano distinto, com maior predominância de bactérias produtoras de ácidos e redutoras de nitrato. Em ambos os casos, foram observadas

importantes associações sintróficas entre os grupos sulfidogênicos, metanogênicos, produtoras de ácidos e redutoras de nitrato.

No entanto, a dominância desses grupos microbianos específicos nos campos de petróleo é determinada pelas características físico-químicas do ambiente. Vale ressaltar que a influência antropogênica, como o uso indiscriminado de produtos químicos, pode causar a exclusão de microrganismos relacionados à corrosão, mas que não estão, necessariamente, envolvidos no processo biocorrosivo no momento. Além disso, o uso indiscriminado de produtos químicos pode favorecer o desenvolvimento de microrganismos resistentes a antibióticos. Essa observação é reforçada pelo fato de que as regiões petrolíferas avaliadas apresentaram um microbioma com uma significativa abundância de metabolismo de terpenoides e de compostos aromáticos, os quais desempenham papéis importantes na sobrevivência microbiana em condições estressantes e como mecanismos de defesa.

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Corrosion-influencing microorganisms in petroliferous regions on a global scale: systematic review, analysis, and scientific synthesis of 16S amplicon metagenomic studies

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ABSTRACT

The objective of the current systematic review was to evaluate the taxonomic composition and relative abundance of bacteria and archaea associated with the microbiologically influenced corrosion (MIC), and the prediction of their metabolic functions in different sample types from oil production and transport structures worldwide. To accomplish this goal, a total of 552 published studies on the diversity of microbial communities using 16S amplicon metagenomics in oil and gas industry facilities indexed in Scopus, Web of Science, PubMed and OnePetro databases were analyzed on 10th May 2021. The selection of articles was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Only studies that performed amplicon metagenomics to obtain the microbial composition of samples from oil fields were included. Studies that evaluated oil refineries, carried out amplicon metagenomics directly from cultures, and those that used DGGE analysis were removed. Data were thoroughly investigated using multivariate statistics by ordination analysis, bivariate statistics by correlation, and microorganisms' shareability and uniqueness analysis. Additionally, the full deposited databases of 16S rDNA sequences were obtained to perform functional prediction. A total of 69 eligible articles was included for data analysis. The results showed that the sulfidogenic, methanogenic, acid-producing, and nitrate-reducing functional groups were the most expressive, all of which can be directly involved in MIC processes. There were significant positive correlations between microorganisms

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Additional Information and Declarations can be found on page 36

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in the injection water (IW), produced water (PW), and solid deposits (SD) samples, and negative correlations in the PW and SD samples. Only the PW and SD samples displayed genera common to all petroliferous regions, *Desulfotomaculum* and *Thermovirga* (PW), and *Marinobacter* (SD). There was an inferred high microbial activity in the oil fields, with the highest abundances of (i) cofactor, (ii) carrier, and (iii) vitamin biosynthesis, associated with survival metabolism. Additionally, there was the presence of secondary metabolic pathways and defense mechanisms in extreme conditions. Competitive or inhibitory relationships and metabolic patterns were influenced by the physicochemical characteristics of the environments (mainly sulfate concentration) and by human interference (application of biocides and nutrients). Our worldwide baseline study of microbial communities associated with environments of the oil and gas industry will greatly facilitate the establishment of standardized approaches to control MIC.

Subjects Bioinformatics, Microbiology
Keywords Metabarcoding, Oil, Microbiologically influenced corrosion, Microbial communities, Worldwide

INTRODUCTION

Microorganisms are present in virtually all oil and gas industry environments containing or interacting with free water. These environments range from the network of pores inside the rocks of oil reservoirs, to the surface and subsurface facilities and equipment that constitute the infrastructure of the oil and gas industry, such as injection wells, production wells, pipelines, valves, membranes, tanks, pumps, separators, water and effluent treatment units, and cooling towers (*Ollivier & Magot, 2005*; *de Waldow, 2018*). The microbial activities that occur in oil and gas industry environments can generate benefits (*e.g.*, bioremediation) or harms (*e.g.*, microbiologically influenced corrosion or MIC), depending on the microbial species and the prevailing environmental conditions (*Augustinovic et al., 2012*).

The global annual costs of the corrosion of pipelines and other industrial equipment for oil and gas production and transport have been estimated at 2.5 trillion US dollars per year, according to the *NACE International (2016)*, and it is estimated that between 10% and 20% of corrosion occurrences involve MIC (*Machuca & Salgar-Chaparro, 2019*). MIC refers to corrosion that is affected by the presence and activity of microorganisms, which involves the establishment of biofilms that initiate and/or accelerate corrosive processes of electrochemical nature (*Eckert, 2014*; *Videla, 1996*; *Zarasvand & Rai, 2014*). MIC is difficult to predict and monitor due to the heterogeneity of biofilms distribution within industrial systems and because it induces localized corrosion, whose monitoring is much more challenging than general corrosion (*Little & Lee, 2007*; *Roche, 2007*). Operating expenditures for mitigating MIC include mechanical cleaning, pigging, injection of chemical products (*e.g.*, biocides, biodispersants, corrosion inhibitors, O_2 and H_2S scavengers), line inspection, and microbiological and physicochemical analyses (*Skovhus, Eckert & Rodrigues, 2017*).

Microbiological analyses allow the evaluation of the microbiota present in a given environment, including those that are capable of causing or accelerating corrosion. Although cultivation-dependent methods are still the most widely used in the oil and gas industry, they are limited because cultivable lineages usually do not exceed 1.0% of all microbial lineages present in environmental samples (*Pedrós-Alió, 2011*). In contrast, molecular microbiological methods (MMM) are culture-independent methods that have attracted the interest of the oil and gas industry for monitoring microbial communities. These methods are based on biomolecules such as adenosine triphosphate (ATP), deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, and metabolites (*Skovhus, Eckert & Rodrigues, 2017*). The advent of MMM in the oil and gas industry is making it possible to obtain a more comprehensive and accurate picture of the microbial communities occurring in different environments related to the oil and gas industry (*Larsen et al., 2009*).

The term "metagenomics" (first coined and used in 1998) refers to the sequencing of DNA molecules extracted directly from environmental samples, allowing a genomic investigation of microbial communities and bypassing the need of cultivation in the laboratory (*Handelsman et al., 1998*). For many years since its first experimental use, metagenomic analysis has relied on extracting community DNA from a sample, using restriction enzymes to cleave the DNA molecules, followed by cloning DNA segments into vectors and scanning the inserts in thousands of clones from metagenomic libraries to explore new products and biological activities (*Handelsman et al., 1998*). The development of the so-called Next-Generation Sequencing (NGS) technologies, combined with improvements in software for bioinformatics analyses, allowed an immediate increase in the number of sequenced metagenomes and at lower costs, contributing to the dissemination and improvement of metagenomics (*Scholz, Lo & Chain, 2012*; *Stark, Giersch & Wünschiers, 2014*).

One limitation of metagenomics through analysis of 16S rRNA gene amplicons (also known as metabarcoding) is the absence of information about the functional and metabolic capabilities of the evaluated communities (*Douglas et al., 2020*). The prediction of metabolic functions can assist in the interpretation of metagenomic analysis of various environments and their respective microbial communities (*Ijoma et al., 2021*). One tool that has been used is software that can predict metabolic and functional profiles of microbial lineages based on taxonomic identification through 16S rRNA sequences and retrieval of published studies on metabolic characterization of microbial lineages. An example of this kind of software is PICRUST2, that includes a vast and updated database of gene families and reference genomes, allowing phenotypic predictions from amplicon metagenomes (*Douglas et al., 2020*). In complement to metagenomic analyses, physicochemical analyses allow us to understand which factors trigger the proliferation or demise of microbial lineages in a specific environment (*Gao et al., 2019*).

Nevertheless, although those tools provide us with information that can predict and diagnose MIC problems, it is still far to thoroughly understand all the intricate interactions comprising MIC. Literature data are heterogeneous, and it is often not possible to find a common denominator that can explain the putative driving factors that may be influencing

the community dynamics of distinct microorganisms in each environment. In addition, obtaining data related to microbial communities present in industrial infrastructure is limited due to logistic difficulties of access to facilities and sample collection in pipelines, submarine, and subsurface equipment (*Rachel & Gieg, 2020*). Furthermore, the lack of communication between different departments within the same company, particularly in large companies (*Kunsch, 2012*), hinders the access to information by scientists and engineers.

Given that scenario, it is highly relevant to establish a standardized study methodology, so that it is possible to compile and analyze the bulk of information already published, since there are still many knowledge gaps regarding the microbial communities' structure and dynamics in oil and gas infrastructure. Therefore, the present systematic review aimed to evaluate the composition and relative abundance of bacteria and archaea associated with MIC, as well as the prediction of metabolic functions present in different samples from oil production and transport systems worldwide.

METHODS

Data collection

This systematic review was based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (*Moher et al., 2009*). All records from all types of documents, of all languages, available as digital media since the beginning of Scopus, Web of Science (main collection), PubMed and OnePetro (focused on the oil and gas industry) databases up to 10th May 2021, were retrieved. The study selection process was performed in three steps: (1) identification of records in Scopus, Web of Science, PubMed and OnePetro databases; (2) screening of documents; and (3) evaluation of eligibility and inclusion of selected studies.

In order to identify the records, a text search evaluating all the documents in all the three databases was carried out, using adequate and standardized search patterns. The keywords searched for were: petroleum, crude oil, oilfield, oil, reservoir, pipeline, biocorrosion, microbiologically influenced corrosion, microbial corrosion, MIC, corrosion, metagenomics, and 16s rRNA. All keyword combinations and the Boolean operators are detailed in Supplementary Material 1.

Identified documents were automatically exported from Scopus, Web of Science and PubMed in *csv* text file format, whereas those from OnePetro were manually exported (Supplementary Material 2). Using the script *format_input.py* (Supplementary Material 3), Excel spreadsheet files were generated for each of the three databases, including the following variables: Title, Year, Digital Object Identifier (DOI), Document Type, Language, and Author(s). The script identified records without DOI and removed those ones with identical Title or DOI.

Excel spreadsheet files of each database were unified, and duplicated records were removed using the script *remove_duplicates.py* (Supplementary Material 4). Besides the initial variables, the unified spreadsheet file was added with the variable Repository, which describes from which database the record was retrieved. Furthermore, when the same record was identified in more than one database, this information was maintained in the

Table 1 Eligibility criteria for inclusion of studies in the systematic review.

Eligibility criteria	
Collection point	Reservoirs, pipelines, and tanks
Sample	Oil, produced water, injection water, biofilm, sediment, sludge and pig residue
Result	Composition and abundance of microorganisms present in oil field samples by 16S amplicon metagenomic analysis
Exclusion criteria	
Collection point	Refinery and petrochemical industry
Sample	Obtained from cultures
Result	Composition and abundance of microorganisms present in oil field samples by molecular analysis of the Denaturing type Gradient Gel Electrophoresis (DGGE).

description. The documents were downloaded as *pdf* files by their DOIs using a software based on permissions of ICB/UFMG internal network. Inaccessible documents were categorized as *Not Available (NA)* whereas the documents without DOI were manually downloaded.

The selection of studies was performed by Joyce Dutra (J.D.) and Rosimeire Gomes (R. G.) and the divergences were solved by Mariana Santos Cardoso (M.S.C.). The divergences were solved as follows: After obtaining the documents (J.D. and R.G.), selected and non-selected files were merged, and the discrepancies were identified. Thus, these divergent documents were separated in one spreadsheet and forwarded to the third reviewer (M.S.C.), who judged whether the document would enter or not. In case of doubt, there was a census in the group of authors to decide whether the document should be remained in the final *corpus* or removed.

Titles and abstracts of the documents were reviewed, and only studies related to both oil fields and metagenomic analyses were retrieved. All these studies were completely re-evaluated based on the eligibility and exclusion criteria related to collection area, sample type, and obtained results: inclusion and exclusion criteria are listed in Table 1.

The following data were extracted from selected documents: (i) sampling design, (ii) physicochemical characterization of samples, (iii) DNA extraction, amplification, and high-throughput sequencing features, and (iv) taxonomic composition (all taxa from Domain to Genera) and relative abundance of microorganisms obtained by both 16S rRNA amplicon and shotgun metagenomics.

Data analyses

Overview

In order to standardize the data, all analyses encompassing the relative abundance of microbial taxa in all samples were performed at the genus level (even if there were documents including classification at lower-level taxonomic ranks, such as the species level).

For data extraction, all archaeal and bacterial genera were considered, regardless of their originally described relative abundance or absence of this information (in case there was only the report of their presence). After data extraction, absolute and relative frequencies for each genus were calculated. Data analyses were carried out for each sample type separately (injection water—IW, produced water—PW, oil— OIL, oil and water—OW, and solid deposits—SD, which include biofilms, sludge, and pigs). Each set of sample types was evaluated for each distinct world petroliferous region, following the United States Geological Survey classification (*USGS, 2003*): 1—Former Soviet Union, 2—Middle East and North Africa, 3—Asia-Pacific, 4—Europe, 5—North America, 6—Central and South America, 7—Sub-Saharan Africa and Antarctica, and 8—South Asia.

Shareability and uniqueness patterns

Results of analyses of shareability and uniqueness patterns were illustrated as Venn diagrams, which were built with Venn v1.10 R package (*Dusa, 2017*). In the current study, this analysis was used to quantitatively demonstrate the shareability and uniqueness of microbial genera in each petroliferous region.

Bivariate statistical analysis

The existence of possible statistically significant quantitative associations between pairs of microbial genera among petroliferous regions, for each sample type, was evaluated by Bivariate Statistics, using correlation analysis. Hellinger data transformation was performed (*Legendre & Gallagher, 2001*), and the non-parametric Spearman correlation test was used. Results were depicted as correlograms, for each sample type, where only significantly correlated microbial pairs were exhibited ($p \leq 0{,}05$). The Spearman correlation test and corresponding correlograms were performed using PAST v. 4.08 (*Hammer, Harper & Ryan, 2001*).

Multivariate statistical analysis

A principal component analysis (PCA), which is a multivariate statistical analysis of ordination, was used to integratively explore all data. PCA summarizes the information of a very high number of original variables in a small set of statistical variables. In the current study, the interrelationships among sets of microorganisms were analyzed using the first two principal components (PC1 and PC2). After Hellinger transformation, data were analyzed by PCA, and visualized by two-dimensional scatter plots using ggplot2 v3.3.5 and factoextra v1.0.7 R packages (*Kassambara & Mundt, 2020*; *Wickham, 2011*).

Functional prediction analysis

The functional prediction analyses were performed on all the available 16S rRNA amplicon metagenomes. Initially, SRA (Sequence Read Archive) or similar codes of all samples of all the selected documents were retrieved, considering at least one access code for each sample (or more than one in case of technical replicates). A total of 23 documents had to be discarded since the access codes were not provided. Therefore, all samples from a total of 35 documents were automatically extracted using the SRA-Toolkit (*Leinonen, Sugawara & Shumway, 2011*) for those with SRA codes, and manually for those with non-SRA codes.

The metagenomes were treated with VSEARCH v2.17.1 (*Rognes et al., 2016*), and pair-end metagenomes were joined (–fastq_mergepairs) in a same *FASTQ* file.

In case of having technical replicates in the samples, these were concatenated in the same *FASTQ* file to be treated as the same sample. For each unique *FASTQ* file, low-quality sequences were removed (–fastq_filter) and identical sequences were fused (–derep_fulllength), and all the resulting processed sequences were concatenated in a single *FASTQ* file. Using this single *FASTQ* file, singleton and chimeric sequences were removed, both without and with reference to the scripts *–uchime_denovo* and *–uchime_ref*, respectively, with the databases of SILVA alignments prepared with MOTHUR (https://mothur.org/wiki/silva_reference_files). Finally, operational taxonomic units or OTUs (–cluster_size) were generated in both *FASTA* and *BIOM* file formats.

Functional inference of metabolic pathways based on MetaCyc database (*Caspi et al., 2018*) was performed using the standard pipeline of the software PICRUSt2 (*Douglas et al., 2020*). The software STAMP v2.1.3 was used to infer significant statistical differences in the functional composition among all samples (*Parks et al., 2014*). The differences in functional composition were analyzed by pairs of sample types (IW-PW, IW-SD, IW-OIL, PW-SD, PW-OIL e SD-OIL), using Welch's t-test with the method of Benjamini–Hochberg FDR for the correction of multiple tests. A p-value > 0.05 was used as a threshold to discard statistically non-significant correlation between metabolic pathways.

RESULTS

Study selection

Among the 547 documents retrieved, 69 articles were selected because they could be directly used to respond to the main question of our study "What is the composition of bacterial and archaeal communities putatively associated with MIC found at distinct locations along the process of oil producing and transporting systems worldwide?" (Supplementary Material 5). Figure 1 depicts the PRISMA flowchart describing all stages of study selection, screening, and inclusion.

A total of 552 records from the Web of Science, Scopus, PubMed, and OnePetro databases were retrieved; and 99 documents without DOI from the Web of Science and Scopus databases were removed. The *format_input.py script* and the *remove_duplicates.py* removed duplicate records (59 articles), according to identical DOI or identical title, and six documents were not available and thus defined as *Not available*. The remaining 388 documents were then downloaded in *pdf* format. We found 309 original articles and 79 other types of documents (book chapters, books, reviews, and letters), which were excluded.

Initially, the remaining 309 documents were manually screened, based on their title, abstract, and entire documents. When necessary, the exclusion criteria described in Table 1 were applied: (1) oil refinery and petrochemical industry; (2) samples obtained from lab cultures, and (3) molecular analysis by Denaturing Gradient Gel Electrophoresis (DGGE) instead of metagenomics.

After this initial screening, 207 documents were excluded since they did not meet these eligibility criteria. Despite the application of the inclusion and exclusion criteria, a total of

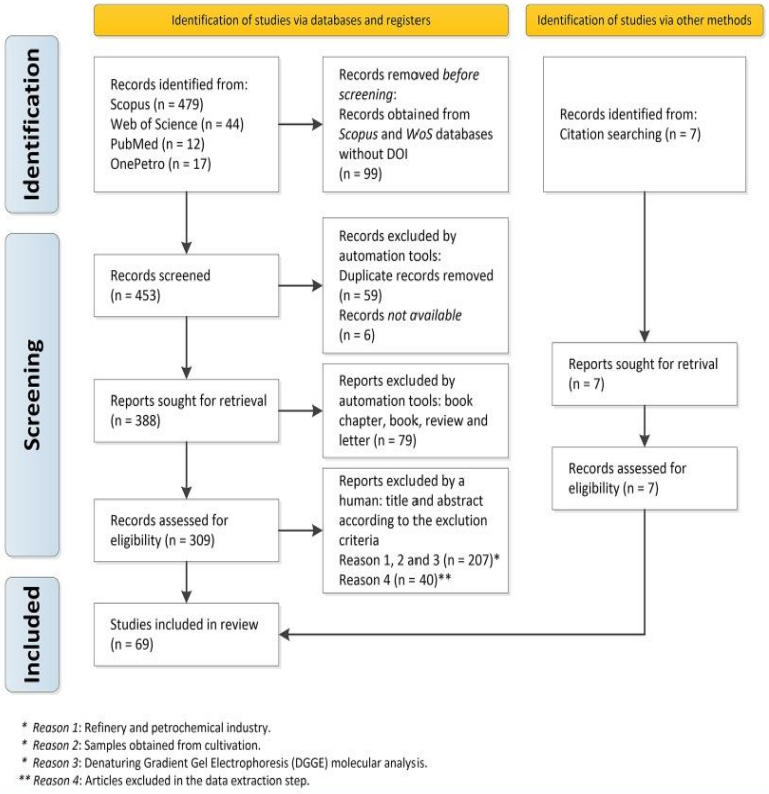

Figure 1 PRISMA flowchart describing all the steps of inclusion of the documents in detail: selection, sorting, and inclusion. Full-size DOI: 10.7717/peerj.14642/fig-1

40 documents did not comply with these criteria in the data extraction stage. Thus, in the step of data extraction, they were excluded. A total of 7 articles considered important for the review, but which had not been found in the database search, were also included: these articles were identified from the bibliographic references of previously selected documents. Therefore, a total of 69 articles were included in our systematic review for data analysis and synthesis.

Geographic location and sampling

Data was extracted and analyzed for each petroliferous region and each sample type. This method allowed the intrinsic analysis of the microbial profile of each variable (sample types and petroliferous regions). Figure 2 shows the distribution of petroliferous regions on the world map, and the number of samples for each region is depicted. A total of 368 samples were evaluated: 33 (8.97%) of IW, 25 (6.79%) of O, 36 (9.78%) of OW, 155 (42.12%) of PW, and 119 (32.34%) from SD (Supplementary Material 6). IW samples were analyzed from seven petroliferous regions, comprising a total of 18 articles, whereas PW was the sample type with the highest number of samples, described in 41 articles and distributed in seven petroliferous regions. O and OW samples were analyzed in eight and

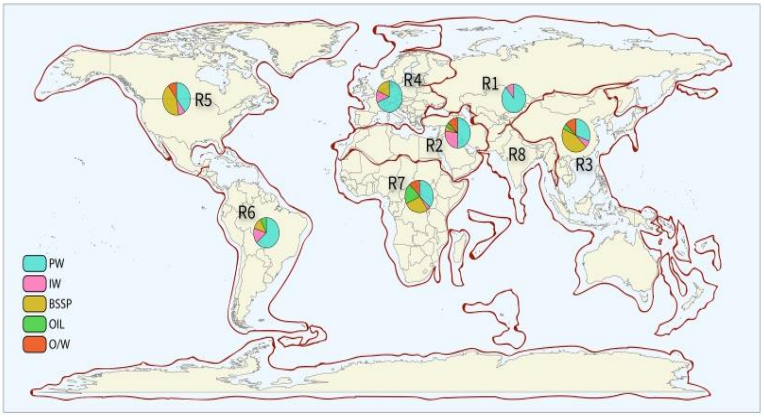

Figure 2 Sampling points by petroliferous region. The pie charts indicate the type and number of samples. PW—produced water, IW—injection water, BSSP—biofilm/sediment/sludge/pig, Oil—Oil, and O/W—oil/water mixture. Petroliferous regions are indicated by: R1—Former Soviet Union, R2—Middle East and North Africa, R3—Asia-Pacific, R4—Europe, R5—North America, R6—Central and South America, R7—Sub-Saharan Africa and Antarctica, and R8—South Asia. All the sector (pizza-like) graphics and the legend were derived from authors' study, and the map of petroliferous regions (in the background) where the sector graphs and legend were plotted are completely redrawn from: USGS World Petroleum Assessment 2000, publicly available at https://pubs.usgs.gov/fs/fs-062-03/FS-062-03.pdf, using R and Illustrator by the authors. Full-size DOI: 10.7717/peerj.14642/fig-2

nine articles, respectively. Finally, SD were characterized from 34 articles by both sample type and petroliferous regions.

The pie charts indicate the type and number of samples. PW—produced water, IW—injection water, BSSP—biofilm/sediment/sludge/pig, Oil—oil, and O/W—oil/water mixture. Petroliferous regions are indicated by: R1—Former Soviet Union, R2—Middle east and North Africa, R3—Asia-Pacific, R4—Europe, R5—North America, R6—Central and South America, R7—Sub-Saharan Africa and Antarctica, and R8—South Asia.

Table 2 shows the location, petroliferous regions, and countries of the oil fields reported in the selected articles. Samples from the 69 selected articles were distributed among seven petroliferous regions as follows: Region 1 (one article), Region 2 (six), Region 3 (18), Region 4 (eight), Region 5 (18), Region 6 (six), and Region 7 (12). No studies performed in the Region 8 were found. Moreover, each region displayed at least one country figuring in the ranking of the top 15 largest oil producers in the world (*IBP, 2020*).

Description of molecular biology data and physicochemical features

Molecular biology

To determine the composition of microbial communities and relative abundance of microbial lineages present in samples, the authors of selected documents extracted the community DNA and performed mainly amplicon (16S rRNA) metagenomics or, more rarely, shotgun metagenomics.

A total of five methods of metagenomic DNA extraction were identified: (1) commercial kits; (2) TRIzol-based; (3) phenol-chloroform-based; (4) QuickGene semi-automated

Table 2 Number of articles selected by petroliferous region, including the country or region corresponding to the samplings.

Petroliferous region	Country or geographic region	Articles (*n*)
1	Kazakhstan—12th	1
2	Saudi Arabia—2nd	4
	Algeria	1
	Tunisia	1
3	Australia	4
	China—7th	11
	Japan	1
	New Zealand	1
	Papua New Guinea	1
4	Germany	1
	Denmark	2
	Norway—15th	1
	England	1
	North Sea	3
5	United States—1st	12
	Canada—4th	4
	Mexico—13th	2
6	Argentina	1
	Brazil—10th	2
	Colombia	2
	Gulf of Mexico	1
7	Nigeria—11th	11
	Guinea	1
Total		**69**

platform; and (5) Maxwell Automated Platform (Supplementary Material 7). Among the 69 articles, 57 used one of these methods, one (1) described its own methodology, six (6) referred to other authors, which are not included in the selected documents, and six (6) did not report the extraction method applied. The most cited methods in the selected articles were commercial kits (43) and the Maxwell automated platform (2). The articles mentioned commercial kits for DNA extraction from different sample types: soil (i); biofilm (ii); water (iii), and general (iv). In the present systematic review, the kits manufactured and indicated for DNA extraction from plants, animals, bacteria, algae and fungi were grouped and named "General". The biofilm kits were used for SD samples, and the water kits for IW and PW samples. The TRIzol reagent was referenced by an article for extracting DNA from PW samples.

Metagenomic DNA extracted from the samples was amplified using specific primers for the 16S rRNA gene from bacteria and archaea. In 14 studies, the universal pair of primers 926F (5′-AAA CTY AAA KGA ATT GAC GG-3′) and 1392R (5′-ACG GGC GGT GTG TRC-3′) were used to amplify the V6–V8 region of 16S rRNA genes from bacteria and

archaea. Considering sample types, these primers were used for amplification in 27 of them: IW (4), PW (8), SD (13), and O (2).

The articles reported Sanger (1st generation) and NGS (new generation) sequencing platforms. The Sanger method, carried out in the ABI PRISM 310, ABI PRISM 377, ABI PRISM 3730 (ThermoFisher, Waltham, MA, USA) sequencers, were described in six studies, while 454 (Roche, Basel, Switzerland) was reported in 23 studies, and MiSeq (Illumina, San Diego, CA, USA) in 35 studies. Article 212 (*Hernández-Torres et al., 2016*) cited the sequencing service provider Macrogen (Seoul, South Korea) (https://dna.macrogen.com/), and articles 245 (*Gittel et al., 2009*) and 249 (*Aktas et al., 2017*; *Duncan et al., 2009*) mentioned the Joint Genome Institute (JGI) of the United States Department of Energy (https://jgi.doe.gov/). Articles 42 (*Kamarisima et al., 2018*) 65 (*Sharma et al., 2018*) and 177 (*Lenhart et al., 2014*) did not describe the sequencing platforms that were used.

Physicochemical features

In order to better understand the microbial communities, present in the studied samples, the physicochemical conditions of the sampled environments were analyzed, as well as the application (or not) of biocides and other possibly interfering chemical products. Microbial communities are dynamic, and the conditions prevailing in the systems will determine the community structure.

Therefore, in order to perform these analyses, data related to sampling point, temperature, pH value, salinity, sulfate and nitrate concentrations were collected, as well as data regarding the application of biocides and other chemical products (Supplementary Material 8). Among the included articles, 68.11% described the temperature, 63.77% the pH value, 56.52% the salinity, 68.11% the sulfate concentration, 28.98% the application of biocides and other chemical products, and 24.63% the nitrate concentration.

Integrative analyses of microbiological data by sample type and petroliferous region

Injection water

A total of 33 IW samples was characterized, Fig. 3. These analyses were carried out based on the relative abundances of microbial genera present in the IW samples.

The principal components (PC1 and PC2) of the ordination analysis (PCA) jointly explained approximately 40% of data variability (Fig. 3A). In addition, 17 genera that most influenced this variability were identified. Seven petroliferous regions were distributed in the four quadrants, as follows: group a (R3, R6 and R7), group b (R2), group c (R5) and group d (R1 and R4). Concerning the correlation analysis, only positive correlations were found (blue dots). Furthermore, groups of genera (G1 and G2) exhibiting strongly positive and statistically significant correlations were formed; however, with low relative abundance (Fig. 3B). There were no genera common to all regions but the R3 region had a higher number of unique microorganisms, followed by the R2 region (Fig. 3C).

Regions R1, R2, and R3 showed the highest number of genera, with high shareability and uniqueness, and relative abundances below 3% (0.34–2.94%)

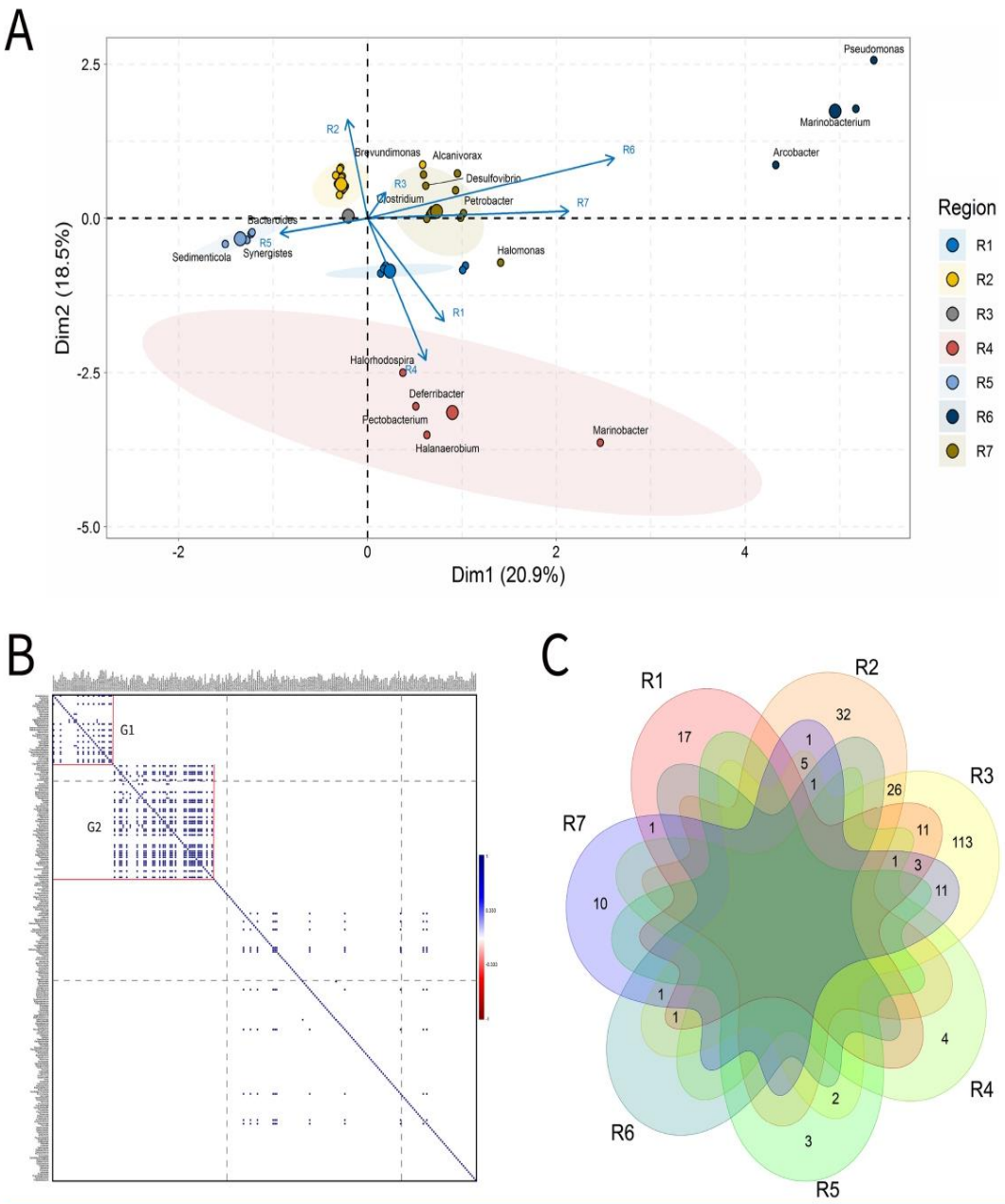

Figure 3 Integrative analyses of microorganisms in Injection Water (IW). (A) Principal components analysis; (B) Pairwise correlation between genera; (C) Shared and unique genera of the petroleum regions. This figure describes the integrative analyses of microorganisms in injection water (IW) (A) Principal components analysis; (B) Correlation of the microorganisms present in the injection water and (C) Venn diagram indicating the shared and unique bacterial and archaeal genera of the oil regions. Full-size DOI: 10.7717/peerj.14642/fig-3

(Supplementary Material 9), Moreover, no statistically significant correlations were identified. The regions R4, R5 and R6 presented genera with high relative abundances (13.33–33.33%), and, among them, only the R5 region displayed statistically significant correlation.

Five genera were retrieved in the R4 region, and four of them were unique to this region; the genus *Halanaerobium* stood out for its/dominance, exhibiting the highest relative abundance (26.67%). In the R5 region, five genera were also detected, three unique and two shared with the R3 region. The dominant genera in this region were *Bacteroides*, *Synergistes*, and *Sedimenticola*, with high relative abundances (16.67–25%) (Supplementary Material 9); however, they are not known to participate directly in MIC processes. Moreover, the genera *Bacteroides* and *Methylophaga* were significantly positively correlated (Fig. 3B). The R5 region was considerably distinct from the other petroliferous regions (Fig. 3A). No significant negative correlations were detected.

The R6 region also displayed a very different community profile, with the largest number of genera that significantly influenced data variability. Three genera dominated the community: *Arcobacter*, *Pseudomonas*, and *Marinobacterium*. In the R7 region, the two most abundant genera were part of those also found in the R6 region: *Pseudomonas* and *Marinobacterium* (6.67%) (Supplementary Material 9), which justifies the proximity between these two regions (Fig. 3A and Supplementary Material 9). Nevertheless, the genera that most influenced data variability in this region were *Halomonas* (4.44%), *Petrobacter* (4.44%), *Alcanivorax* (4.44%), and *Desulfovibrio* (2.22%). There were statistically positive correlations between the nitrate-reducing bacteria (NRB) *Halomonas* and methanogens (MET) *Methanolobus* and *Methanosaeta* (Fig. 3B). Additionally, the genus *Halomonas* also positively correlated with the methanogens of the genera *Methanobacterium* and *Methanocalculus*.

Produced water

PW samples are commonly evaluated to infer the microbial community present in subsurface environments (*Lipus et al., 2019*). For the analysis of PW, a total of 155 samples was characterized. In the ordination analysis, Fig. 4. PC1 and PC2 explained approximately 40% of the data variability with identification of 65 genera that most influenced this variability (Fig 4A). The seven petroliferous regions were distributed in two main groups: group b (R1, R2 and R5), and group c (R4 and R6) and those between these two groups (R3 and R7).

In the correlation analysis (Fig. 4B), both positive and negative statistically significant correlations between pairs of genera were retrieved. A large highly positively and significantly correlated group was formed, and this group comprised approximately 30 genera, some of which had considerably high relative abundances.

R3 region exhibited a higher number of exclusive genera (Fig. 4C). Additionally, two genera common to all regions were identified, which are representatives of the acid-producing (*Thermovirga*) and sulfate-reducing (*Desulfotomaculum*) functional groups.

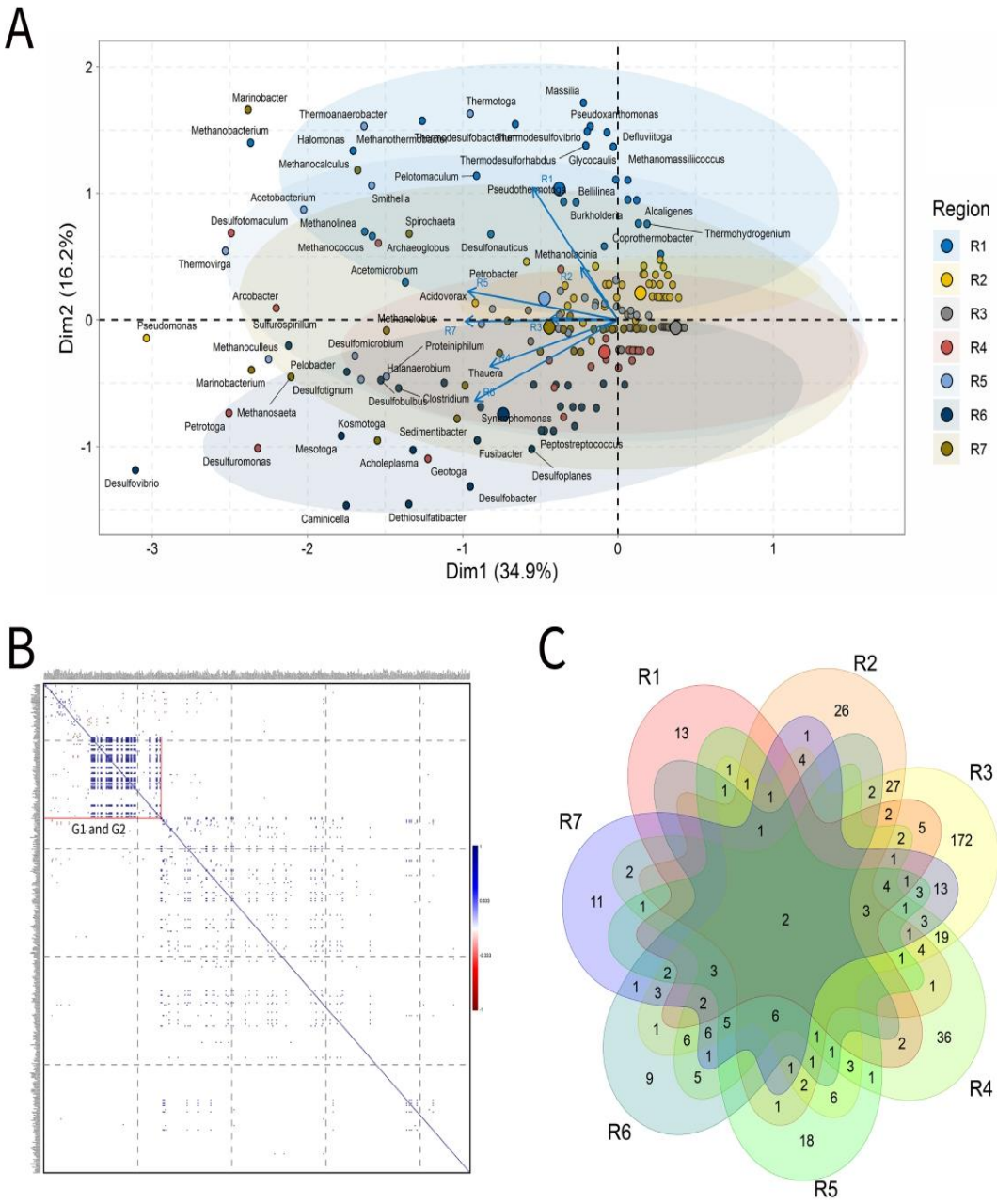

Figure 4 Integrative analyses of microorganisms in Produced Water (PW). (A) Principal components analysis; (B) Pairwise correlation between genera; (C) Shared and unique genera of the petroleum regions. This figure describes the integrative analyses of produced water oil regions (PW). (A) Principal components analysis; (B) Correlation of microorganisms present in produced water and (C) Venn diagram indicating the shared and unique bacterial and archaeal genera of petroleum regions. Full-size DOI: 10.7717/peerj.14642/fig-4

The R1 region was composed of 45 genera, with 13 unique to it (Fig. 4C). All of these genera have a relative abundance between 0.58% and 4.07%. Statistically positive correlations were observed not only between genera within the same functional group, such as sulfate-reducing bacteria (SRB), MET and acid-producing bacteria (APB) but also between different functional groups (MET and SRB), (MET and iron-reducing bacteria—IRB) and (sulfur-reducing bacteria—S^0RB and APB) (Fig.4B). Among the statistically negative correlations, there were also correlations within the same functional group (MET) and between different functional groups (aerobic bacteria of the class Betaproteobacteria, NRB, SRB, and S^0RB), (MET and APB) and genera of the G1 group (*Brevundimonas*, *Sphingomonas*, and *Tistrella*) from the R2 region, with an abundance of less than 3.0%, aerobes of the class Alphaproteobacteria (Fig. 4B and Supplementary Material 9) (Supplementary Material 9). Those genera with the highest relative abundance comprised *Defluviitoga* (4.07), *Thermodesulfovibrio* (4.07%), *Pelotomaculum* (4.07%), *Desulfotomaculum* (4.07%) (Supplementary Material 9).

The R2 region display important genera of the phylum Proteobacteria that correlated significantly and positively, such as *Petrobacter* and *Pseudomonas*, which are NRB. Negative correlations were also retrieved between NRB and APB of the genera *Pseudomonas* and *Acetomicrobium* (2.33%), respectively, from the R1 region.

The R3 region was composed of 322 genera, of which 172 were unique to it (Fig. 4C), and presented only genera with relative abundances below 2.0%. This region exhibited fourteen genera with an abundance above 1.0%, and those with the highest abundance values were *Pseudomonas* (a hydrocarbon-degrader NRB) and *Methanosaeta* (MET) with 1.61%, (Supplementary Material 9). Among the diverse genera that were identified, a total of 18.75% were constituted by genera of the class Deltaproteobacteria, which contains many SRB, and 18.18% of the phylum Euryarchaeota, in which all representatives are MET.

The R4 region was composed of 123 genera, with 36 unique to it (Fig. 4C). The genera that effectively influenced data variability were: sulfidogenic archaea, *Archeoglobus* (5.00%); NRB, *Arcobacter* (2.73%); SRB, *Desulfotomaculum* (4.09%) and *Desulfuromonas* (3.64%); S^0RB, *Petrotoga* (3.18%) and *Geotoga* (2.27%), (Fig. 4A and Supplementary Material 9). In addition, there were significant positive and negative correlations between some genera (Fig. 4B). Two sulfidogenic genera *Desulfuromonas* and *Geotoga* were positively correlated. *Desulfuromonas* was also positively correlated with another sulfidogenic genus abundant in the R6 region, *Desulfovibrio* (SRB). These sulfidogenic genera (*Desulfuromonas* and *Geotoga*) were correlated negatively with the thermophilic and hydrogenotrophic methanogen *Methanothermobacter*, which was the one that most contributed to explain data variability in the R1 region. In turn, the genus *Geotoga* was also correlated with other genera, highlighting negative correlations with the thermophilic *Thermoanaerobacter*, an iron-reducing genera (*Slobodkin et al., 1999*), and S^0RB *Thermotoga* from the R5 region (*Davey et al., 1993*). A negative correlation was also found between *Petrotoga* and the thermophilic fermentative and aerobic NRB *Halomonas* from the R1 region.

The R5 region was composed of 72 genera and, among them, 18 unique to that region (Fig. 4C). The genera that significantly influenced the ordering of the R5 region were: *Methanoculleus* (5.21%), *Acetobacterium* (4.86%), *Desulfomicrobium* (4.17%), *Smithella* (3.12%), *Thermotoga* (2.78%), *Thermoanaerobacter* (2.43%), and *Proteiniphilum* (2.08%) (Fig. 4A and Supplementary Material 9). The genera in this region were positively correlated (Fig. 4B). The APB *Acetobacterium* and *Smitella* were positively correlated between them and also with other genera from different petroliferous regions. *Acetobacterium* positively correlated with *Methanocalculus*, a hydrogenotrophic methanogen abundant in the R7 region. Additionally, *Smitella* was positively correlated with *Desulfonuticus*, an elemental sulfur or thiosulfate reducer with high abundance in the R1 region. There were also correlations between thermophilic anaerobic genera with less than 3.0% abundance, such as *Thermoanaerobacter*, with iron reducers, and *Thermotoga*, an S^0RB and APB. These aforementioned genera were also correlated with MET, S^0RB, and APB.

The R6 region presented 59 genera, with nine unique to this region (Fig. 4A). Several genera that significantly influenced the ordering of this region were represented; however, those with the highest relative abundance were: *Desulfovibrio* (5.45%), *Desulfobacter*, *Caminicella*, *Mesotoga*, and *Dethiosulfatibacter* (all these genera with 4.55%). This region has statistically significant positive and negative correlations (Fig. 4B). The genera SRB *Dethiosulfatibacter* and *Desulfobacter* (*Lien & Beeder, 1997*; *Takii et al., 2007*) were positively correlated with each other and with the genus *Geotoga* (S^0RB) in the R4 region. Additionally, *Dethiosulfatibacter* and *Desulfobacter* were also negatively correlated with *Marinobacter*, a NRB that was dominant in the R7 region. The S^0RB *Caminicella* was positively correlated with other S^0RB genera in the R4 and R7 regions; and negatively with the thermophilic S^0RB genera, the most relevant in the R5 region, as well as with thermophilic MET *Methanothermobacter* in the R1 region. The SRB *Desulfovibrio*, displayed significant positive correlations with NRB and other SRB genera that are resistant to pollutants, such as *Sulfurospirillum* from the R7 region (*Goris & Diekert, 2016*) and S^0RB genera from the R4 region. Furthermore, it also negatively correlated with the aerobic genus *Massilia*, which is influential in the R1 region.

The R7 region was composed of 84 genera, 11 of which were unique to it (Fig. 4A). The most important genera that were central in the ordination analysis of this region were: *Thauera* (3.05%), *Petrobacter* (3.06%), *Methanobacterium* (3.07%), *Methanocalculus* (3.05%) (Fig. 4) and *Methanosaeta* (3.32%), *Spirochaeta* (3.60%), *Thermanaerovibrio* (3.88%), *Marinobacter* (4.16%), *Pseudomonas* (4.16%), *Methanolobus* (4.43%), and *Marinobacterium* (4.71%) (Fig. 4A and Supplementary Material 9). Four groups of positively correlated genera were observed (Figs. 4B and 4C). The first one included the APB *Spirochaeta*, and the MET *Methanococcus*, (with higher abundance in the R1 region) and the SRB *Desulfomicrobium*, which was the most relevant in the R5 region. The second one comprised the MET *Methanocalculus*, and the hydrocarbon-degrader NRB *Halomonas* (R1) and *Marinobacter* (R7). The third one was composed of the MET *Methanobacterium* and the APB *Acetobacterium* (R1). Finally, the fourth one, which displayed correlation between the NRB *Marinobacterium* and the MET *Methanoculleus*,

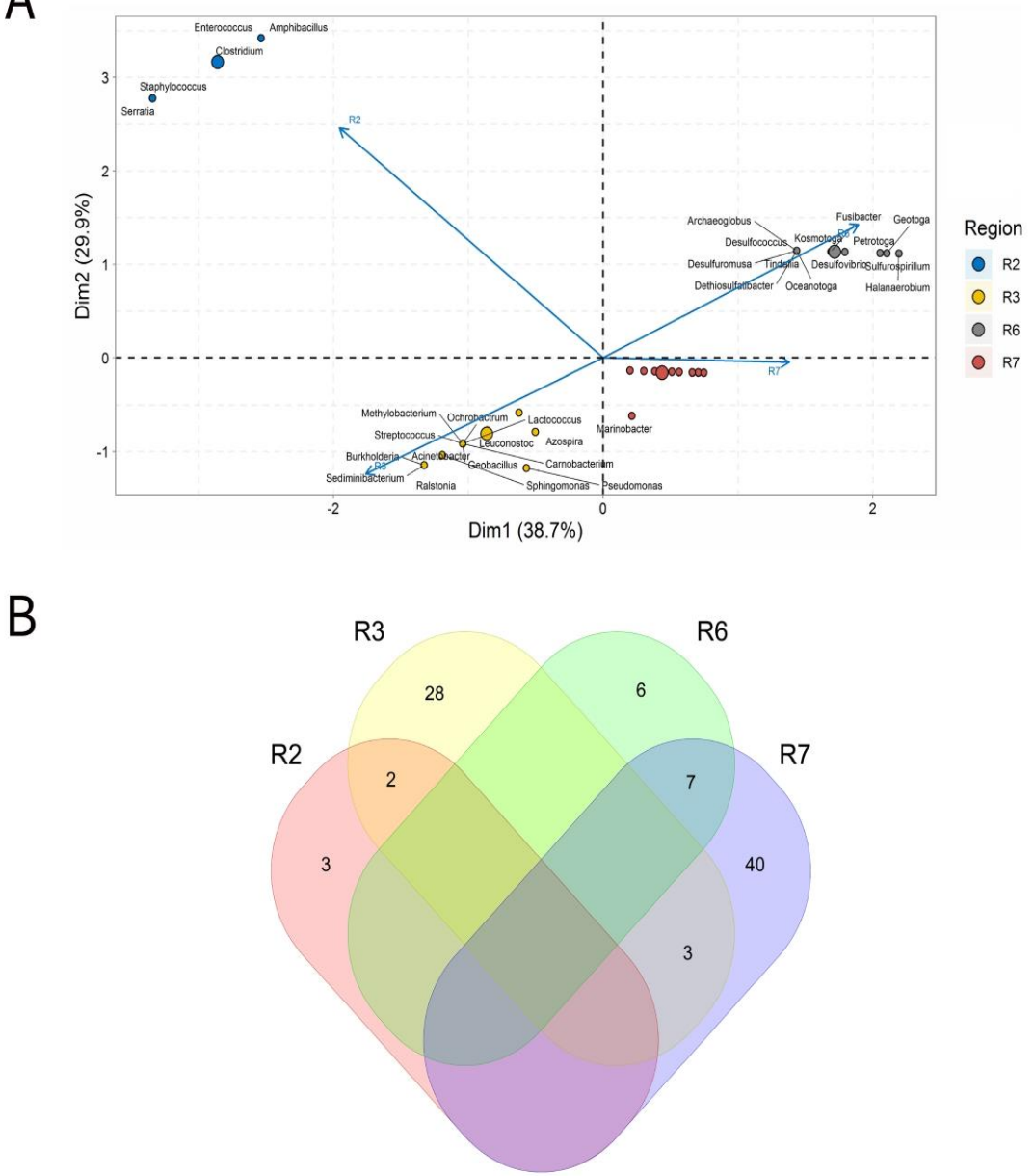

Figure 5 Integrative analyzes of oil samples in oil fields. (A) Principal component analysis and (B) Venn diagram indicating the shared and unique bacterial and archaeal genera of petroleum regions. Full-size DOI: 10.7717/peerj.14642/fig-5

the most relevant in the R5 region, *Methanosaeta* and *Methanolobus*, the most relevant in this R7 region. Regarding the negative correlations, the genus *Marinobacterium* was correlated with the aerobic genus *Massilia*, which was the most relevant in the R1 region.

Oil (O) and Oil/Water

In the present study, 25 O samples and 36 OW samples were analyzed; and both did not exhibit genera that were statistically significantly correlated (Fig. 5). In the ordination analysis, PC1 and PC2 explained almost 69% of the data variability (Fig. 5A). This type of sample was represented by 33 most relevant genera, grouped in four distinct groups: group a (R6), group b (R2), group c (R3), and group d (R7). It is observed that the vectors of regions R3 and R6 were in opposite directions, which indicates possible negative correlations between the genera of these regions, but these correlations were not significant. Genera shared by all the petroliferous regions were not identified, and the R3 region had a high number of unique genera when compared to the other ones (Fig. 5B).

The R2 region (Fig. 5A), had a greater distance in relation to the other regions. A total of 90% of the genera was from Bacilli and Clostridia classes of the Firmicutes phylum, such as *Amphibacillus*, *Clostridium*, *Enterococcos*, *Serratia*, and *Staphylococcus* (Fig. 4C). These classes contain APB, such as *Clostridium*, that accounted for a relative abundance of 20% (Supplementary Material 9). In the R3 region, 14 genera were considered those that most contributed to the ordination of this region; however, the genera with the highest relative abundance were *Ralstonia*, *Burkholderia*, *Sediminibacterium*, and *Pseudomonas* (all of them with 6.49%) (Fig. 4A and Supplementary Material 9).

The R6 region had 13 genera, of which 6 are unique (Fig. 5B), and all genera of this region were represented in the ordination analysis (Fig. 5B) displayed a relative abundance of 7.69%. In this region, a clear dominance of SRB and high abundance of APB and S^0RB was observed. On the other hand, in the R7 region, only one genus that influenced the data variability was highlighted, *Marinobacter* (NRB), with a relative abundance of 4.58%. Regarding the analysis of shareability and uniqueness, this region exhibited 50 genera with 40 unique ones (Fig. 5B).

The results of the integrative analyses related to the OW samples are depicted in Fig. 6. In ordination analysis, PC1 and PC2 explained approximately 69% of the data variability (Fig. 6A), and a total of 47 genera were the most relevant for the ordination in three groups: group a (R3), group b (R2) and group c (R5 and R7). Unlike the other samples types (IW, PW), there were OW data only for four petroliferous regions (R2, R5, R3 and R7) (Fig. 6B). Moreover, genera shared with all regions were not identified, and the R3 region displayed a high number of unique genera when compared to the other regions.

The R2 and R3 regions were found to be distant from each other and from the R5 and R7 regions. In the R2 region, 16 genera were described, and 6 of them were unique to this region (Fig. 6B). The genera with the highest relative abundances were the sulfidogens *Pelobacter*, *Petrotoga*, *Acetomicrobium*, *Clostridium*, *Flexistipes*, *Thermoanaerobacterium*, and *Thermovirga*.

The regions R2 and R3 (Fig. 6B) were far from each other and from the other regions, which may indicate that they have unique features. In the R2 region, 16 genera were

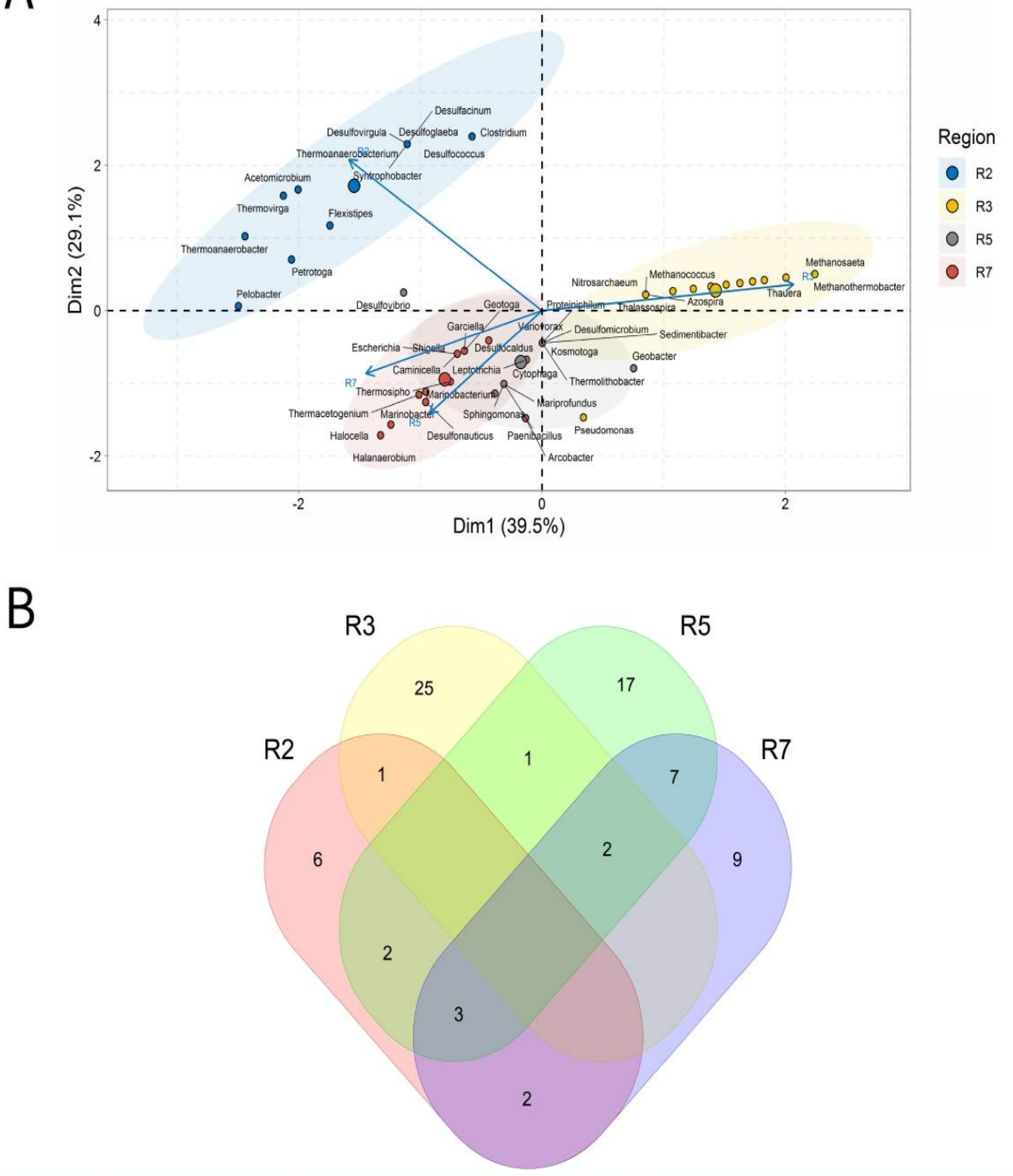

Figure 6 Integrative analysis of oil and water mixture samples (OA). (A) Principal component analysis and (B) Venn diagram indicating the shared and unique bacterial and archaeal genera of petroleum regions. Full-size DOI: 10.7717/peerj.14642/fig-6

retrieved, and 6 of them were unique (Fig. 6A). Relative abundances varied between 2.63% and 7.89%, and only *Desulfovibrio* (2.63%) exhibited an abundance below 3.0% (Supplementary Material 9). In the R3 region, 29 genera were described, and 25 of them were unique (Fig. 6B). Nine genera were the most relevant to data variability (Fig. 6A), including representatives of the functional groups NRB, S^0RB, and MET, with *Methanosaeta* and *Methanothermobacter* (8.33%) as the most abundant genera. The R5 and R7 regions were relatively close to each order in the ordination analysis when compared to the other regions (Fig. 6A). These regions displayed genera with relative abundances between 1.14–5.68% (Supplementary Material 9).

The R5 region presented 32 genera, with 17 of them unique to this region (Fig. 6B). The most important genera for the ordination of the R5 region werethr IRB *Geobacter* and the SRB *Desulfovibrio* (Fig. 6A), besides 17 genera with an abundance greater than 3.0%, including those from NRB (Supplementary Material 9). In the R7 region, 23 genera (of which six unique ones) were described (Fig. 6B): there was dominance of APB (*Pelobacter* e *Acetomicrobium*) NRB (*Marinobacter* e *Marinobacterium*), thiosulfate-reducing bacteria —TRB, and S^0RB (*Thermacetogenium*, *Thermosipho*, *Geotoga, Desulfonauticus*), as well as a smaller number of SRB were also detected, as well as in the R5 region (Fig. 4A).

Solid deposits

For BSLP, a total of 155 samples was retrieved and analyzed, Fig. 7. In the ordination analysis, PC1 and PC2 explained 52.5% of data variability, and the petroliferous regions were distributed in two groups: group b (R2 and R5) and group c (R3, R4, R6 and R7) (Fig. 7A). In the correlation analysis, there were significant positive correlations and a small number of significant negative correlations (Fig. 7B). Nonetheless, extensive groups of correlated genera were not formed, as happened in IW and PW samples. There were representatives in six petroliferous regions: R2, R3, R4, R5, R6 and R7 (Fig. 7C). Additionally, only one genus was shared among all regions: the NRB *Marinobacter*. As occurred for IW, PW and OW samples, the R3 region of SD exhibited the highest number of unique microorganisms.

The R2 region (group b) was segregated from all the other regions in the ordination analysis (Fig. 7A), and showed only 10 genera, with four of them unique to this region (Fig. 7B). The NRB *Chromohalobacter*, (*Aguilera et al., 2007*) and the TRB *Halanaerobium* were significantly positively correlated (Fig. 7C). On the other hand, the NRB *Halomonas* was negatively correlated with the MET *Methanosarcina*, which was more abundant in the R6 region: these genera showed a relative abundance equal to 10% (Supplementary Material 9).

In the ordination analysis, the R3, R4, R5, R6 and R7 regions were relatively closer to each other than with R2 region (Fig. 7A). The R3 region had 173 genera, of which 117 unique to it (Fig. 7B), with an abundance of less than 3.0%. This region showed genera from the functional groups: hydrocarbon-degraders, APB and IRB, and the most abundant was *Pseudomonas* (2.78%), followed by the IRB *Shewanella* (2.20%), and APB, such as *Proteiniphilum* (2.22%) (Supplementary Material 9).

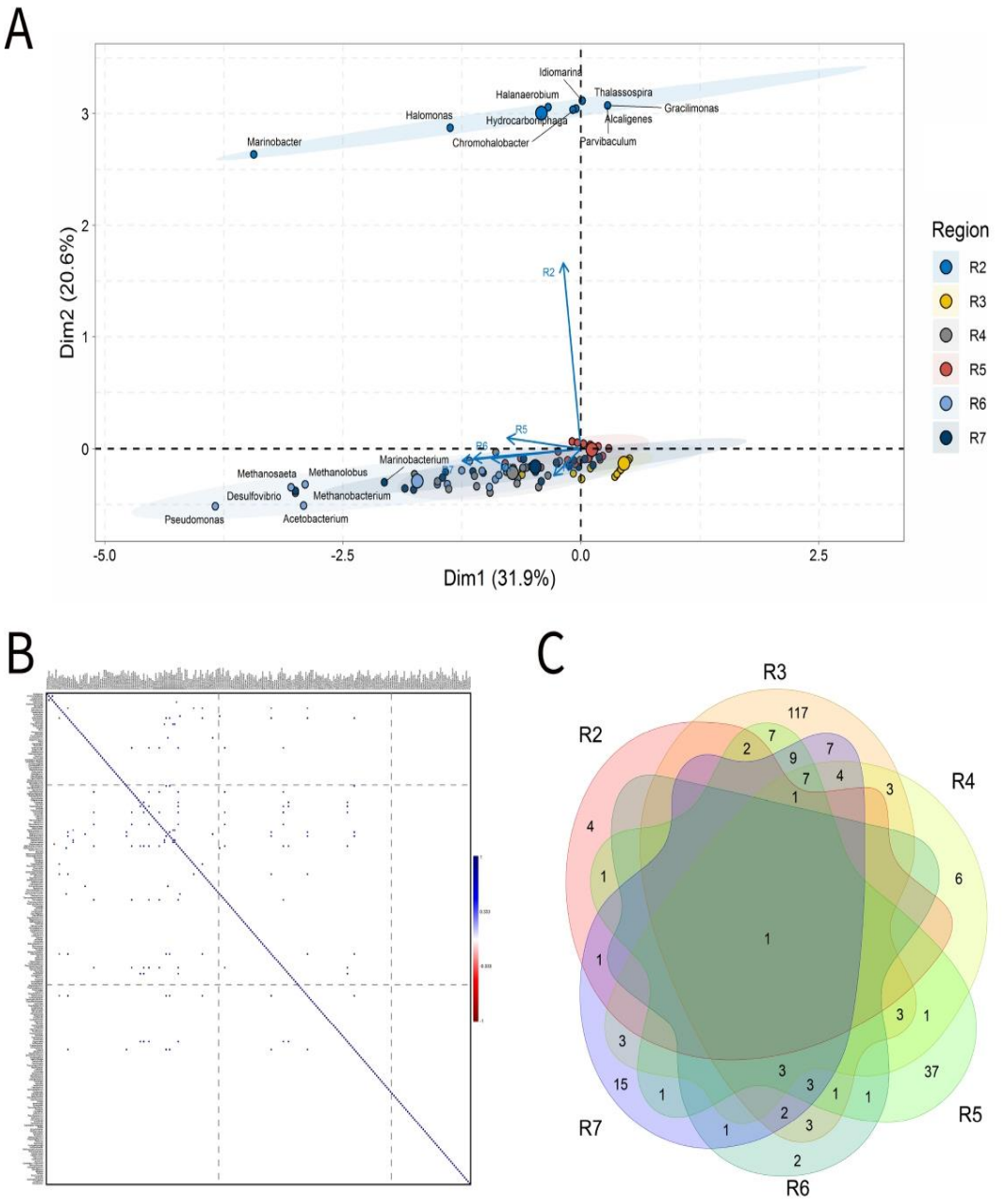

Figure 7 Integrative analyses of microorganisms in solid deposit samples (BSLP). (A) Principal components analysis; (B) pairwise correlation between genera; (C) shared and unique genera of the petroleum regions. This figure describes the integrative analyses of solid deposit samples (BSLP) in oil fields. (A) Principal components analysis; (B) correlation of microorganisms present in produced water and (C) Venn diagram indicating the shared and unique bacterial and archaeal genera of petroleum regions. Full-size DOI: 10.7717/peerj.14642/fig-7

The R4, R5, R6, and R7 regions displayed similar genera and shared many functional groups. The R4 region had 32 genera, of which six unique to it (Fig. 7B), with relative abundances between 1.61% and 6.45%. This region was dominated by sulfidogenic thermophilic genera (S^0RB and SRB), NRB, and MET, with greater abundance of *Thermococcus* and *Desulfonatrospira* (6.45%), followed by the *Methanothermococcus* (4.84%).

There were statistically significant positive correlations between the MET *Methanothermobacter* and genera with an abundance above 3% in the NRB, APB, S^0RB and IRB functional groups. In turn, the R5 region had 78 genera, 37 of which are unique (Fig. 7B). The relative abundance varied between 0.52% and 4.17%, with *Pseudomonas* (4.17%) as the most abundant, followed by *Desulfomicrobium*, *Desulfovibrio*, and *Methanosaeta* (3.65% each).

The R6 region exhibited 17 genera, with two unique to it (Fig. 7C). This region displayed genera associated with NRB, SRB, APB, and MET functional groups (Figs. 7B and 7C). Two groups were significantly positively correlated: the first one between MET *Methanococcus* and the APB *Acetobacterium*; and the second one between MET *Methanolobus* and *Methanosaeta* and the hydrocarbon-degrader NRB *Pseudomonas*. Furthermore, the later correlated positively with the most abundant genera in the R7 region: the MET *Methanobacterium*.

The R7 region had 58 genera, of which 15 unique to it (Fig. 7B), with relative abundances between 0.38% and 6.82%. Six main genera were detected, and only the S^0RB *Thermanaerovibrio* did not significantly correlate with the other genera. In the R7 region, there were positive correlations between genera within the region and with genera of the R4 region, which had an abundance greater than 3.0%. The MET *Methanoculleus* and *Methanocalcullus* correlated positively each other and also with *Thauera* (3.41%), anaerobic hydrocarbon degrader. The NRB *Marinobacterium* and the S^0RB *Kosmotoga* were positively correlated with each other and also with APB *Sedimentibacter*. Moreover, *Marinobacterium* and *Kosmotoga* (3.23%) also correlated with genera of S^0RB, APB, and MET groups in the R4 region (*Desulfuromonas*, *Anaerophaga Methanothermobacter* and *Thermosipho*). The S^0RB *Kosmotoga* correlated with the APB *Spirochaeta* and *Geoalkalibacter* (3.23%) from the R4 region. *Geoalkalibacter* is strictly anaerobic, and there are species that can utilize Fe(III), Mn(IV), nitrate, elemental sulfur and trimethylamine *N*-oxide as terminal electron acceptors, and a wide variety of organic acids as electron donors. The most abundant genus is *Methanobacterium* (6.82%), followed by *Methanolobus* and *Methanosaeta* (5.68%). This region was dominated by the hydrogenotrophic methanogens *Methanobacterium*, *Methanocalculus*, *Methanoculleus*, and *Methanosaeta*.

Functional prediction

Among the 16S rRNA metagenomes recovered from different sample types, a total of 440 metabolic pathways was identified in Metacyc at the highest classification level using PICRUSt2. Removal of statistically non-significant metabolic pathways ($p \geq 0.05$) resulted in a total of 167 pathways, Fig. 8. Subsequently, these pathways were manually classified according to Metacyc level 2 in order to facilitate understanding, totaling 26 significant

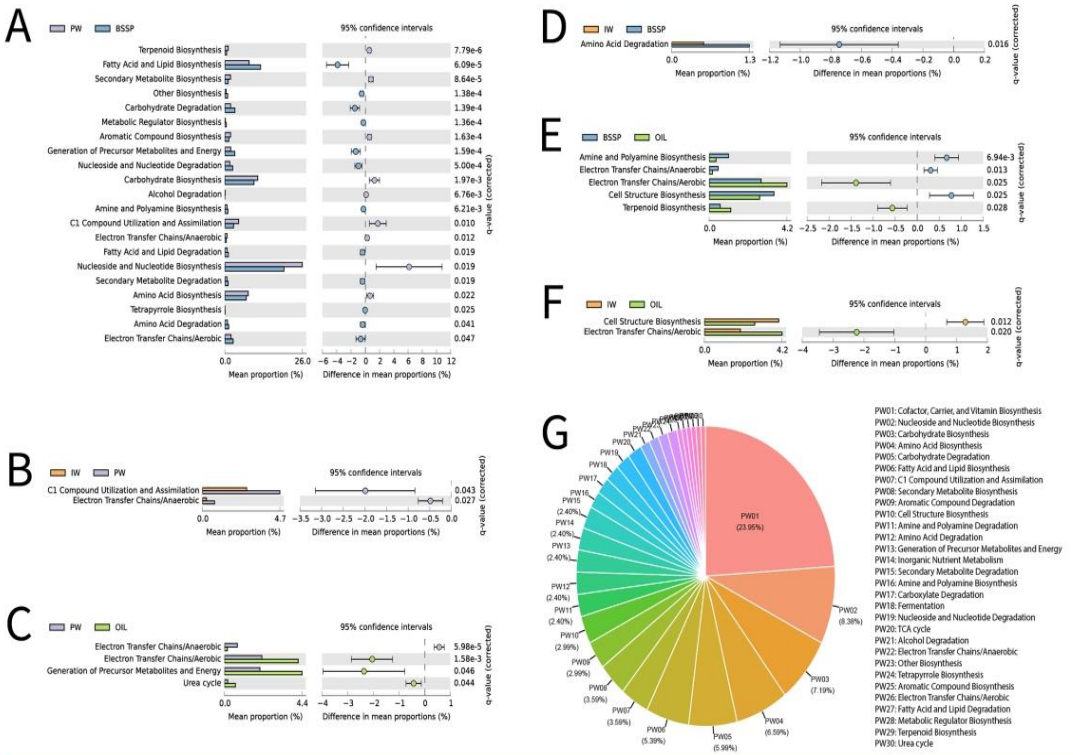

Figure 8 (A–G) Extended error bar graph for comparing analysis module of two groups of MetaCyc functional data predicted by PICRUSt2, and the pie graph illustrating the predicted functions in the different sample types. Full-size DOI: 10.7717/peerj.14642/fig-8

metabolic pathways. The most frequent functions in these samples were (in this order): (1) Cofactor, Carrier, and Vitamin Biosynthesis, (2) Nucleoside and Nucleotide Biosynthesis, (3) Carbohydrate Biosynthesis, (4) Amino Acid Biosynthesis, (5) Carbohydrate Degradation, and (6) Fatty Acid and Lipid Biosynthesis (Fig. 8G).

Extended error bar graph was used for the comparison between two samples. The bar graphs on the left display the average proportion of each MetaCyc pathway, while the dot plots on the right show the differences in the average proportions between two samples: A—produced water (PW) and solid deposits (BSSP); B—injection water (IW) and produced water (PW); C—produced water (PW) and oil; D—injection water (IW) and solid deposits (BSSP); E—solid deposits (BSSP) and oil; F-injection water (IW) and oil. Only predicted functions with $p < 0.05$ are shown; G—pie chart of predicted functions and substrate types.

A pairwise comparative analysis of the metabolic prediction of the different sample types were carried out to identify the greater or lesser similarity between sample types and functional categories from the different petroliferous regions (Fig. 8). Since PW and SD retrieved a higher number of 16S rRNA metagenomes when compared to the other sample types, the combination of these sample types (PW and SD) also exhibited higher amounts

of common functions (21), followed by the combination of SD and O (five), PW and O (four), IW and PW (two), IW and O (two) and IW and SD (one) (Figs. 8A–8F).

For PW and SD, Nucleoside and Nucleotide Biosynthesis was the most expressive functional category, followed by Fatty Acid and Lipid Biosynthesis, Carbohydrate Biosynthesis, and Amino Acid Biosynthesis (Fig. 8A). The most relevant difference between these samples was the higher abundance of Nucleoside and Nucleotide Biosynthesis in PW and Fatty Acid and Lipid Biosynthesis in SD. In contrast, in the combination of PW, IW and O, the metabolic pathways were quite distinct. Between PW and IW, C1 Compound Pathway Utilization and Assimilation stood out, with higher abundance in PW (Fig. 8B). In relation to PW and O, the most expressive pathways were Electron Transfer Chains/Aerobic, and Generation of Precursor Metabolites and Energy, which were more abundant in O samples (Fig. 8C). Regarding the combinations of SD with IW, there was greater prominence of the Amino Acid Function Degradation (Fig. 8D). On the other hand, in the combination between SD and O, the Electron Transfer Chains/Aerobic were observed with higher abundance in O samples, and Cell Structure Biosynthesis was more abundant in SD (Fig. 8E). These functional categories also stood out in the combination of IW and O: Cell Structure Biosynthesis was more abundant in IW whereas Electron Transfer Chains/Aerobic was more abundant in O (Fig. 8F).

DISCUSSION

This study evaluated the microbial profile of samples associated with the oil industry (PW, IW, OW, OIL, and SD) in seven petroliferous regions, comprehending several countries all over the world. Many of these countries figure in the ranking of the top 15 oil producers in the world (*IBP, 2020*) and each petroliferous region has at least one country classified in this ranking (Table 2). This demonstrates, in general, that the current systematic review constitutes a representative sample of the world scenario, referring to the composition and abundance of microorganisms present in petroliferous regions. The countries with the highest number of articles were the USA, China, and Nigeria. USA is the largest oil producer in the world, China occupies the seventh place, and Nigeria, the eleventh place. Thus, this scenario can corroborate the growing interest of these countries in research related to the oil sector.

The PW and SD sample types were the most numerous, with 155 (42.12%) and 119 (32.34%) samples, respectively, out of a total of 368. PW is one of the most common sources of microbiological samples (*Rachel & Gieg, 2020*), mainly because it is considered a representative sample of the oil field, for having had direct contact with the oil reservoir and injection water, making it possible to predict circulating (planktonic) microorganisms in the water column, besides the logistic facilitation if compared to collections carried out directly in subsurface. On the other hand, SD allows the analysis of sessile microorganisms, which form biofilms, responsible for localized corrosion (*Carrascosa et al., 2021*). These samples are obtained through pig residues and/or swabs of metallic coupons, commonly used in oil industries to monitor corrosion rates (*Sliem et al., 2021*). Furthermore, pigs are the only equipment that can access the entire length of a pipeline, for cleaning and inspection purposes, and can be complemented by metallic coupons, which are fixed at

points (located at the beginning and end of pipelines due to logistic facilitation) on the walls of the ducts to be exposed to internal conditions. Nevertheless, as many ducts are not possible to be inspected by pigs, it is not always possible to obtain this type of sample in oil industries.

Regarding molecular biology methods, we detected that 62.3% of the documents performed DNA extraction using commercial kits specific for each sample type. This is justified by the complexity of the material, as they may have elements that influence cell lysis, isolation of nucleic acids or polymerase activity (*Oldham et al., 2012*; *Wilson, 1997*). For DNA sequencing, 84% of documents used NGS platforms. Regarding the data obtained from physicochemical characteristics and application of chemical products, we realized that, although usually most of the articles offered information on at least one parameter, there was no priority in reporting this information, or there was even a lack of such information. This scarcity of such kinds of data may have occurred due to the difficulty to obtaining them.

Concerning the IW, PW and SD samples, the region R3 presented the smallest number of genera able to explain data variability (Figs. 3C, 4C, 5B, 6B and 7C), with relative abundances inferior to 3.0%. Additionally, in all sample types, except for O, there was a higher number of unique genera (Figs. 3C, 4C, 5B, 6B and 7C). One of the probable explanations for this pattern would be the high number of studies (18) distributed just in five countries with a greater prominence in China, with 11 articles (Table 2). This likely favored data heterogeneity, and thus a high variability of the physicochemical characteristics and application of chemical products. Therefore, this scenario resulted in an increase in the diversity of organisms with different metabolisms (*Liu et al., 2019*) evaluated the changes in microbial diversity in petroleum exploration, and concluded that environmental variation (temperature, O_2 concentration, pH value, and salinity) (Supplementary Material 8) alters the diversity of microbial communities during oil production.

In IW samples, the R6 region displayed the largest vector, indicating greater importance in explaining data variability, highlighting the genera *Arcobacter*, *Pseudomonas*, and *Marinobacterium*. The regions R6 and R7 were the closest ones, which suggests a higher similarity between the variables, sharing the genera *Pseudomonas* and *Marinobacterium*. The R4 also highlighted, exhibiting the *Marinobacter* genus, which is present in relative abundances above 2% in the R1 and R7 regions.

The genus *Arcobacter* participates in elemental sulfur oxidation, nitrogen metabolism, and iron and manganese reduction. Moreover, many studies suggest that these bacteria may not be native of oil reservoirs, but artificially introduced (*Bedoya et al., 2021*). Bacteria of the genus *Pseudomonas* are found in diverse terrestrial and aquatic environments, including oil reservoirs (*Dellagnezze et al., 2016*). *Pseudomonas* is a versatile, heterotrophic, facultative aerobic genera with evidence of fermentation (*Jeon, Yi & Park, 2014*; *Ramos, 2018*) and capable of degrading high fractions of crude oil constituents in the presence of O_2 but it can also prevail under anaerobic facultative conditions in oil/water mixtures (*Bedoya et al., 2021*). On the other hand, the genus *Marinobacterium* is facultative anaerobic (*Conlette & Nnameka, 2018*) and consists of nitrate-reducing species. In turn,

Marinobacter species, besides being nitrate reducers, are halophilic, mesophilic, and tolerant to pH variations from 6.0 to 9.5; they are aerobic with non-fermentative metabolism and can grow anaerobically in the presence of nitrate and acetate, but do not grow in the presence of glucose. They use nitrate as terminal electron acceptor, generating N_2, and can utilize acetate and other organic acids as the sole carbon source. Furthermore, they are capable of degrading a wide range of hydrocarbons (aliphatic and aromatic) in anaerobic conditions (*Duran, 2010*).

The dominance of the NRB group may have been favored by the application of biocides. *Bedoya et al. (2021)* evaluated the microbial community and biocide resistance profile in PW and IW of an oil reservoir in Colombia: the injected water was treated with biocides glutaraldehyde and tetrakis (hydroxymethyl) phosphonium sulfate (THPS). The authors reported a dominance of the genera *Arcobacter* and *Pseudomonas* found more than 20 genes associated with antibiotic and biocide resistance. One of these genes was *opr*N (outer membrane efflux protein), which encodes a multidrug efflux pump related to glutaraldehyde resistance (*Vikram, Bomberger & Bibby, 2015*). They concluded that the biocides applied in the IW probably had induced significant changes in the community structure, since long-term treatment with biocides can induce a selection process, which reduced diversity and strongly favored the genera *Arcobacter* and *Pseudomonas*.

The presence of SRB was also detected. According to *Sokolova et al. (2020)* the constant reinjection of PW results in contamination with sulfidogens, which can promote equipment corrosion and cause oil biodegradation.

In PW, we found that there were two genera common to all petroliferous regions (*Desulfotomaculum* and *Thermovirga)* (Fig. 4C). These genera are thermophilic and commonly found in oil reservoirs. The genus *Desulfotomaculum* comprises SRB that have been implicated in MIC processes (*Liu et al., 2019*) and are members of the class Clostridia of the phylum Firmicutes (*Whitman, 2009*). This genus has also been shown to use aromatic hydrocarbons (*e.g.*, toluene, *m*-xylene, *o*-xylene) as carbon and energy sources (*Morasch et al., 2004*). *Thermovirga* is a genus of the class Synergistia of the phylum Synergistetes, with a fermentative metabolism, using proteins, some amino acids, organic acids and alcohols as carbon and energy sources, and cystine and elemental sulfur as terminal electron acceptors (*Dahle & Birkeland, 2006*). These genera may be native of oil reservoirs, as they are common to all described petroliferous regions.

The greater distance of the R1 region when compared to the other regions indicates its unique features (Fig. 4A). This region exhibited the largest number of relevant microorganisms for the ordination, followed by R5, R6, and R7. The physicochemical characteristics, especially sulfate concentration, strongly influenced the microbial structure of these regions. The regions R1, R5 and R7 showed lower sulfate levels, while the R6 region showed higher sulfate levels (Supplementary Material 8).

R1 and R5 regions showed similar patterns: despite the low content of sulfur compounds, there was a dominance of genera reported in the literature as corrosion enhancers, such as SRB and S^0RB. There was also a high abundance of syntrophic and methanogenic archaea. Low concentrations or absence of sulfur compounds in PW may possibly decrease sulfate generation but do not prevent the development of

microorganisms that are involved in the carbon cycle (such as fermenting or syntrophic bacteria) (*Sokolova et al., 2020*).

There were positive correlations between SRB and MET. Some known SRB can syntrophically degrade organic substances such as lactate, ethanol, and propionate, in close association with hydrogenotrophic methanogens by means of H_2 (electron) transfer between species (*Plugge, Balk & Stams, 2002*), in the absence of sulfate. There may also be a syntrophic relationship between APB with methanogens and sulfidogens, since they consume the organic acids produced by APB. Correlations between genera in the same functional group may suggest that there are no competitive relationships. On the other hand, negative correlations may indicate that environmental characteristics can cause the selection of organisms that are both more and lesser adapted to the environment.

The R7 region also showed low sulfate content. Nonetheless, it was dominated by MET, APB and facultative hydrocarbon-degraders, with a significant number of mesophilic organisms. According to *Okoro, Samuel & Lin (2017)* methanogens dominate pipeline corrosion in low sulfate environments: some studies show that, in the absence of sulfate or nitrate, the fermentation of soluble organic compounds and oils into methane and carbon dioxide becomes the dominant metabolic processes (*Youssef, Elshahed & Mcinerney, 2009*).

Conversely, the R6 region had a low abundance of MET and this may be associated with the high relative abundance of SRB, since these organisms use and compete for the same substrates, hydrogen and acetate (*de Sousa Pires et al., 2021*; *Shelton et al., 2016*). The most relevant genus in this region was *Desulfovibrio*, a slow-growing aerotolerant SRB (*Crispim et al., 2018*), which are described as incomplete oxidants and responsible for souring and MIC (*Bedoya et al., 2021*; *Crispim et al., 2018*; *Semenova et al., 2019*). In addition, *Desulfuvibrio* species have already been detected in the PW samples from oil wells in China (*Menon & Voordouw, 2018*).

Positive correlations show that there is no competition between sulfidogenic groups and APB. In contrast, negative correlations indicate an inhibitory relationship between the SRB and NRB functional groups. This pattern is expected, since the injection of water treated with nitrate into oil reservoirs favors the development of NRB and, although the control mechanism is unclear, many proposed mechanisms include the competition between NRB and SRB for electron donors and carbon sources, inhibition of SRB by nitrite, changes in environmental redox potential, or even switching from conventional SRB metabolism to nitrate reduction (*Johnson, 2015*).

In the OW samples, R2 and R3 regions are located far from each other and from the other regions, which may indicate that they have unique characteristics (Fig. 5A). In addition, they also presented the largest vector, with the largest number of relevant genera. R2, R5, and R7 regions showed similar microbial patterns, with dominance of sulfidogens (SRB) and APB. In the R5 region there was also an expressive presence of IRB, and NRB were present in the R5 and R7 regions. This profile may have been defined by the physicochemical characteristics, mainly by the low sulfate levels.

Nasser et al. (2021) carried out a study in Saudi Arabia evaluating 11 samples, three of them of OW. The authors found that the composition of the microbial communities was

not determined by the facilities, but by the physicochemical conditions, specifically the sulfate concentration, since the reservoirs were rich in sulfate. Nevertheless, they also observed that other factors also influenced microbial dynamics. The presence of SRB in abundance in sulfate-poor environments was detected and could be explained by the likely syntrophy between these groups and methanogens. Furthermore, SRB can obtain energy using other metabolic pathways, such as fermentation and anaerobic respiration with alternative terminal electron acceptors. Otherwise, the scarcity of SRB in environments with high sulfate contents can be explained by the fact that when water is injected into wellhead, a mesophilic and possibly aerobic environment is created (*Liu et al., 2019*), or even by possible leaks in the facilities, which causes the presence of O_2 and can suppress the development of SRB (*Vigneron et al., 2016*).

Summer et al. (2014) evaluated four samples of OW originating from a rapidly corroding piping system and stored in tanks for a long period in the Gulf of Mexico. The tanks exhibited mesophilic temperatures, circumneutral pH, and low sulfate content. They observed dominance of genera of the IRB functional group and significant presence of the APB group, and both groups can participate in MIC processes. Nonetheless, the most dominant species was *Halanaerobium congolense* from the TRB group. Therefore, the authors concluded that the dominance of this functional group in rapidly corroding piping systems is an interesting finding, and it is necessary to consider the role of non-SRB sulfidogens in oil field corrosion.

Duncan et al. (2017) analyzed the microbial communities of OW samples at different points of the oil processing facility in the Gulf of Guinea and pointed out a similar microbial profile at all sampled points. The most abundant microorganisms are described as potential corrosion inducers. According to the authors, the low incidence of SRB suggests that souring in oil fields may be originated from other sulfidogens besides SRB. They concluded that monitoring at different points of oil facilities is an efficient way to identify corrosive organisms in problematic locations and thus to guide operational practices in order to control MIC.

In turn, the R3 region was dominated by methanogens, probably due to the application of chemical products, such as alkali-surfactant-polyacrylamide (ASP). *Gao et al. (2019)* performed a microbial profile comparison in reservoirs flooded with ASP and untreated IW, and reported a reduction in microbial diversity in ASP-flooded wells. This pattern was due to pH values above 11.5, representing extremely alkaline environments, which exceeds the survival limits of most microbial species. Therefore, that specific and highly stressful environment promoted the selection of alkali-tolerant organisms, such as NRB *Halomonas*, MET *Methanobacterium* and properly alkaliphilic genera that can use sulfur compounds as electron acceptors, such as *Anoxynatronum* (*Toh et al., 2011*). Regarding the wells not flooded with ASP, a dominance of genera commonly found in oil reservoirs flooded with water was observed. They concluded that the microbial profile of reservoirs treated with ASP was determined by the high pH of the environment, causing a limitation of microbial growth to a certain extent, with a selection of organisms resistant to high pH values. Hence, these ASP-flooded reservoirs would, at first, be unfeasible for nitrate

application to favor microorganisms which can compete with those who have a corrosive metabolism.

In O samples, the distance between the regions indicates that each one presented a distinct microbial profile. Furthermore, the opposition between R3 and R6 suggests that they may be completely negatively correlated, and there are no shared genera between these regions. The R3 region was dominated by aerobic microorganisms, with the vast majority not known to directly participate in MIC, whereas the R6 region was dominated by microorganisms that are known to cause MIC (sulfidogens).

Liu et al. (2019) evaluated two samples in replicates of a virgin field (without IW nor recirculation of PW) and one at the wellhead of a well with continuous water injection, in an oil field located in Northeast China. The authors reported a dominance of Proteobacteria in both samples. In the virgin well, there was a higher abundance of the genera *Ochrobactrum* and *Acinetobacter*, while at the wellhead of the other well, there was a higher abundance of genera such as *Lactococcus* and *Pseudomonas*. Among these genera, *Pseudomonas* is the most mentioned in oil fields, probably due to the water injection into the reservoirs, and by the fact its species are potential hydrocarbon degraders. Strictly aerobic species of the genus *Sediminibacterium* were isolated from a Chinese reservoir and display interesting characteristics, with species capable of producing hydrogen sulfide (H_2S) and organic acids from sugars. Regarding the other genera, there is not much information related to their metabolism in the oil field; however, some species of these genera are aerobic or facultative anaerobes, antibiotic-resistant and are also considered pathogens, as well as important organisms in biotechnology.

In contrast, the R6 region was dominated by SRB *Archaeoglobus* and *Desulfovibrio* and showed a high abundance of APB and S^0RB, indicating that there may be a syntrophic relationship between these functional groups. *dos Santos et al. (2020)* evaluated the microbial profile of an oil sample from a Brazilian field and also analyzed the metabolic profile of these samples. The authors pointed out the dominance of sulfate-reducing *Archaeoglobus* and *Desulfovibrio*. The genus *Archaeoglobus* comprises hyperthermophilic sulphate-reducing archaea, frequently found in hydrothermal vents and oil wells, with temperatures around 85 °C, which develop in environments with high salinity (*Birkeland et al., 2017*). And, despite the authors having found dominance of SRB, the dominant metabolic pathway was sulfite metabolism.

R7 region had the lowest number of genera that explain data variability data (Fig. 6A). The high uniqueness of the genera (Fig. 6B) can be explained by data heterogeneity, representing, only in this region, 60% of all studies. That is, in addition to the intrinsic differences in the physicochemical conditions existing between the samples from the same oil field (Supplementary Material 8), there were also differences between oil fields, which displayed very different microbial profiles. This region exhibited dominance of S^0RB, APB, NRB, and hydrocarbon degrading genera. The high availability of nutrients, together with the probable decrease in pH due to the excess of byproducts of the metabolism of both APB (organic acids) and S^0RB species (H_2S), may have excluded SRB and MET. Moreover, sulfate unavailability may have been an important factor in the low abundance of SRB (Supplementary Material 8).

In the SD samples, the presence of the genus *Marinobacter* in all regions may indicate that this genus actively participates in the biochemical processes that occur in oil fields. The presence of hydrocarbon-degrading bacteria and biofilm-forming syntrophic bacteria allied to MET is expected to facilitate hydrocarbon degradation in oilfields, since it is likely that, while syntrophic bacteria degrade hydrocarbon substrates to products such as acetate, hydrogen or carbon dioxide, methanogens use these substrates to produce methane (*Fowler, Toth & Gieg, 2016*).

The R2 region was contemplated by only one study (*Albokari et al., 2014*), including only one sample from an oil field of the Eastern Province of Saudi Arabia. The correlations between genera of versatile, hydrocarbon-degrading, nitrate-reducing, and sulfidogenic bacteria (*Chromohalobacter*) with genera from the TRB group (*Halanaerobium*) may indicate that there is no competition between these genera and that the environment is providing necessary conditions for the development of both genera.

In contrast, the negative correlation between the genus *Halomonas*, with facultative aerobes, nitrate-reducing, halophilic and alkane-degrading species, and the genus *Methanosarcina*, a methanogen that can use three metabolic pathways (acetoclastic, hydrogenotrophic and methylotrophic) to produce methane (influential in the R6 region) may indicate inhibition by toxicity. Nitrite is an intermediary compound in denitrification by NRB, and (*Detlef & Conrad, 1998*) evaluated the effect of nitrite application on methanogenesis in pure cultures of *Methanosarcina*, demonstrating that this compound inhibits methanogenesis.

Albokari et al. (2014) reported dominance of a non-culturable genus, *Flavobacterium* (phylum Bacteroidetes), with a relative abundance of 66%, and this phylum has chemolithotrophic and anaerobic representatives. Furthermore, the authors only reported their results at the phylum level, and, thus, it was not included in our initial data analysis (see Materials and methods section). The abundance of *Flavobacterium* may be due to the high availability of organic material rich in dimethyl sulfide (DMS) originated from the degradation of the compatible solute dimethylsulfonyiopropionate (DMSP). DMS can be co-metabolized, generating a flow of electrons that is probably used in energy metabolism and thus in biomass formation (*Green et al., 2011*).

The R3, R4, R5, R6 and R7 regions had in common the genera *Marinobacter*, *Pseudomonas* and *Acetobacterium*. In these regions, the high abundance of *Pseudomonas* suggests that these organisms triggered the formation of biofilms, which harbor diverse microorganisms with metabolic capability of promoting or potentiating MIC processes (*Hamzah et al., 2014*). *Salgar-Chaparro et al. (2020)*, who analyzed metabolism products in corroded gaskets on an offshore platform, observed a dominance of *Pseudomonas* and *Shewanella*. *Pseudomonas* species are pioneering colonizers in biofilm formation and are capable of synthesizing extracellular polymeric substances (EPS), such as adhesive exopolysaccharides, and these compounds promote the adherence of other microbial groups, establishing a cooperative relationship, in addition to promoting protection against biocides, disinfectants, and other environmental stresses (*Alena, Schreiberova & Masa, 2014*). In these biofilms, metal cations are bound by the EPS, which increases ionization and ion accumulation, and, thus, altering the properties of metals and

promoting corrosion. Additionally, the production of electron mediators may favor the transfer of electrons between cells and metals and stimulate corrosion rates by the extracellular electron transfer MIC mechanism (EET-MIC). This mechanism has been recently discovered and is an electrical biocorrosion performed by chemolithotrophic microorganisms that use metallic iron (Fe0) as an electron donor, oxidizing it to Fe2+ (*Kato, 2016*; *Kato, Yumoto & Kamagata, 2014*). This electron extraction can occur through direct contact with the metallic surface—through cytochrome multi-heme enzymes linked to the cell membrane or to nanowires—or indirectly—through the diffusion of H2 or soluble redox carriers (such as flavins, phenanzines and quinones) to the extracellular medium (*Kato, 2016*). Nevertheless, *Pseudomonas* bears a metabolism capable of promoting corrosion under anaerobic conditions, such as iron oxidation combined with nitrate reduction or nitrite production. Nitrate reduction could explain the low abundance of SRB in this region since SRB are sensitive to the presence of nitrite (*Johnson, 2015*; *Salgar-Chaparro et al., 2020*).

R4 and R6 regions showed an abundance of thermophilic S^0RB and SRB. In both regions there were statistically positive correlations between methanogens, BPA and BNR. In the R4 region, the positive correlations between methanogens and NRB, APB, S^0RB and IRB may indicate that local microbiota are engaged in syntrophies. Nonetheless, despite the methanogens being abundant in these sample types, when specifically analyzing the documents, it is possible to observe that in this region they were not so relevant as the sulfidogens and hydrocarbon degraders.

Bonifay et al. (2017) performed a metagenomic and metabolomic analysis on pig solid deposits at different depths in two adjacent pipeline systems with different corrosion rates (high and low) in a North Sea oil field. Both systems were similar in terms of physicochemical characteristics, which suggests that abiotic processes may not be the differentiating factor, but that differences in corrosion rates can be explained by the biological activity that occurs in both systems. The authors used the electron transport chain to explain these differences, because they observed a dominance of genera capable of reducing nitrate in pipelines with low corrosion rates and dominance of sulfidogens in pipelines with high corrosion rates. Although this study clarified the metabolic activity of organisms in pipelines with different corrosion rates, the process that triggered the dominance of different organisms in apparently similar environments remained unclear. In the R6 region, the negative correlation between *Methanosarcina* and *Halomonas* (more abundant in the R2 region) may indicate exclusion by toxic metabolites. As previously discussed, in the R2 region, NRB may release nitrite, which is toxic to methanogens, such as *Methanosarcina*, causing their inhibition (*Detlef & Conrad, 1998*).

The R7 region displayed the largest vector (Fig. 7A). It was represented by 11 articles, all of them from Nigeria, with characterization of 21 SD samples. This region was the one with the highest number of physicochemical information (Supplementary Material 8). This region was dominated by hydrogenotrophic methanogens (*Methanobacterium*, *Methanocalculus*, *Methanoculleus*, and *Methanosaeta*), commonly found in environments of low salinities and mesophilic temperatures. There was also the presence of methylotrophs, such as *Methanolobus*, which use methanol and methylamine to produce

methane, which are not used by SRB (*Duffes, Jenoe & Boyaval, 2000*; *Jeon, Yi & Park, 2014*). Therefore, the high abundance of hydrocarbon degraders and methanogens, and their positive correlation, may indicate a syntrophic relationship between nutrient availability by hydrocarbon degraders and consumption by methanogens.

Several factors can influence methanogenesis, such as salinity, temperature, pH value, substrate availability, microbial interactions, and anthropic interventions (*Blake et al., 2015*). *Okoro, Samuel & Lin (2017)* evaluated the effects of temperature and pH on corrosion rates and methane production using metallic coupons. The authors reported that methane production strongly correlated with corrosion rates and moderately with pH. The rise in temperature can cause a shift from acetoclastic methanogenesis to hydrogenotrophic methanogenesis (*Fey & Conrad, 2003*). Although temperatures above 55 °C inhibit methanogenesis, it is still unclear how temperature influences methane production pathways in different ecosystems (*Okoro, Samuel & Lin, 2017*). High abundances of methanogens are uncommon in environments with high concentrations of saline sulfate (*Gittel et al., 2009*), since SRB can develop and compete with MET for electron donors and, therefore, prevailing sulfate scarcity, methanogens thrive. In Nigeria's oil fields, THPS is commonly used as biocide (*Okoro, Samuel & Lin, 2016*), and a direct relationship between THPS use and the predominance of methanogens is highly probable. At least three hypotheses capable of explaining this phenomenon are described.

First, limitations regarding the effectiveness of THPS against methanogens were detected. The THPS dosage allows the elimination of part of the microbial population, but some survive and recover, and may develop resistance. Resistance of some groups of methanogens to this compound has already been verified, such as the genus *Methanolobus* (*Okoro, Nwezza & Lin, 2018*). Second, continuous nitrate and THPS dosage may have suppressed the SRB population due to exclusion by competition for NRB groups, even in sulfate-rich environments, or by sensitivity to THPS. And third, THPS can serve as a precursor in methanol production and, thus, enhance the proliferation of methanogens (*Machuca & Salgar-Chaparro, 2019*).

Machuca & Salgar-Chaparro (2019) conducted a study to identify the diversity and metabolic capabilities of the microbiota of solid deposits in a pipeline in an Australian oil field. They used THPS and acrolein as biocides and reported dominance of methanogens and non-SRB sulfidogens. Additionally, they pointed out the presence of genes referring to the energy metabolism of methane, that is, a high capacity of these microorganisms to use methane pathways for oil degradation. The authors explained that methanol is the most likely byproduct of THPS degradation, and this compound may be a potential carbon source for methanogens. Among the four samples evaluated by the authors, three described the dominance of methanogens and non-SRB sulfidogens. High sulfate content was reported only in one, which may have created a competitive relationship between the groups, since there was an overlap of the SRB in relation to the methanogens; both with high abundances of S^0RB, since all samples exhibited high levels of other forms of sulfur. There were pathways related to primary metabolism (Biosynthesis of Carbohydrates, Amino Acids, Nucleotides, Fatty acids, Cofactor, Transporters and Vitamins and Cellular

structure, Glycolysis, Fermentation, Electron transport chain, among others) and secondary (Biosynthesis of Terpenes, Precursors, Tetrapyrrole, among others) (Fig. 8).

Primary metabolism pathways are responsible for maintaining bacterial survival (*Pereira, Pilz-Junior & Corção, 2021*). Cofactor, Carrier and Vitamin Biosynthesis, Nucleoside and Nucleotide Biosynthesis, Carbohydrate Biosynthesis, Amino Acid Biosynthesis and Fatty acid and Lipid biosynthesis were dominant in the oil fields (Fig. 8). Among them, only the Cofactor, Carrier and Vitamin Biosynthesis refer to secondary metabolism (*Pereira, Pilz-Junior & Corção, 2021*). These are produced for survival under certain conditions, such as microbial competition for nutrients and/or space, and protection from abiotic stress (*Pereira, Pilz-Junior & Corção, 2021*; *Singh et al., 2019*). Nevertheless, the products generated from this pathway are related to the enzymatic reactions of amino acid and carbohydrate synthesis (*Alves et al., 2013*; *Santos et al., 2018*), which are also related to life maintenance (primary metabolism). The biologically active variants of vitamin B_{12} contain rare organometallic bonds, which are used by enzymes in a variety of central metabolic pathways, such as amino acid (L-methionine) synthesis (*Santos et al., 2018*). In turn, vitamin B_1 is important in carbohydrate metabolism, besides being an essential growth factor for some bacteria and archaea (*Alves et al., 2013*).

In contrast, pathways related to nucleotide and carbohydrate metabolism are directly involved in the hydrolysis and initial breakdown of complex compounds and substrates (*Yang, 2020*). Amino acids can be used as a nitrogen source by microorganisms (*Xia et al., 2021*). Moreover, some synthesis pathways of sulfur-containing amino acids (L-methionine and L-lysine) were found, which participate in sulfur metabolism; many organisms use this pathway, such as sulfidogens (*Pereira, Pilz-Junior & Corção, 2021*), which may explain the high activity of amino acid biosynthesis in oil fields. Finally, the synthesis of some fatty acids is critical for the maintenance of membrane structure and function in many bacterial groups (*Yang, 2020*), and this metabolic profile is supported by the findings of *Roy et al. (2018)*.

Roy et al. (2018) evaluated the metabolic profile of hydrocarbon-rich oil refinery sludge in Northeast India and found a community similar to the present study, with an abundance of anaerobic and fermentative organisms. The authors highlighted microbial populations of sulfate reducers, syntrophic microbes, and methanogens. They also observed high activity of metabolism of carbohydrates, amino acids, energy, cofactors and vitamins, nucleotides, lipids, biodegradation of xenobiotics, terpenoid metabolism, metabolism and biosynthesis of secondary metabolites.

The pairwise combination of sample types from the oil fields was also evaluated to biologically verify the statistically significant differences between the metabolic groups. The high number of statistically different pathways in the combination between PW and SD can be explained by the greater number of articles and samples obtained, which led to a greater number of 16S rRNA sequences for these sample types. Carbohydrate, amino acid, and nucleotide metabolism are associated with hydrolysis, the initial breakdown of complex compounds and substrates (*Ijoma et al., 2021*). In the PW and SD samples, the intense activity of these pathways may be associated with the existing syntrophic relationships between APB, sulfidogens, and methanogens. Initially, the complex

compounds are hydrolyzed by fermentative organisms (such as APB). This group uses only the glycolytic pathway to metabolize organic compounds. Hence, they release organic acids (acidogenesis), acetate and H_2 (acetogenesis) as end-products of their metabolism, which are used as substrates by the chemolithotrophic SRB and methanogenic bacteria and archaea, through the incomplete reductive tricarboxylic acid cycle (TCA) cycle pathway.

Incomplete reductive pathways of the TCA cycle can still transform pyruvate into biosynthetic intermediates needed in response to anaerobic or microaerophilic growth conditions (*Wood, Aurikko & Kelly, 2004*). Most anaerobic autotrophic bacteria and archaea do not have the ability to carry out a complete oxidative or reductive TCA cycle (*Goodchild et al., 2004*; *Wood, Aurikko & Kelly, 2004*). This pathway was grouped in class 2 as C1 Compound Utilization and Assimilation. This pathway is also statistically significant between IW and PW samples. Nonetheless, only between PW and SD, the incomplete reductive TCA cycle occurred (Fig. 8). This does not mean that this pathway is not active in other substrate types. It is necessary to consider the limitations of the current study, such as the low number of IW, OW and O samples.

Electron transfer chains/aerobic metabolic pathway was significant among the combinations of IW/O, SD/O, PW/O, and PW/SD samples. This pathway is essential for aerobic respiration in microorganisms, and it is the pathway that provides the highest energy yield. In oil fields, high activity of this pathway is not very common, since they are environments deficient in O_2. In IW samples, the statistical significance of this pathway was already expected due to the origin and processes of this sample type in oil fields, due to water injection in wellhead, creating an aerobic environment (*Liu et al. 2019*) or by pits along pipelines (*Vigneron et al., 2016*).

In turn, there was a low abundance of this pathway (Electron transfer chains/aerobic) in PW when compared to IW. Nevertheless, the presence of this metabolic pathway in both sample types was probably due to the recirculation of PW together with IW, which can promote contamination of organisms such as *Pseudomonas*, which are versatile and adapt to anaerobic environments (*Bedoya et al., 2021*).

In contrast, this metabolic pathway is less abundant in O samples and SD. Nevertheless, the explanation for their occurrence may be due to the pits along the pipelines, which can cause an aerobic microenvironment and permit the development of organisms with this kind of metabolism (*Vigneron et al., 2016*). Another possible explanation would be the sampling location, as explained by *Liu et al. (2019)*, who found a high amount of *Lactococcus* and *Pseudomonas* at the wellhead of an oil well with continuous injection of IW.

CONCLUSION

The microbial composition of oil fields in different sample types and petroliferous regions, along with the production and transport processes worldwide, showed that most of detected microorganisms are potentially involved in MIC processes (sulfidogenic, biofilm forming, acidogenic, acetogenic, and methanogenic microbes). Furthermore, these microbial functional groups, jointly with nitrate reducers, probably established syntrophic or inhibitory relationships.

The PW and SD samples had the largest data volume and, thus, more consistent results were obtained from these samples. In PW samples, despite high microbial diversity, sulfidogenic groups were more abundant, with the presence of the *Desulfotomaculum* and *Thermovirga* genera in all petroliferous regions. Moreover, there was an emphasis on the syntrophic relationships between sulfidogens, methanogens, nitrate-reducing and acid-producing microorganisms. Otherwise, in SD samples, despite also presenting high diversity, a distinct microbial profile was observed: higher abundance of APB and NRB, and important syntrophic associations between sulfidogens, methanogens and NRB were retrieved. The latter group includes biofilm-forming organisms, such as *Pseudomonas*. In SD samples, the NRB *Maribacterium* was present in all petroliferous regions. Presence and abundance of these groups and correlations between them were influenced by environmental physicochemical characteristics, especially, sulfate concentration, as well as anthropic interferences, such as the application of biocides. High sulfate content favored the sulfate reduction metabolism by SRB, while in sample types with low sulfate content, there was a metabolic deviation of SRB, which probably ally with methanogens as a survival strategy. The application of chemical products caused several changes in the environment. In many cases this strategy is efficient in causing the inhibition of potential organisms involved in MIC (such as SRB), but it induced the selection of: (i) antibiotic resistant organisms, such as *Pseudomonas*, (ii) organisms whose metabolism uses the applied products (THPS) as substrate, such as *Methanolobus*, and (iii) organisms tolerant to extreme conditions, mainly high pH values, such as *Halomonas*, *Methanobacterium*, and *Anoxynatronum*, among others. Moreover, the petroleum environment was also rich in non-SRB sulfidogens, and these bacteria are probably not affected by common biocides. According to the functional prediction analysis, there are high microbial activity in oil fields, with the highest abundances of Cofactor, and Carrier, and Vitamin Biosynthesis, which plays the role of catalysts for essential reactions in microorganisms, such as the biosynthesis of carbohydrates and amino acids. Additionally, there was also the presence of secondary metabolic pathways, such as terpenoid biosynthesis and aromatic compounds biosynthesis, which are directly involved in survival, under stressful conditions as well as used as a defense mechanism.

Therefore, our worldwide systematic review and analyses of existing data in the specialized literature of microbial communities associated with environments of the oil and gas industry has generated a global scientific synthesis of this theme, which can greatly facilitate the establishment of standardized approaches to control microbial-induced corrosion.

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Vinicius Waldow, Claudia Groposo, Rubens N. Akamine, and Maira Sousa are employees of PETROBRAS.

Author Contributions

- Joyce Dutra conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Rosimeire Gomes conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Glen Jasper Yupanqui García conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Danitza Xiomara Romero-Cale conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
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- Aristóteles Góes-Neto conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data are available in Supplemental Files.

Supplemental Information

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CAPÍTULO 2

CAPÍTULO 2 – PADRONIZAÇÃO DOS EXPERIMENTOS

Capítulo apresentado em 3 etapas

O capítulo 2 desta tese tem como objetivo a padronização dos experimentos e consiste em três etapas. Primeiro, foi realizado um experimento piloto que testou diferentes kits comerciais para extração de DNA de água produzida e petróleo bruto, incluindo o uso de solventes orgânicos como pré-tratamento. Em seguida foi realizada uma análise comparativa entre as abordagens de shotgun e amplicon 16S rRNA para investigar a correlação entre a taxonomia e as funções metabólicas dos microrganismos encontrados em amostras de água produzida de campos de petróleo. Por fim foi proposto um protocolo eficiente e de baixo custo chamado "CostSaver DNA" para extração metagenômica de DNA de amostras complexas, com ênfase na análise de metabarcoding. Além disso, foi realizado um estudo para comparar o desempenho do protocolo CostSaver DNA com o FastDNA Spin Kit for Soil na extração de DNA metagenômico de água produzida obtida de campo de petróleo. Este estudo foi intitulado "CostSaver DNA: a simpler and cost-effective DNA extraction protocol for multiple sample sources for metagenome study" e submetido à revista "Memórias do Instituto Oswaldo Cruz".

ETAPA 1 - PADRONIZAÇÃO DOS PROTOCOLOS PARA EXTRAÇÃO DE DNA DE ÁGUA PRODUZIDA E ÓLEO

O presente estudo descreve as atividades relacionadas ao teste de diferentes protocolos para extração de DNA a partir de amostras de campo de petróleo, visando obter um protocolo padronizado para as etapas seguintes do projeto.

1 INTRODUÇÃO

Apesar da indústria de petróleo e gás utilizar equipamentos altamente sofisticados e tecnológicos, os acidentes envolvendo falhas em dutos de exploração e transporte de petróleo e gás têm sido recorrentes (Nace, 2016). A causa mais comum dessas falhas são corrosões, as quais podem ser desencadeadas ou potencializadas por microrganismos (Skovhus et al., 2017). Esse processo é conhecido como CIM (corrosão influenciada por microrganismos) (Al Abbas et al., 2013; S. Y. Li et al., 2016; Videla, 1996). Dessa forma, é importante que toda a etapa de produção e transporte em indústrias de petróleo seja monitorada.

Atualmente, o método de controle mais aplicado em indústrias petrolíferas são dependente de cultivo como o NMP (número mais provável), no entanto, esse método não é eficaz uma vez que menos de 1% de todos os organismos existentes são cultiváveis (Pedrós-Alió, 2011). Assim, a utilização de métodos moleculares para o monitoramento dos dutos pode ser interessante. Existem diversificados protocolos de extração de DNA descritos na literatura e a escolha do mais adequado deve levar em consideração a molécula desejada (DNA e/ou RNA), o tipo de amostra, o grau de pureza e o rendimento desejado, além da disponibilidade de recursos para a sua realização (Nonohay & Hepp, 2017).

O grande desafio para a implantação de métodos moleculares para monitoramento em dutos de petróleo é a padronização das técnicas de extração do DNA desse composto, devido às suas características físico-químicas e alta impureza. Assim, o objetivo do presente estudo foi analisar alternativas para preparação de amostras de petróleo e padronizar técnicas de extração do DNA de petróleo e água produzida, visando obtenção do maior rendimento de ácidos nucléicos, pureza e integridade das amostras de campos de petróleo para análise metagenômica.

2 MATERIAL E MÉTODOS

2.1 LOCAL DE COLETA

A coleta foi realizada pela equipe da Petrobras, no dia 01 de julho de 2019, entre 08:30 e 10:45 horas no Terminal Campos Elíseos (TECAM), terminal do Transpetro localizado em

Duque de Caxias (RJ) (Figura 1).

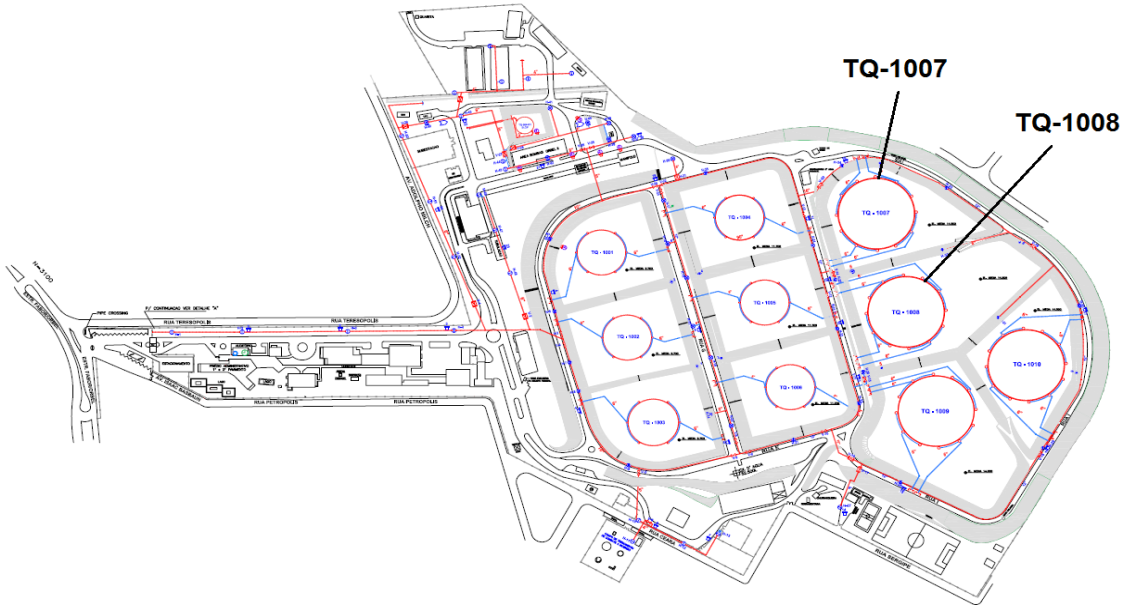

Figura 1 Mapa do Terminal Campos Elíseos (TECAM) com a indicação dos dois tanques de amostragem.

2.2 LOCAL DE AMOSTRAGEM

A coleta foi realizada em dois tanques de armazenamento (Figura 2), ambos com altura total de 14,63 m, por meio do preenchimento de frascos opacos previamente esterilizados por autoclave e preenchidos com os fluidos do interior dos tanques, os quais foram obtidos por meio de torneiras localizadas nas laterais.

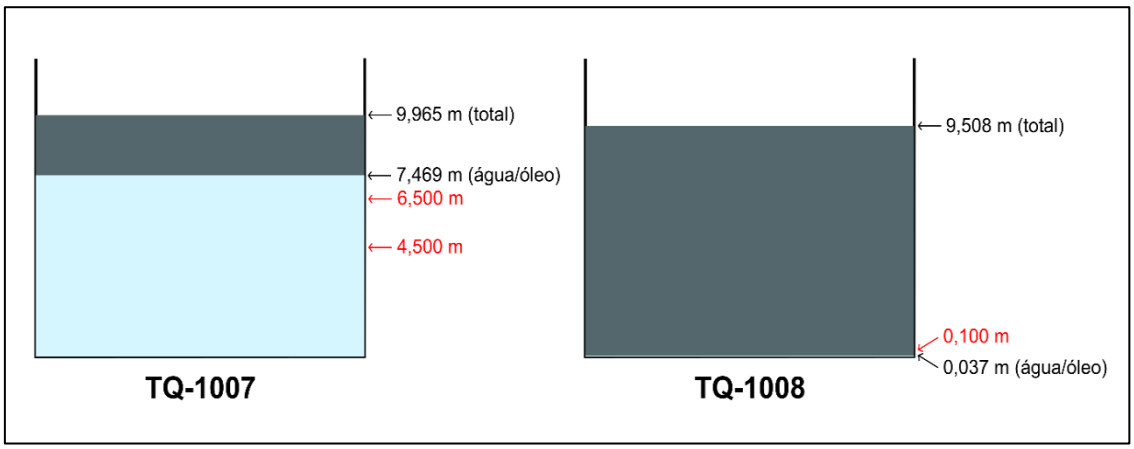

Figura 2 Esquema ilustrando o conteúdo e os pontos amostrais nos dois tanques do Terminal Campos Elíseos (TECAM). As setas e textos em vermelho mostram os três pontos de amostragem.

TQ-1007 - É o tanque que vinha sendo utilizado como "dreneiro", o qual é o que recebe a "raspagem" de outros tanques (o residual de água e óleo após o esvaziamento de um

tanque) e, portanto, é um tanque com alto teor de água, e consequentemente propício à proliferação microbiana. A fase água estava atingindo uma altura de 7,469 m conforme a última medição realizada em 30/06/2019 às 16:30, e a altura total dos líquidos estava em 9,955 m. Realizou-se a coleta de dois frascos de 500 mL de cada uma de duas torneiras localizadas nas seguintes alturas: (1) 6,5 m, a qual era a torneira mais próxima da interface água/óleo, e (2) 4,5 m, a qual era a segunda altura mais próxima da interface água/óleo.

TQ-1008 - Um tanque que estava armazenando praticamente somente petróleo cru: a última medição foi realizada em 30/06/2019 às 18:30 e a altura da fase água estava em 37 mm enquanto a altura total dos líquidos era de 9,508 m. Realizou-se a coleta de dois frascos de 500 ml a partir da torneira localizada numa altura de 0,1 m. O petróleo presente no tanque possui predomínios dos petróleos "1PB - Lula Extremo Sul P - 69" (86% do volume total) e "1NG- Tartaruga Verde FPSO Campos" (7% do volume total).

Cerca de 15 a 20 min depois das coletas, os frascos foram colocados dentro da caixa de isopor e o gelo seco foi derramado no interior da caixa. Após a chegada ao CENPES, as amostras foram colhidas pela equipe do transporte ao redor de meio-dia para o envio para a UFMG.

2.3 PROCESSAMENTO DAS AMOSTRAS

2.3.1 Processamento das amostras de óleo

Após a descongelação e homogeneização das amostras, 20 mL de cada amostra foram transferidos para tubos Falcon. Em seguida, um volume igual de solvente orgânico foi adicionado aos tubos. A mistura foi realizada por agitação em vórtex (Yamane et al., 2008; Yoshida et al., 2005).

Foram realizados testes utilizando três solventes orgânicos altamente hidrofóbicos: hexano, heptano e iso-octano. Após a adição desses solventes às amostras de petróleo, as misturas foram submetidas a centrifugação a 5.000 g (12.500 rpm) a uma temperatura de 4 °C por 1 hora. O sobrenadante contendo o DNA foi cuidadosamente removido, e 20 mL de solvente fresco foram adicionados aos tubos. Esse procedimento de adição de solvente e centrifugação foi repetido três vezes.

Posteriormente, o material resultante das lavagens foi congelado a -80 °C por 2 horas para facilitar a formação de um precipitado. Em seguida, o material foi liofilizado por 24 horas para remover a umidade e obter um precipitado liofilizado contendo o DNA. Esse precipitado foi armazenado a -80 °C até o momento de sua utilização. A Figura 3 apresenta o resultado visual do material obtido após a terceira lavagem com solvente orgânico (Figura

3A) e a representação do descarte da mistura do solvente com os resíduos oleosos (Figura 3B).

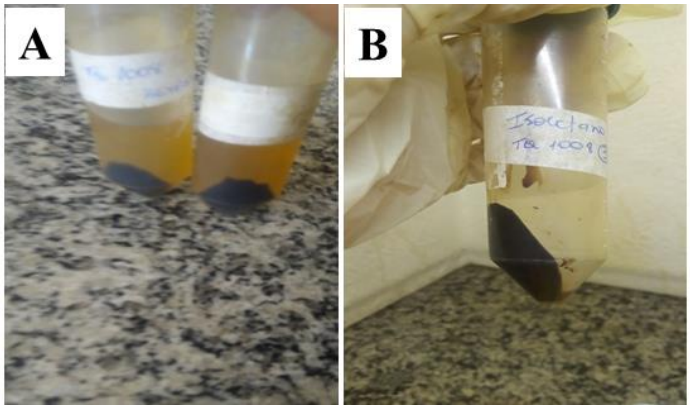

Figura 3 Aspecto das amostras após processamento com os solventes orgânicos.

2.3.2 Processamento das amostras de água produzida

Para concentrar a biomassa presente nas amostras de água, as amostras foram descongeladas e filtradas em membranas estéreis de 0,22 µm, livres de DNAse e RNAse. Essas membranas, feitas de polietersulfona (Pes) com diâmetro de 75 mm, tinham capacidade de 500 mL. A filtração foi realizada utilizando uma bomba de vácuo para succionar o líquido através das membranas.

Após a filtração, os filtros foram cuidadosamente removidos do sistema de filtração e transferidos para tubos plásticos estéreis de 15 mL. Esses tubos foram armazenados a -80 °C até serem submetidos aos protocolos de extração de DNA. A Figura 4 ilustra o processo de filtração a vácuo.

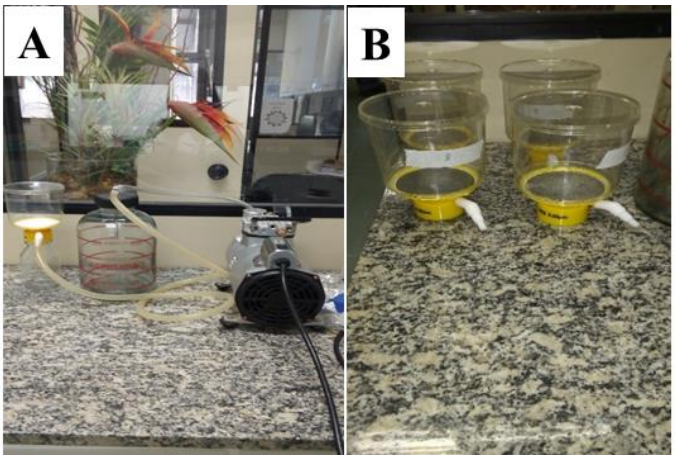

Figura 4 Filtração das amostras de água. (A) Sistema de filtração montado e conectado à bomba de vácuo; (B) Material filtrado no sistema de filtração a vácuo.

2.4. EXTRAÇÃO DE DNA

Três protocolos de extração de DNA foram testados. Cada amostra teve o seu DNA

extraído em duplicata, e a qualidade dos extratos de DNA obtidos foram avaliados por meio de (1) eletroforese em gel de agarose, (2) eletroforese capilar, e (3) leitura espectrofotométrica pelo equipamento NanoDrop® 2000. Após esses procedimentos, os extratos de DNA foram armazenados a -80 °C para utilização posterior.

O primeiro protocolo testado foi um método de extração automatizada de DNA com o Maxwell® 16 System, que é constituído pelo Maxwell® 16 Blood DNA Purification Kit (N.º Cat. AS1015) e pelo Maxwell® 16 Instrument (N.º Cat. AS2050, AS3050). Uma quantidade de 50 mg foi utilizada em cada reação de extração, sendo essa a capacidade máxima do equipamento.

Os outros dois protocolos testados foram o FastDNA™ SPIN Kit (extração a partir de 100 a 200 mg de material) e o FastDNA™ SPIN Kit for Soil (extração de até 500 mg de material). Em ambos os protocolos, foram utilizadas 100 mg das amostras de petróleo, e 200 mg de fragmentos cortados das membranas de 0,22 µm com a biomassa retida das amostras de água.

2.5 DELINEAMENTO EXPERIMENTAL E ESTATÍSTICA

2.4.1 Delineamento experimental e estatística das amostras de petróleo cru

Um planejamento experimental consiste em um conjunto de experimentos projetados de acordo com uma matriz contendo diferentes combinações de níveis das variáveis em estudo. O objetivo desse planejamento é obter estimativas confiáveis dos coeficientes da função que será ajustada aos dados experimentais, reduzindo assim o número de experimentos necessários. Isso permite uma avaliação eficiente dos coeficientes e do modelo matemático utilizado (Novaes et al., 2018).

No presente estudo, utilizou-se o planejamento experimental Box-Behnken, que requer um número reduzido de combinações de fatores. Nesse delineamento, os fatores são avaliados em três níveis (-1, 0, 1). O objetivo foi realizar uma análise multifatorial com superfície de resposta para amostras de petróleo. O delineamento experimental Box-Behnken 3^2 foi empregado para avaliar os efeitos principais, os efeitos de interação e os efeitos quadráticos das variáveis do processo sobre o rendimento de DNA.

Para realização da estatítica utilizou-se um modelo de regressão quadrática não-linear, gerado a partir do delineamento experimental. Esse modelo visa descrever a relação entre as variáveis do processo e o rendimento de DNA obtido.

Eq (1)

$$Y = b0 + b1X1 + b2X2 + b11X1^2 + b22X2^2 + b12X1*X2$$

Onde: Y é a resposta encontrada associada com cada nível de fator combinado; b0 é uma constante; b1 a b22 são coeficientes de regressão calculados a partir dos valores experimentais observados de Y e X1 e X2 são os níveis codificados de variáveis independentes. O termo X1* X2 e X_i^2 (i = 1, 2) representam a interação e os termos quadráticos, respectivamente. As superfícies de resposta e análise foram realizadas usando o software Minitab 17. Na Tabela 1 é mostrada as combinações realizadas no planejamento experimental Box-Behnken 3^2 para amostras de petróleo.

Tabela 1 Combinações do planejamento experimental Box-benken 3^2 (análises dos resultados de extração de petróleo).

Variáveis	**Símbolos**	**Níveis**		
		-1	**0**	**1**
Método de extração	X1	Maxwell	FastDNA Spin	FastDNA Spin Soil
Tipo de reagente	X2	Heptano	Hexano	Isooctano

X1- Métodos de extração; X2- Tipos de solventes orgânicos

2.4.2 Delineamento experimental e estatística das amostras de água produzida

A análise dos resultados de extração de DNA da água produzida foi realizada separadamente de forma unifatorial (Tabela 2). Para análise estatística, inicialmente foi verificada a normalidade dos dados através do teste de Shapiro Wilks. Constatada a normalidade dos dados, foi aplicado o teste de ANOVA, seguido do teste de Tukey, para comparação das médias.

Tabela 2 Planejamento experimental unifatorial (análises dos resultados para extração de DNA de água de petróleo).

Variáveis	**Símbolos**	**Níveis**
Tanque de coleta	X3	AP 4,5; AP 6,5
Métodos de extrações	X4	Maxwell; Spin e Spin for Soil

X3- Tanque de coleta; X4- Métodos de extração; AP 4,5 - Água de petróleo a 4,5 metros; AP 6,5- Água de petróleo a 6,5 metros. Spin = FastDNA™ SPIN Kit; Spin for Soil = FastDNA™ SPIN Kit for Soil.

3 RESULTADOS E DISCUSSÃO

3.1 QUALIDADE E RENDIMENTO DOS EXTRATOS DE DNA DAS AMOSTRAS DE DNA DE PETRÓLEO E ÁGUA PRODUZIDA

Os resultados dos parâmetros mensurados nos extratos de DNA metagenômico para avaliação da qualidade e do rendimento são mostrados na Tabela 3.

Tabela 3 Avaliação da qualidade e do rendimento dos extratos de DNA obtidos com

diferentes protocolos de extração.

Parâmetro	**$A_{260/280}$**			**Rendimento (ng/mL)**		
Variáveis	Maxwell	SPIN	SPIN Soil	Maxwell	SPIN	SPIN Soil
Hexano	3,02	1,74	1,55	1,2	19,8	47,1
	3,04	1,65	1,35	1,4	14,2	38,4
Heptano	1,92	1,48	1,45	3,8	27,6	48,8
	1,54	1,63	-	3,8	11,7	-
Iso-octano	1,48	1,46	1,48	1,7	19,7	45,4
	1,42	1,36	1,34	1,5	17,8	50,5
4,5 m	1,70	1,78	1,36	3,6	52,0	68,8
	1,54	1,75	1,49	5,3	65,3	76,6
6,5 m	1,83	1,60	1,41	3,2	20,2	51,5
	1,59	1,66	1,42	4,2	19,8	59,2

3.2 ANÁLISES ESTATÍSTICAS

3.2.1 Análises estatísticas das amostras de petróleo cru

O planejamento experimental Box-Behnken 3^2 em forma de matriz é mostrado na Tabela 4, juntamente com os valores de rendimento de DNA obtidos pelos diferentes protocolos de extração combinados aos diferentes solventes orgânicos usados no pré-processamento das amostras de petróleo.

Tabela 4 Matriz do delineamento experimental Box-Behnken 3^2 para obtenção da superfície de resposta em relação ao rendimento das extrações de DNA de amostra de petróleo.

	Variáveis independentes		Variável dependente
Corrida	Protocolo (X1)	Solvente (X2)	Rendimento (ng/mL)
1	Maxwell (-1)	Heptano (-1)	3,8
2	Spin for Soil (1)	Heptano (-1)	48,4
3	Maxwell (-1)	Iso-octano (1)	1,7
4	Spin for Soil (1)	Iso-octano (1)	45,4
5	Maxwell (-1)	Heptano (-1)	3,8
6	Spin for Soil (1)	Heptano (-1)	-
7	Spin (0)	Iso-octano (1)	19,7
8	Spin (0)	Iso-octano (1)	17,8
9	Spin (0)	Heptano (-1)	27,6
10	Spin (0)	Heptano (-1)	11,7
11	Maxwell (-1)	Iso-octano (1)	1,5
12	Spin for Soil (1)	Iso-octano (1)	50,5
13	Maxwell (-1)	Hexano (0)	1,2
14	Spin for Soil (1)	Hexano (0)	47,1
15	Maxwell (-1)	Hexano (0)	1,4
16	Spin for Soil (1)	Hexano (0)	38,4
17	Spin (0)	Hexano (0)	19,8
18	Spin (0)	Hexano (0)	14,2

Uma análise de regressão foi realizada utilizando-se um modelo quadrático (Tabela 5).

Observando-se os resultados dessa análise, nota-se que somente os coeficientes do termo linear X1 (protocolo) e do termo quadrático $X1^2$ (protocolo*protocolo) foram estatisticamente significativos ($p < 0,05$): ambos apresentaram um sinal positivo, indicando que apenas o protocolo de extração influenciou significativamente no rendimento do DNA, e que o FastDNA™ SPIN Kit for Soil foi o protocolo que resultou nos maiores rendimentos.

Tabela 5 Análise de regressão do rendimento de ácidos nucléicos

Termos	**Rendimento (ng/mL)**		
	Efeito	Coeficiente	p-Valor
Constante	-	16,54	0,000*
X1	43,89	21,94	0,000*
X2	-0,94	-0,47	0,731
$X1^2$	11,42	5,71	0,026*
$X2^2$	5,77	2,21	0,219
X1*X2	1,24	0,62	0,719
% Coeficiente de determinação (R^2)		96,27	
% Coeficiente de determinação ajustado		94,57	
% Coeficiente de determinação previsto (Pred R^2)		91,94	
Coeficiente de variação (CV)		4,32	

Equação de regressão

$$RAN = 16,54 + 21,94.X1 – 0,47.X2 + 5,71\ X1^2 + 2,89.X2^2 + 0,62.X1*X2$$

RAN = rendimento de ácidos nucléicos; * = valores estatisticamente significativos ($p < 0,05$).

A qualidade do ajuste do modelo foi analisada a partir do coeficiente de determinação ($R^2 = 0,9627$), o qual indicou que 96 % da variância total foi atribuída às duas variáveis contempladas. O coeficiente de determinação previsto (Pred $R^2 = 0,92$) foi próximo ao coeficiente ajustado (Adj $R^2 = 0,946$), o que é considerado satisfatório para validar o modelo. Além disso, o baixo coeficiente de variação (CV = 4,32) mostrou que os experimentos realizados foram precisos e confiáveis. A superfície de resposta evidencia graficamente que o protocolo de extração teve o maior efeito sobre o rendimento de DNA (Figura 5).

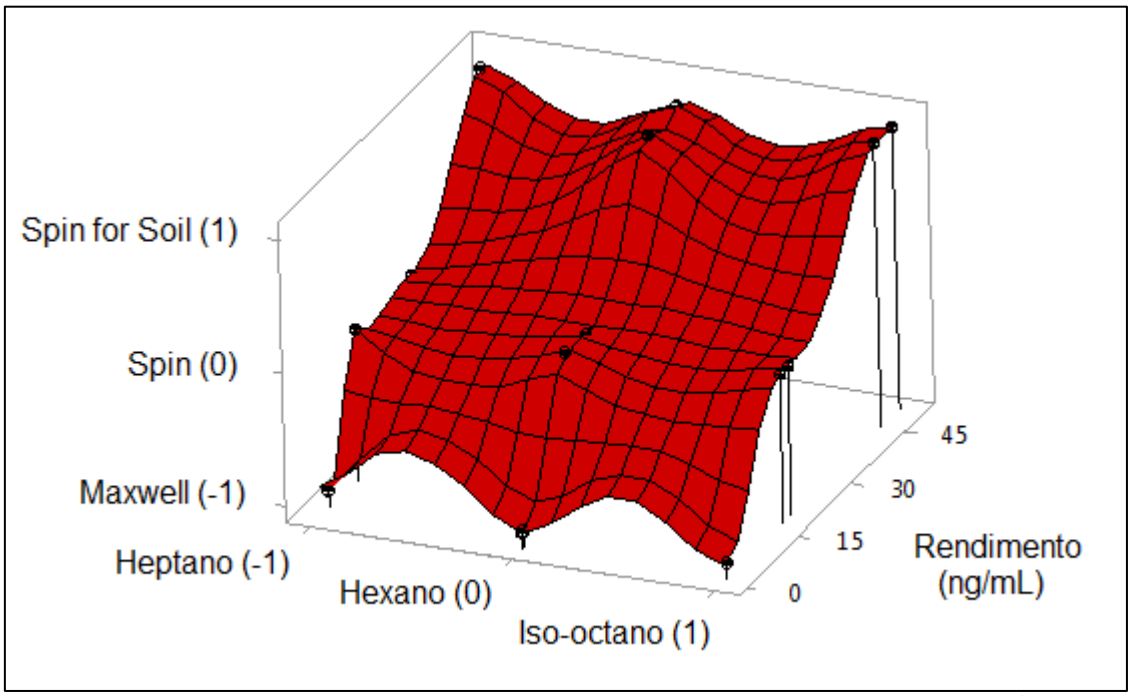

Figura 5 Superfície de resposta do delineamento experimental Box-Behnken 3^2 utilizado para analisar o rendimento da extração de DNA a partir de amostras de petróleo.

3.2.2 Análises estatíticas das amostras de água produzida

A análise dos resultados de extração de DNA da água produzida foi realizada separadamente de forma unifatorial com utilização de testes estatísticos, considerando apenas duas variáveis para comparação das médias. Na Tabela 6 é mostrado os resultados.

Tabela 6 Resultado do teste de normalidade Shapiro-Wilks.

Protocolo	Maxwell	Spin	Spin for Soil
N	4	4	4
Shapiro-Wilk W	0,9506	0,8447	0,9837
p(normal)	0,7201	0,2095	0,9236

Spin = FastDNA™ SPIN Kit; Spin for Soil = FastDNA™ SPIN Kit for Soil. * Diferenças significativas entre os grupos, p≤0,05.

Os resultados mostraram que os dados seguiram uma distribuição normal e assim foi aplicado o teste de ANOVA como mostrado na Tabela 7.

Tabela 7 Análise de variância (ANOVA) dos três protocolos de extração de DNA metagenômico para as amostras de água.

Termos	**Soma dos quadrados**	**GL**	**Quadrado médio**	**F**	**p(igual)**
Entre grupos	7.261,57	2	3.630,8	16,79	0,000917*
Dentro dos grupos	1.945,95	9	216,22	Permutação p (n = 99.999)	
Total	**9.207,52**	**11**	**0,0009**		

* Diferenças significativas entre os grupos, , p≤0,05.

Após a realização do teste de ANOVA, foi identificado que existem diferenças estatisticamente significativas entre os grupos avaliados, que correspondem aos diferentes kits de extração de DNA utilizados. Com o intuito de investigar de forma mais detalhada qual protocolo apresentou maior eficiência em relação ao rendimento de DNA nas amostras de água produzida, aplicou-se o teste de Tukey.

O teste de Tukey é uma ferramenta estatística utilizada para comparar todas as combinações de médias entre os grupos, permitindo identificar quais são estatisticamente diferentes entre si. Os resultados do teste de Tukey estão apresentados de forma clara e concisa na Tabela 8, possibilitando a análise comparativa entre os protocolos de extração de DNA em relação ao rendimento obtido nas amostras de água produzida.

Tabela 8 Teste de Tukey para comparação de médias pareadas.

Protocolo	**Maxwell**	**Spin**	**Spin for Soil**
Maxwell	-	0,01975*	0,0007084*
Spin	4,794	-	0,09536
Spin for Soil	8,154	3,36	-

Spin = FastDNA™ SPIN Kit; Spin for Soil = FastDNA™ SPIN Kit for Soil.
* Diferenças significativas entre os grupos

3.3 ANÁLISE DA INTEGRIDADE DO DNA EM AMOSTRAS DE PETRÓLEO CRU E ÀGUA PRIDUZIDA

Para fins de avaliação qualitativa da integridade do DNA, os extratos de DNA foram submetidos à eletroforese em gel de agarose 0,8 % (Figura 5). A eletroforese em gel de agarose 0,8 % não acusou a presença de bandas em nenhuma das amostras de petróleo (poços 1-6), independentemente do protocolo de extração e do solvente orgânico utilizado no pré-processamento. Em relação às amostras de água produzida, bandas foram observadas quando o DNA foi extraído com os kits FastDNA™ SPIN Kit e FastDNA™ SPIN Kit for Soil (Figuras 5 A e B); entretanto, o arraste observado nessas bandas indicam que houve degradação do DNA. Portanto, os resultados obtidos na eletroforese em gel de agarose 0,8 % apontam que os protocolos de extração de DNA utilizados não foram satisfatórios quanto à integridade do DNA extraído.

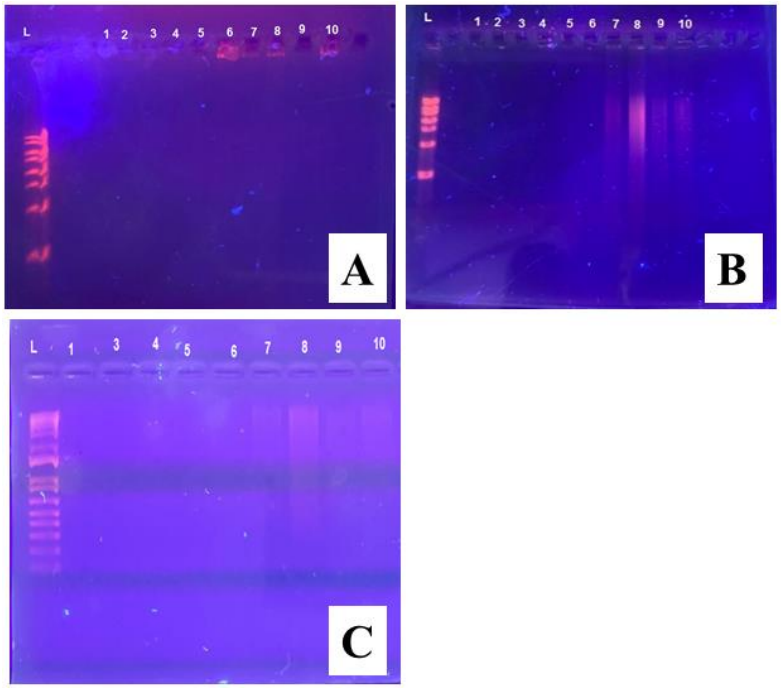

Figura 5 Eletroforese em gel de agarose para avaliação qualitativa da integridade dos extratos de DNA. Extrações com Maxwell® 16 System (A); FastDNA™ SPIN Kit (B); e FastDNA™ SPIN Kit for Soil (C). As amostras de petróleo foram pré-processadas com heptano (1 e 2), iso-octano (3 e 4) e hexano (5 e 6). As amostras de água coletadas nas alturas de 4,5 m (7 e 8) e 6,5 m (9 e 10). "L" é o marcador de peso molecular.

Por ser mais sensível que a técnica de eletroforese em gel de agarose, a técnica de eletroforese capilar em gel foi utilizada para analisar a qualidade dos extratos de DNA (Figuras 6, 7 e 8).

Entre os extratos obtidos com o Maxwell® 16 System (Figura 6), a presença de DNA só foi detectada numa das amostras de petróleo pré-processada com hexano (poço 6) e em uma das amostras de água coletada na altura de 4,5 m (poço 7); mas em ambos os casos, o arraste das bandas indica a degradação do DNA.

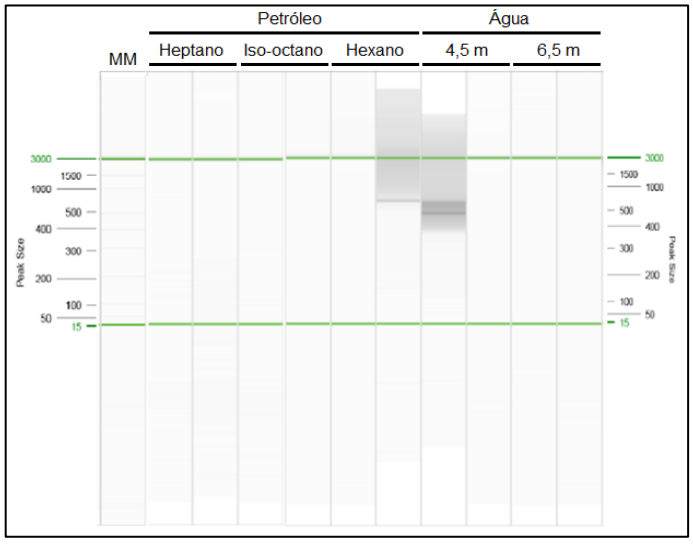

Figura 6 Eletroforese em gel de agarose capilar para avaliação qualitativa da integridade do

DNA extraído com o Maxwell® 16 System. Sigla: MM = marcador molecular.

Entre os extratos obtidos com o FastDNA™ SPIN Kit (Figura 7), a presença de DNA foi detectada em três extratos (poços 1, 8 e 9), sendo que, somente uma das amostras pré-processada com heptano (poço 1), o padrão de bandas indicou um DNA com integridade satisfatória.

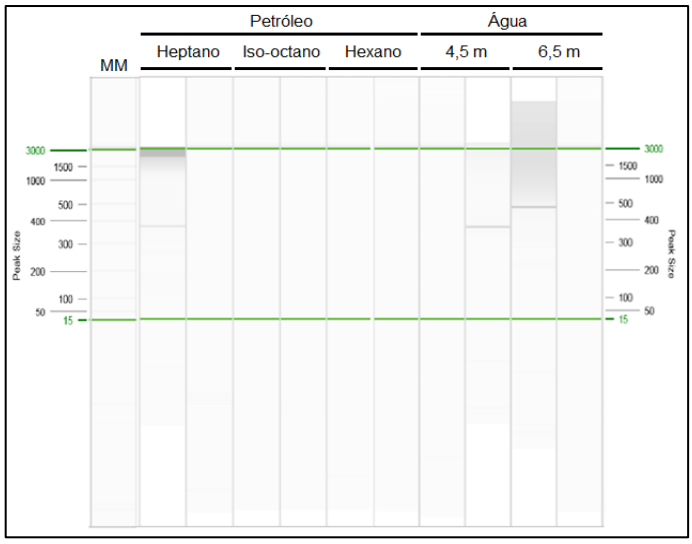

Figura 7 Eletroforese em gel de agarose capilar para avaliação qualitativa da integridade do DNA extraído com o FastDNA™ SPIN Kit. Sigla: MM = marcador molecular.

Por sua vez, entre os extratos obtidos com o FastDNA™ SPIN Kit for Soil (Figura 8), três amostras apresentaram um DNA com boa integridade: amostras de petróleo pré-processadas com iso-octano e hexano (poços 4 e 5, respectivamente) e uma amostra de água coletada na altura de 6,5 m (poço 9).

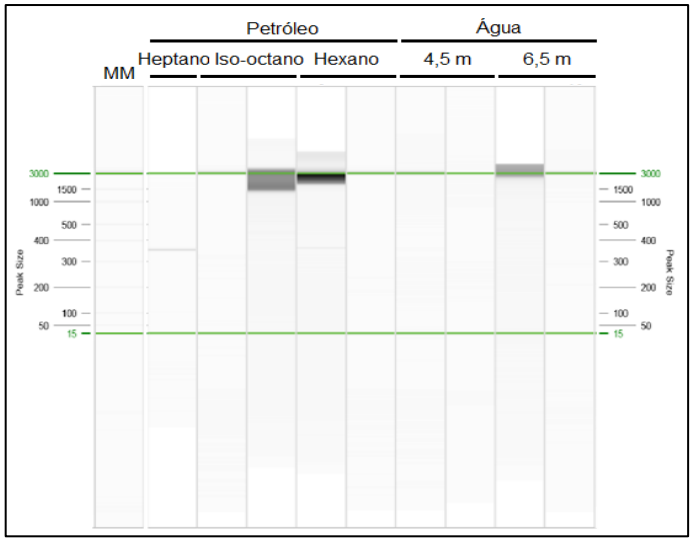

Figura 8 Eletroforese em gel de agarose capilar para avaliação qualitativa da integridade do DNA extraído com o FastDNA™ SPIN Kit for Soil. Sigla: MM = marcador molecular.

4 CONCLUSÃO

Com base nos resultados obtidos, podemos concluir que não houve diferenças significativas entre os pré-tratamentos com solventes orgânicos nas amostras de petróleo. No entanto, ao analisar os protocolos de extração de DNA, observamos que o FastDNA™ SPIN Kit for Soil se destacou, apresentando maiores rendimentos e uma melhor preservação da integridade do DNA metagenômico nas amostras de petróleo.

No caso das amostras de água, não foi possível determinar qual dos kits comerciais (FastDNA™ SPIN Kit for Soil e FastDNA™ SPIN Kit) obteve um desempenho superior em termos de rendimento de DNA. No entanto, o FastDNA™ SPIN Kit for Soil demonstrou uma maior preservação da integridade do DNA nessas amostras.

É importante ressaltar que nenhum dos protocolos de extração produziu de maneira consistente extratos de DNA metagenômico com qualidade satisfatória para análises de biologia molecular em todas as amostras de água. Portanto, ainda é necessário otimizar o protocolo de extração utilizando o FastDNA™ SPIN Kit for Soil a fim de alcançar um rendimento mais consistente e melhorar a qualidade dos extratos de DNA.

Apesar disso, os extratos de DNA obtidos até o momento apresentam qualidade suficiente para serem utilizados em estudos de metagenômica de amplicon do gene 16S rRNA. No entanto, é fundamental realizar testes adicionais para determinar se a qualidade alcançada é adequada para a aplicação da metagenômica shotgun, que envolve análises mais abrangentes do material genético presente nas amostras.

Essas conclusões ressaltam a importância de otimizar os protocolos de extração de DNA, visando obter rendimentos mais consistentes e aprimorar a qualidade dos extratos de DNA metagenômico, permitindo análises moleculares mais completas e confiáveis.

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ETAPA 2 - TAXONOMIC AND FUNCTIONAL COMPARISON BETWEEN THE SHOTGUN AND 16S RRNA AMPLICON APPROACHES FOR THE MONITORING OF BIOCORROSION IN OIL FIELDS

Comparação taxonômica e funcional entre as abordagens shotgun e amplicon 16S rRNA para o monitoramento da biocorrosão em campos petrolíferos.

Apesar das vantagens dos estudos metagenômicos de shotgun em relação aos estudos baseados na abordagem do amplicon 16S rRNA em termos de inferência funcional, existem algumas limitações a serem consideradas. Um dos principais desafios é o custo mais elevado do sequenciamento shotgun, uma vez que requer recursos computacionais e financeiros adicionais devido à grande quantidade de dados gerados e ao processamento necessário. Como alternativa, o software PICRUSt2 foi desenvolvido para prever o metabolismo de uma comunidade bacteriana com base nos perfis de sequenciamento de genes marcadores, como o gene 16S rRNA.

Nesse contexto, o objetivo deste estudo foi avaliar a correlação entre duas abordagens, denominadas shotgun (PW_shotgun) e 16S rRNA (PW_16S), em relação à abundância relativa de taxonomia e funções metabólicas obtidas a partir de informações genéticas. A abordagem shotgun envolveu a análise completa das informações genéticas, enquanto a abordagem 16S rRNA utilizou o gene 16S rRNA para obter informações taxonômicas. O software Picrust2 foi utilizado para inferir as funções metabólicas com base nas informações taxonômicas obtidas pelo gene 16S rRNA.

Os resultados indicam que a abordagem baseada no gene marcador 16S rRNA (PW_16S) possui um alto potencial para abordar questões ecológicas em ambientes específicos, como campos petrolíferos. Tanto a análise taxonômica quanto a análise funcional foram mais direcionadas e coerentes com as características desses ambientes. Comparativamente, a análise de amplicon revelou maior diversidade e uniformidade, identificando mais gêneros relacionados à biocorrosão e vias metabólicas correlacionadas. Esses resultados promissores indicam que a abordagem baseada no gene marcador 16S rRNA pode se tornar um método padrão de monitoramento em campos petrolíferos, devido à sua menor complexidade e custo, permitindo uma avaliação mais eficiente e acessível da ecologia microbiana e dos processos metabólicos envolvidos.

TAXONOMIC AND FUNCTIONAL COMPARISON BETWEEN THE SHOTGUN AND 16S rRNA AMPLICON APPROACHES FOR THE MONITORING OF BIOCORROSION IN OIL FIELDS

ABSTRACT

Microorganism-influenced corrosion (MIC) is a major cause of costs associated with corrosion in the petroleum industry. To monitor and understand the role of these microorganisms in metallic corrosion, it is necessary to use microbial identification strategies through molecular biology techniques. However, isolated taxonomic information is not enough to predict biocorrosion events in oil fields. It is essential to investigate microbial interactions and determine the factors that influence and trigger the corrosive process. Thus, the aim of this study was to the relative abundance of the microbial taxonomy and metabolic functions from produced water (PW) in oil fields using two DNA sequencing approaches, shotgun (PW_shotgun) and 16S rRNA amplicon (PW_16S), and compare these approaches. The PW_shotgun used complete genetic information, while the PW_16S approach used the 16S rRNA gene to obtain taxonomic information and the software PICRUSt2 to infer metabolic functions based on taxonomic information obtained by the 16S rRNA gene. The results indicate that the PW_16S approach is sufficient to address ecological issues in specific environments such as oil fields. Both taxonomic and functional analysis were consistent with the characteristics of these environments. PW_16S analysis revealed greater diversity and uniformity, identifying more genera related to biocorrosion and correlated metabolic pathways. These promising findings indicate that the PW_16S approach may become a standard monitoring method in oil fields due to its lower complexity and cost, allowing a more effective and accessible assessment of the microbial ecology and the metabolic processes involved.

Keywords: WGS; metabarcoding; biocorrosion; gene functional prediction; oil fields

1 INTRODUCTION

Annual global costs from corrosion of pipelines and industrial equipment for producing and transporting oil and gas total about $2.5 trillion (Nace, 2016). It is believed that between 10 and 20% of these corrosion cases are related to Microorganism Influenced Corrosion (MIC) (Machuca & Salgar-Chaparro, 2019). MIC is a phenomenon in which corrosion is initiated and/or accelerate by the presence and activity of microorganisms via corrosive processes of an electrochemical nature (Videla, 1996). This process is difficult to predict and monitor, requiring physical-chemical and microbiological analysis (Skovhus et al., 2017).

In the petroleum industry, the microbiological analyzes are generally performed by the most likely number method (MPN), which depends on culture (Bhagobaty, 2014; Lomans et al., 2016). In the petroleum industry, microbiological analyzes are generally performed by the most likely number method (MPN), which depends on culture (Dutra, Garc, et al., 2023). Another approach is the application of DNA sequencing using Next Generation Sequencing platform for analysis of microbial communities, in which there are two main strategies: shotgun metagenomics and (16S rRNA gene) amplicon sequencing (Laudadio et al., 2018).

In the shotgun metagenomics approach, all environmental DNA is sequenced (without prior amplification of a taxonomic biomarker region) and the sequenced reads are then analyzed using metagenomics databases as a reference, with the aim of assigning each read to a specific taxon (Laudadio et al., 2018; Quince et al., 2017; Truong et al., 2015). On the other hand, the amplicon sequencing (or metabarcoding), hereafter “amplicon”, uses prior DNA amplification followed by large-scale sequencing of genomic biomarker regions, such as the 16S rRNA gene for Bacteria and Archaea (Klindworth et al., 2013; Laudadio et al., 2018; Quince et al., 2017).

Both the shotgun and amplicon approaches can be employed for the taxonomic characterization of bacteria and archaea at microbial levels (Laudadio et al., 2018). However, taxonomic information alone is not enough to predict the occurrence of biocorrosion events in oil fields, because depending on the metabolic activity present in the medium, there may be an increase or inhibition of corrosion reactions in the environment (Bonifay et al., 2017). Thus, inferences of metabolic functions are necessary, aiming at an efficient diagnosis of biocorrosion.

The shotgun approach has the potential to provide information about the metabolic

function of microbial communities, as sequencing is performed on all DNA in the environment, including genes participating in metabolism, which can be used to infer functions and identify new genes and processes unidentified so far (Quince et al., 2017). In contrast, the amplicon approach is limited to providing information about the metabolic function of communities, since the 16S rRNA gene is not directly involved in metabolism and its sequence does not offer references about metabolic processes of microbial communities (Douglas et al., 2020).

Although shotgun metagenomic studies have advantages related to functional inference compared to studies using the 16S rRNA amplicon approach, there are limitations such as the higher price of shotgun sequencing, as it requires more computational and financial resources due to the large amount of data generated and necessary data processing (Quince et al., 2017). Thus, the software PICRUSt2 was developed to predict the metabolism of a bacterial community from the sequencing profiles of marker genes (Ijoma et al., 2021), for example the 16S rRNA gene.

The aim of this study was to compare the shotgun and amplicon approaches for the taxonomy and metabolic function analysis of microorganisms found in samples of water produced in oil fields.

2 MATERIAL AND METHODS

2.1 SAMPLING

Produced water (PW) samples were collected at an oil storage and transfer terminal, located in Duque de Caxias-RJ, Brazil. PW samples were collected from side valves connected at two heights (1.00 m and 2.75 m) of a collector tank, totaling 14.63 m, which receives the residual volume of water and oil from other storage tanks.

The collection was performed in triplicate, using three 5.0 L sterile plastic containers per collection point. The samples were immediately transported at room temperature, for a maximum of 2 h, to the laboratory, where they were subjected to filtration using membranes (Kasvi, Curitiba, Brazil) with a mesh diameter of 0.22 µm, using vacuum pumps for retention of microbial cells. Approximately 450 mL of produced water was filtered from each sampling point. Filters (Kasvi, Curitiba, Brazil) with retained microbial cells were placed in sterile 50 mL tubes and stored in deep freezers (−80 ◦C) for subsequent DNA extraction.

2.2 DNA EXTRACTION

DNA extraction from the PW samples was performed using membrane filters with 100

and 250 mg of microbial cells retained from the PW. DNA extraction was performed in triplicate using the FastDNA™ Spin Kit for Soil (MP Biomedicals, Jacksonville, USA), following the manufacturer's instructions. Quantification and purity (evaluated using the A260/280 and A260/230 ratio) were confirmed using the NanoDrop 2000 (Thermo Fisher Scientific, Waltman, USA). DNA integrity was verified by visualization on a 1.0% (w/v) agarose gel. Half of the DNA was sent for shotgun sequencing and half was sent for 16S rRNA amplicon sequencing.

2.3 16S RRNA GENE AMPLIFICATION

For amplification of the 16S rRNA gene, specific primers for the V3-V4 region were used: 341F (5'-CCTACGGGRSGCAGCAG-3') (Yong Wang & Qian, 2009) and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2010).

2.4 DNA SEQUENCING OF SHOTGUN AND 16S GENE AMPLICONS

Total metagenomic DNA was fragmented by standard shotgun and libraries were constructed and sequenced on a HiSeq 2500 (Illumina, CA, USA). The 16S rRNA gene amplicon sequencing was performed on the MiSeq sequencing platform (Illumina Inc., USA), using the V2 paired-end Kit for 500 cycles (2 × 250 bp).

2.5 DATA PROCESSING

The shotgun data analysis was performed using the SqueezeMeta pipeline (Tamames & Puente-Sánchez, 2019). This pipeline automatically performs a complete analysis, from quality assessment to annotation. Default parameters for Co-assembly mode were used. The pipeline uses Trimmomatic (Bolger et al., 2014) for quality control (Trimming and Filtering), Megahit (D. Li et al., 2015) for assembly, and Prodigal (Hyatt et al., 2010) for ORF (*Open Reading Frame*) prediction. Diamond (Buchfink et al., 2014) is used for ORFs taxonomic classification using Genbank NR database and functional annotation using the databases KEGG (Kanehisa & Goto, 2000), eggNOG (Huerta-Cepas et al., 2016) and PFAM (Finn et al., 2014). The R package SQMtools v1.6.0 (Puente-Sánchez et al., 2020) generated a tabulated file containing the result of absolute abundances of taxons from the shotgun data.

The amplicon data was analyzed using an approach called Amplicon Sequence Variant (ASV) and a custom pipeline developed in Python. The source code for this pipeline is available in the GitHub repository (https://github.com/LBMCF/pipeline-for-amplicon-analysis). The pipeline uses several tools, including USEARCH (Edgar, 2016), VSEARCH (Rognes et al., 2016), Cutadapt (Martin, 2011) e FastQC (Andrews, 2010). Initially, the

readings were subjected to a quality assessment using FastQC. Then the sequences were merged with USEARCH using the "--fastq_merge pairs" option, and the sequencing adapters were removed with Cutadapt. Removal of low-quality reads was performed with VSEARCH using the "--fastq_filter" and "--fastq_maxee=1.0" options. To eliminate duplicates, we use the "--derep_fulllength" option from VSEARCH. Removing chimeras and creating the table of ASVs was performed with the USEARCH unoise3 algorithm using the "--unoise3" option. In addition, we generate an absolute abundance table using the "usearch_global" and "id=0.99" VSEARCH options. Taxonomic identification and classification of ASVs were performed using SINTAX (Edgar, 2016) implemented in USEARCH with the database SILVA SSU NR 138.1 (Quast et al., 2013).

Absolute abundances of both shotgun and amplicon were determined based on Amplicon Sequence Variants (ASVs) and analyzes were performed at domain, phylum, class, order, family, and genus taxonomic levels. The taxonomic table of absolute abundance results from the analysis of shotgun and amplicons were joined using the package's full_join function from the R package dplyr v1.1.0 (Package, 2023). Relative abundances were calculated using the dplyr library (v1.1.0) in the R environment (Package, 2023).

2.6 INTEGRATIVE ANALYSIS

Tho online tool Venny (versão 2.1) (Oliveros, 2021) was used to visualize shared and exclusive taxa at different levels (domain, phylum, class, order, family and genus) among PW water samples using the shotgun approach (PW_shotgun) and amplicon (PW_16S). Alpha diversity data, including Shannon Index (Shannon_H), Simpson Index (Dominance_D) and Equitability Index (Equitability_J), were obtained by comparing PW_shotgun and PW_16S data using PAST 4.08 software (Hammer et al., 2001)**.**

To plot the bar graph by taxonomic categories the R package ggplot2 v3.4.1 was used (Wickham, 2011). The established limit was 1% and the lower relative abundance values were grouped in the “Others” category.

Principal Coordinate Analysis (PCoA) was employed as a methodology to perform an integrative analysis covering the samples with a threshold of 0.1%. Values below this limit were grouped in the "Others" category. In this study, we investigated the interrelationships between sets of microbial genera through PCoA, using the first two principal coordinates (PC1 and PC2). The data were initially subjected to a Hellinger transformation to suit the analysis requirements. To visualize the results, two-dimensional scatter plots were generated using Past 4.08 software (Hammer et al., 2001), using the Euclidean distance as a measure.

These graphs allowed the representation of the samples in a multidimensional space, with the relative position of the samples indicating their similarity or dissimilarity based on the composition of the microbial genera. A taxonomic association analysis was also performed between the PW_shotgun and PW_16S approaches, using Spearman's correlation as a measure of statistical association, with a significance level of $p \leq 0.05$.

2.7 FUNCTIONAL PREDICTION ANALYSIS

Functional prediction analyzes were performed on shotgun metagenomes PW_shotgun and amplicon (PW_16S) from PW samples. The functional annotation on the shotgun was described according to the methodology in section 2.5. Using the R package SQMtools v1.6.0 (Puente-Sánchez et al., 2020), the results of the functional absolute abundances of the shotgun metagenomic analysis, from the eggNOG database (COG) were also exported in a tabbed file (Huerta-Cepas et al., 2016). Functional inference of metabolic pathways based on the MetaCyc database (Caspi et al., 2018) of the abundance table of Amplicon Sequence Variants (ASVs), was carried out using the software PICRUSt2 (Douglas et al., 2020) with the default parameters.

After obtaining the metabolic functions of both approaches (PW_shotgun and PW_16S), manual standardization of the pathways was performed, following level 2 of metabolic classification, to make them comparable. This standardization was performed using Excel tools.

STAMP v2.1.3 (Parks et al., 2014) was used to visualize and compare the metabolic pathways present in the PW_shotgun and PW_16S approaches, considering those with a contribution equal to or greater than 0.1%. The inference of statistically significant differences in functional composition between PW_shotgun and PW_16S was performed. Differences in functional composition were analysed using Fisher's exact test, with Bonferroni's correction method for multiple test adjustment. A *p*-value > 0.05 was used as a cut-off to rule out statistically non-significant correlations between metabolic pathways. In addition, an association analysis was conducted between the PW_shotgun and PW_16S samples, using Spearman's correlation as a measure of statistical association.

3 RESULTS AND DISCUSSION

3.1 TAXONOMY

3.1.1 Taxonomic resolution

Table 1 shows the number of reads of the sequences obtained from the raw data from

the shotgun and amplicon approaches.

Tabela 2 Read number and length from shotgun metagenomes and 16S rRNA gene amplicon sequencing of produced water (PW) samples

Method	Sample	# reads	Read length
Shotgun	PW	85,675,734 ±6,142,443 a	150
Amplicon	PW	532,256 ±136,899 b	205-305

Lower case letters show numbers of reads statistically different from the ANOVA test followed by the Dunn's Post hoc test. $p \leq 0.05$.

We observed that the number of raw sequences obtained in the shotgun approach was statistically higher compared to the 16S rRNA approach. Figure 1 compares the abundance of PW_shotgun and PW_16S.

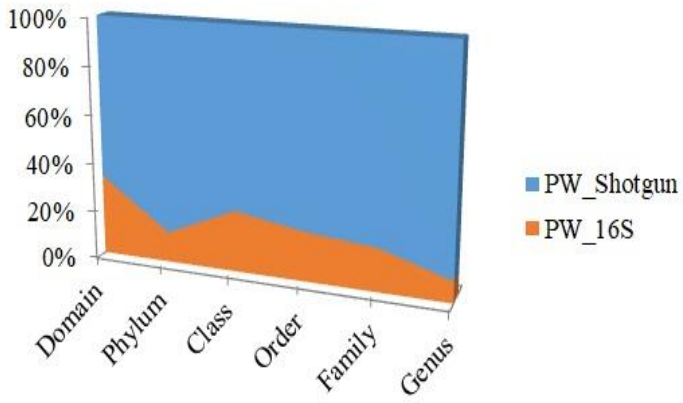

Figure 1 Percent number of organisms found at each taxonomic level in relation to the total abundance calculated for the produced water samples sequenced using the shotgun approach (PW_shotgun) and the 16S rRNA gene amplicon approach (PW_16S).

PW_shotgun demonstrated superior efficiency in identifying and classifying organisms at all evaluated taxonomic levels. In contrast, PW_16S had identification percentages below 40% at all levels, being especially low at the genus level, with less than 20% identification. These results highlight the limited ability of the 16S rRNA amplicon approach to provide more accurate taxonomic resolution compared to the shotgun approach. This result is especially advantageous for studies that demand broader genomic coverage and higher resolution (Durazzi et al., 2021; Laudadio et al., 2018), However, it is important to note that these genera may be present in low abundance (Durazzi et al., 2021), which may not be ideal in targeted studies (Di Segni et al., 2018).

3.1.2 Patterns of shared, exclusive and relative abundance of different taxonomic levels

By analyzing the patterns of exclusivity and sharing at different taxonomic levels (Figure 2), we found that there was a low rate of sharing of organisms between the PW_shotgun and PW_16S samples, ranging from 3.3% to 12%. Furthermore, we observed that the PW_shotgun sample had a significantly higher percentage of unique organisms at all taxonomic levels. These findings corroborate the study carried out by Laudadio et al. (2018), who also observed a higher taxonomic resolution in the shotgun approach compared to 16S rRNA sequencing. The authors used the number of taxa identified in each sample as a measure of performance. Similarly, Durazzi et al. (2021) highlighted this pattern, noting that shotgun sequencing is indeed more sensitive than 16S rRNA sequencing. However, the authors highlighted that the genera detected in the shotgun approach were not very abundant and even with a detection rate of 75%, only 9% of the total readings found were associated with these genera.

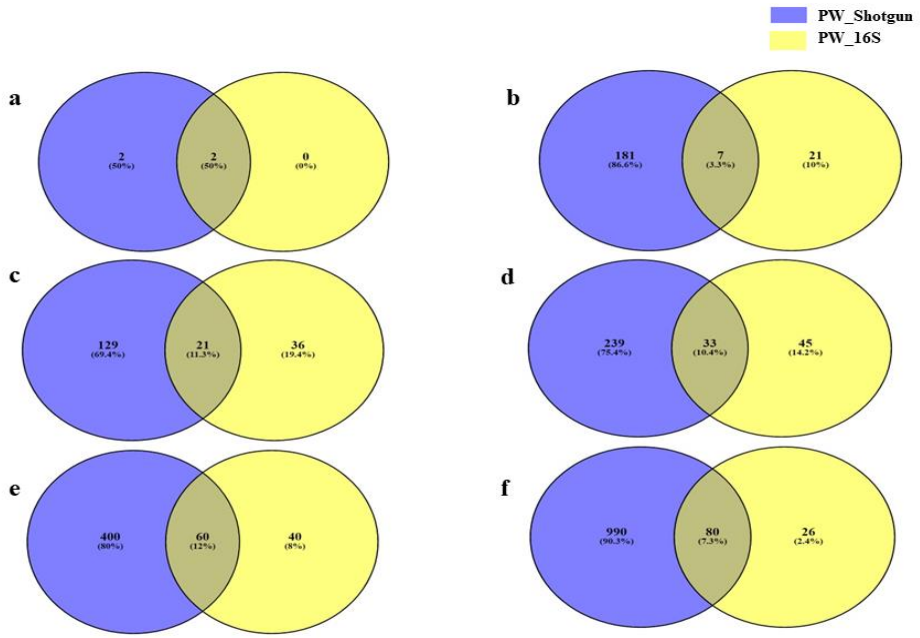

Figure 2 Uniqueness and sharing of microorganisms at different taxonomic levels among produced water (PW) DNA samples sequenced by the shotgun (PW_shotgun) and 16S rRNA amplicon approaches. (PW_16S rRNA)
(a) Domain; (b) Phylum; (c) Class; (d) Order;family; and (f) genus

When comparing the abundance ratios between PW_shotgun and PW_16S (Figure 3), it was found that the shotgun approach identified four main domains (Figure 3a), in addition to unmapped sequences (Unmapped) and unclassified organisms (Unclassified). Surprisingly,

these unmapped sequences and unclassified organisms exhibited a higher abundance compared to the domain of viruses and eukaryotes, which exhibited an abundance of less than 1% in the shotgun approach. At the taxonomic level considered, the proportions of bacteria and archaea between the two approaches were similar, with a predominance of bacteria and a significant presence of archaea.

In this study, we chose to use the V3-V4 hypervariable region in the amplicon sequencing of the 16S rRNA gene. It is important to emphasize that this choice may have resulted in an underestimation of the abundance of archaea. This is because the V4 region is generally considered more suitable for short reading sequencing, which is crucial for detection of archaea, as suggested by previous studies (Bachran et al., 2019; Ghyselinck et al., 2013). However, it is interesting to mention a study conducted by Yang et al. (2022) who observed greater efficiency in detecting bacteria and archaea when using the V3-V4 region in ocean trench sediments. This ecosystem is little explored and can harbor rare organisms, so that the complexity of microorganisms can be comparable to the environment analyzed in the present study.

When comparing the abundance proportions at lower levels of taxonomy, it was observed that at all levels (phylum, class, order, family and genus), the results obtained from the PW_16S were more satisfactory in detecting organisms abundant above 1 %.

At the phylum level, some inconsistencies in the taxonomic classification were identified (Figure 3b). The phylum Candidates Cloacimonetes was found in the sample sequenced by shotgun, representing 12.01% of the abundance. However, this phylum is also known as Cloacimonadota (L. A. Johnson & Hug, 2022), and presented an abundance of 18.13% in the PW_16S samples. These inconsistencies in nomenclature, resulting from continuous updates in databases, can introduce biases in obtaining and interpreting taxonomic results (Schoch et al., 2020).

A greater number of genera with an abundance equal to or greater than 1% were observed in PW_16S samples compared to PW_shotgun (Figure 3f). This is due to a significant proportion of organisms detected in the shotgun still do not have a taxonomic classification, being classified as "Unclassified", representing a relative abundance of 70.78%.

Regarding organisms of relevance in monitoring oil fields, it was observed that both sequencing approaches detected a relative abundance above 1% of sulfidogenic genera unrelated to sulfate-reducing bacteria (SRB), such as *Dethisulfatibacter* and *Halanaerobium*. These genera are capable of reducing thiosulphate (BRT), playing an important role in sulfur

cycling in environments rich in hydrocarbons (Dutra, Garc, et al., 2023; Dutra, Gomes, et al., 2023). Furthermore, the genus *Syntrophotalea* was identified, which may engage in a syntrophic relationship with other metabolic groups. In this syntrophic relationship, *Syntrophotalea* produces hydrogen and acetate through the partial oxidation of organic compounds, providing these metabolites as substrate for methanogenic groups. This symbiotic interaction is characterized by the exchange of metabolites between species, in which hydrogen and acetate are food sources for methanogenic bacteria (Pires et al., 2021).

In PW_shotgun, as mentioned earlier, it was possible to obtain a higher taxonomic resolution for archaea, as illustrated in Figure 3f, in which the genera *Methanothrix* (6.38%), *Methanocalculus* (2%) and *Methanoplanus* (1.53%) were identified %). These genera are known for their ability to use hydrogen, carbon dioxide or acetate as substrates for methane production (Dutra, Gomes, et al., 2023). Methane, in turn, has a high correlation with corrosion processes in oil fields (Okoro et al., 2017).

However, curiously, PW_shotgun did not detect the genus *Methanosaeta*, hydrogenotrophic methanogenic archaea, which presented an abundance of 5.61% in the PW_16S samples. This genus has been identified in oilfields in Africa (Dutra, Gomes, et al., 2023), that present oil with low sulfur content, representing an important indicator of possible syntrophy with diverse organisms, and thus importantly varied metabolisms in the monitoring of petroleum industries.

In addition to the previous observations, it is important to mention that the genus *Fusibacter* was also not identified in the shotgun approach. *Fusibacter* is a genus of halotolerant fermentative anaerobic bacteria, which have the ability to produce sulfur compounds through the reduction of thiosulphate (Serrano et al., 2017).

In addition, it is relevant to emphasize the importance of the genus *Desulfovibrio* in the corrosion process. In PW_shotgun, the genus *Desulfovibrio* was detected with an abundance of 0.06%, while in PW_16S it was found with an abundance of 4.91%. These bacteria can use sulfate as an electron acceptor, resulting in the production of hydrogen sulfide (H_2S) as a metabolic product. They are widely discussed in the specialized literature on microbiologically influenced corrosion (MIC) processes and play a significant role in oilfields (Clark et al., 2012; H. Li et al., 2015; Zumelzu et al., 2016).

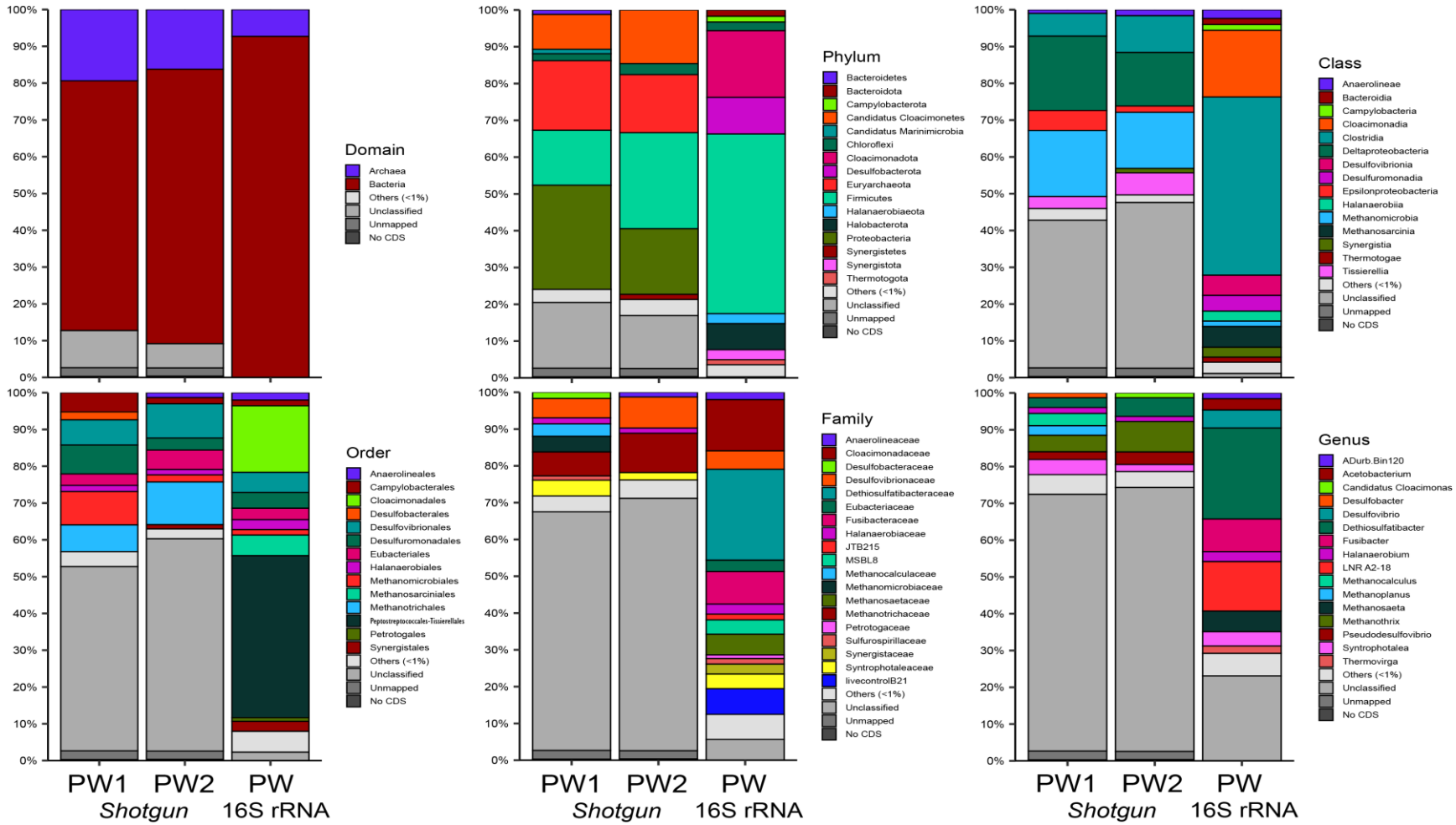

Figure 3 Relative taxon abundance of produced water (PW) microbiomes estimated using shotgun (PW_shotgun) and 16S rRNA amplicon sequencing (PW_16S).

Bar chart. Each letter indicates classification at a different taxonomic level. Each color indicates a different organism according to its taxonomic level and the proportions indicate abundance above 1%. Taxonomic levels (a) Domain; (b) phylum; (c) Class; (d) Order and (e) Genus.

3.1.3 Diversity and similarity

Analysis of taxonomic diversity at the genus level (Figure 4) revealed important differences between samples sequenced using both approaches (PW_shotgun and PW_16S).

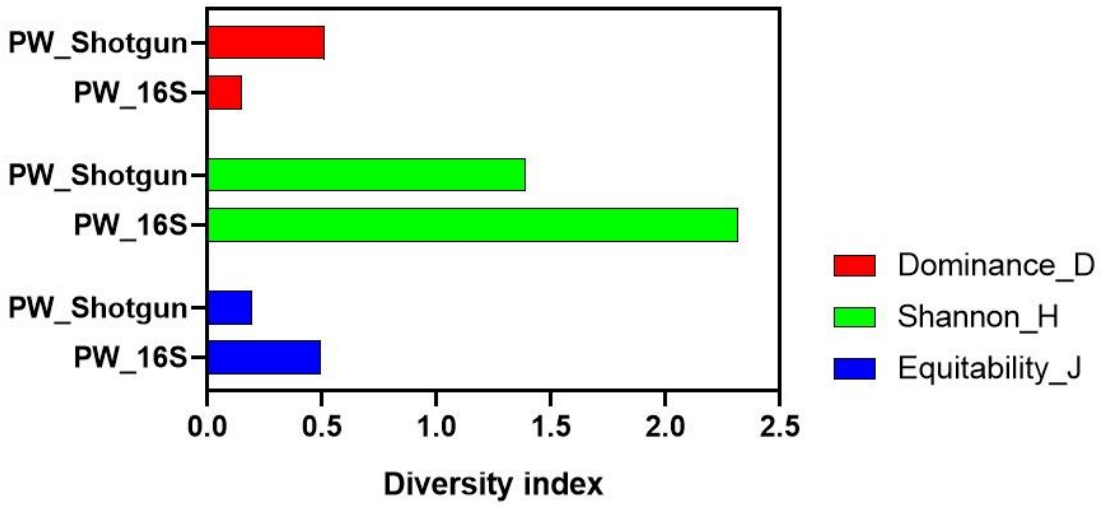

Figure 4 Alpha-diversity analysis and comparison Shannon_H, Dominance_D, and Equitability_J in the level genus;

Higher dominance, lower diversity and lower uniformity were observed in the PW_shotgun samples compared to the PW_16S samples. This pattern can be attributed to the high abundance of Unclassified genera in the shotgun approach, representing 70.74% of the total. These results corroborate a previous study conducted by Tessler et al. (2017) who compared amplicon and shotgun sequencing in 49 samples from various floodplain river systems in Brazil. The study by Tessler et al. (2017) demonstrated that amplicon sequencing was superior in assessing diversity indices such as the richness index, indicating an advantage of this approach to investigate specific ecological issues.

The results of the PCoA analysis, represented in Figure 5, showed a tendency of grouping of the genera detected both in PW_shotgun and in PW_16S, inside the circle, indicating a considerable similarity between the approaches. This proximity suggests the existence of common associations in terms of biological or environmental characteristics shared by the genera.

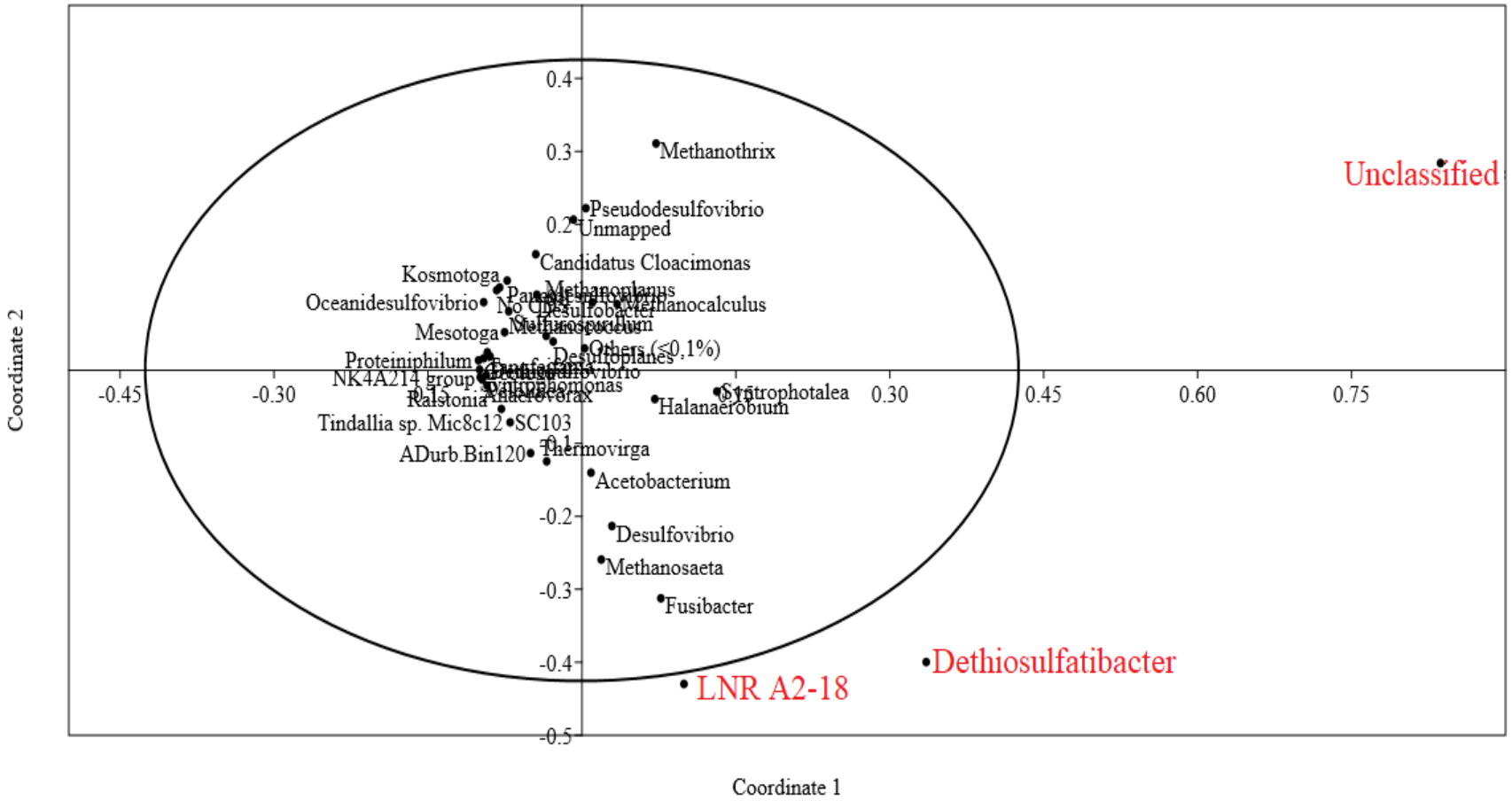

Figure 5 Similarity and dissimilarity between the composition of the produced (PW) water microbial community in the shotgun and 16S rRNA approaches. The graph was constructed using Euclidean distance. The black dots represent the microorganisms grouped in the circle. The red dots indicate the groups with the greatest dissimilarity.

However, two genera were positioned outside the circle, denoting a dissimilarity in relation to the others. Additionally, the unclassified organisms (Unclassified) showed an even more pronounced dissimilarity. Despite these differences, a statistically significant but weak correlation (rho = 0.12, $p \leq 0.05$) was found between the approaches used. These findings corroborate the study by Tessler et al. (2017) who also observed a weak correlation between the shotgun and 16S rRNA approaches, despite taxonomic overlap.

3.2 METABOLISM

By grouping the metabolic pathways at level 2 of the PW_shotgun and PW_16S samples, a total of 30 metabolic pathways were found, equal to or greater than 0.1%. A low coefficient of determination was observed between these metabolic pathways ($R^2 = 0.013$), as shown in Figure 6. When performing the Spearman correlation analysis, a statistically significant correlation was not detected (rho = -0.011, $p = 0, 54$).

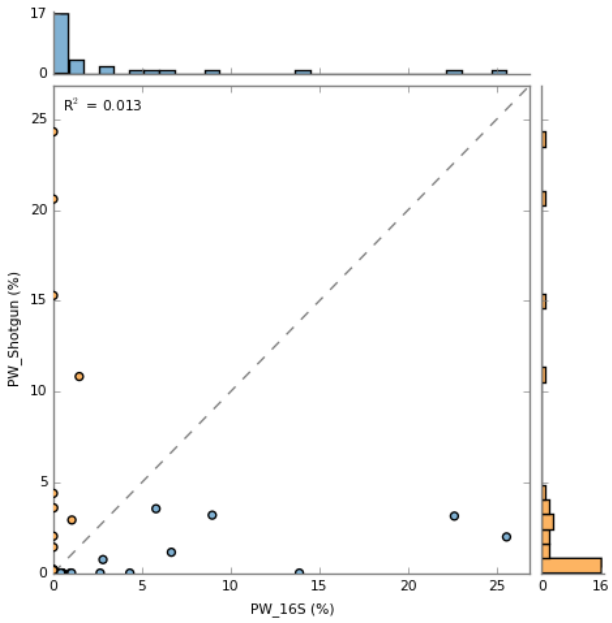

Figure 6 Correlation between level 2 metabolic functions found in produced water (PW) samples in the shotgun and 16S approaches.
Histograms display stacked bars. Each column is divided into a part corresponding to the abundance of metabolic functions detected by the shotgun approach (PW_shotgun) in orange color and the other part corresponding to the genera detected by the 16S rRNA approach (PW_16S) in blue color. The coefficient of determination (R2) indicates the proportion of variability in abundance that can be explained by differences between approaches. Values close to 1 indicate a greater contribution of the differences between the approaches to the variation in abundance.

All 30 metabolic pathways were statistically significant, at an effect size of 0.1. Figure

7. Of these, 10 level 2 metabolic pathways are shared between PW_shotugun and PW_16S, 10 pathways only in PW_shotgun, and 10 are present only in PW_16S (Figure 7).

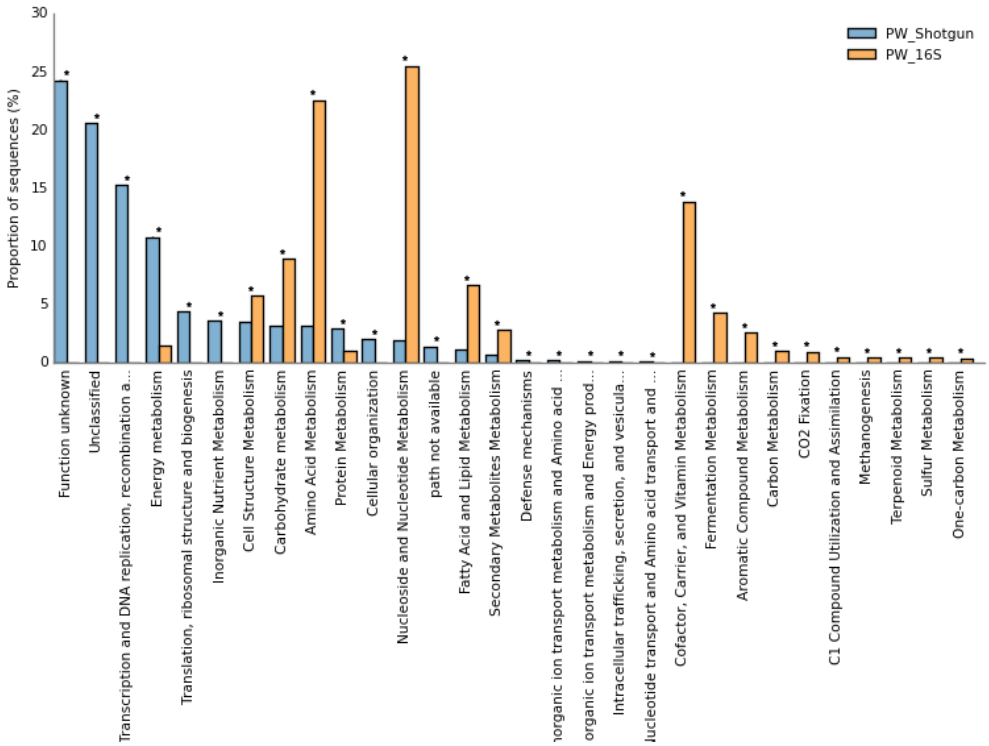

Figure 7 Metabolic profile of PW_shotgun and PW_16S samples.

Bar profile chart. Each bar in the graph represents a metabolic pathway; the height of the bar indicates the relative abundance of the routes; o (*) indicates that the difference observed between the groups is statistically significant, that is, it is not the result of chance.

Ten metabolic pathways shared between PW_shotgun and PW_16 are related to primary metabolism (Figure 7). These pathways are essential for cell survival and maintenance (Pereira et al., 2021). However, the shotgun analysis (PW_shotgun) showed a greater abundance of these pathways compared to the 16S rRNA gene-based approach (PW_16S).

The most abundant metabolic pathways found in PW_shotgun are biogenesis and ribosomal structure (4.25%) and translation and cell organization (1.96%), related to primary metabolism (Pereira et al., 2021). However, a significant part of the pathways has unknown (23.57%) or unclassified (19.98%) functions, and there is also a considerable percentage of unassessed pathways (1.38%).

The 16S rRNA (PW_16S) approach, using PICRUSt2, inferred the presence of metabolic pathways with greater abundance related to primary metabolism, such as the

metabolism of vitamins, transporters and cofactors (13.41%) (Pereira et al., 2021). In addition, metabolic pathways directly related to biocorrosion, and hydrocarbon degradation processes were found, such as fermentation (4.17%) and the metabolism of aromatic compounds (2.55%). Pathways with lesser abundance were also identified, but which may play an important role in the biocorrosion process, such as methanogenesis, carbon metabolism, sulfetogenic metabolism, CO2 fixation and single carbon metabolism (Dutra, Gomes, et al., 2023; R. Liang et al., 2016; Santos et al., 2022).

These results reflect the different approaches used to obtain these metabolic pathways. In the shotgun approach, sequencing the total DNA allows access to genes, enzymes, and proteins, which increases the reliability of functional inference (Sharpton, 2014). However, due to the extensive genomic diversity in nature compared to the number of genes annotated in sequence databases, many predicted genes may not show homology with previously annotated sequences (Sharpton, 2014), resulting in a large number of unknown, unclassified, or unevaluated metabolic pathways. On the other hand, the metabolic inference from the 16S rRNA marker gene is based on reference taxonomic sequences (Douglas et al., 2020). This means that the presence of functional relationships is inferred based on taxonomic homology with the database, provided that the genus has been functionally characterized.

4 CONCLUSION

Our results point to the high potential of the approach based on the 16S rRNA amplicon to answer ecological questions in specific environments, such as oil fields, which have peculiar characteristics. Both the taxonomic analysis and the functional analysis proved to be more targeted and consistent with what is usually observed in these environments. When comparing the approaches, we observed that the amplicon analysis, although presenting lower taxonomic resolution, revealed greater diversity and uniformity, in addition to having identified a greater number of genera already described in the literature as potential in the biocorrosion process. Consequently, it was also possible to detect a greater number of metabolic pathways that correlate with the identified taxonomy. These findings are promising for the development of monitoring methods based on the 16S rRNA marker gene, given its lower complexity and cost compared to the shotgun approach. This approach has the potential to become a standard method of monitoring in oilfields, allowing for a more effective and accessible assessment of the microbial ecology and the metabolic processes involved.

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ETAPA 3 - COSTSAVER DNA: A SIMPLER AND COST-EFFECTIVE DNA EXTRACTION PROTOCOL FOR METAGENOME STUDIES OF MULTIPLE SAMPLE SOURCES

CostSaver DNA: um protocolo de extração de DNA mais simples e econômico para estudos de metagenoma de múltiplas fontes de amostra.

Artigo submetido na revista "Anais da Academia de ciências"

Diversos métodos têm sido empregados na extração de DNA de campos petrolíferos, especialmente os destinados à extração de DNA de solos. No entanto, esses kits comerciais frequentemente possuem um custo relativamente elevado, o que pode inviabilizar a extração em larga escala de amostras, dificultando seu uso em monitoramentos rotineiros, como em campos de petróleo.

Diante dessa questão, este estudo propôs testar um protocolo de extração metagenômica de DNA de baixo custo e eficiente, adequado para amostras diversas e complexas, utilizando água produzida de um campo de petróleo. Para efeitos comparativos, utilizou-se também o Kit comercial FastDNA Spin Kit for Soil, desenvolvido especificamente para solos.

Os resultados obtidos revelaram diferenças significativas na abundância absoluta do DNA extraído com o kit comercial em comparação com o protocolo caseiro. No entanto, a proporção entre os grupos microbianos foi semelhante, e houve uma correlação significativa e alta entre ambos os métodos. Isso indica que o protocolo caseiro apresenta uma boa relação custo-benefício e é viável para a análise de diferentes tipos de amostras, pois permite a extração de DNA de qualidade a um custo reduzido.

Dessa forma, o protocolo caseiro se mostra como uma alternativa viável para a extração de um grande volume de amostras, sendo uma opção atrativa para o monitoramento em campos de petróleo. Ele oferece uma boa solução, tanto em termos de custo quanto de eficiência, permitindo a análise de amostras de forma mais acessível e econômica.

CostSaver DNA: a simpler and cost-effective DNA extraction protocol for metagenome studies of multiple sample sources

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ABSTRACT

The development of high-throughput DNA sequencing technology has enabled a more comprehensive understanding of microbial community profiles in various hosts and habitats, including air, soil, water, gastrointestinal tract, skin, and complex samples, such as those extracted from oil residues and wastewater. Sequencing quality depends on the DNA isolation method, which should allow quality and sufficient yield. To reduce DNA extraction costs while maintaining the necessary quality for different sample types, our study developed an adapted protocol named "CostSaver DNA" for metagenomic DNA extraction. Moreover, the present study aimed to evaluate the performance of this protocol for extracting metagenomic DNA from produced water obtained from an oil field storage tank in comparison to a commercial kit for DNA extraction of soil samples, which represents the predominant method employed for DNA extraction from oil field-derived samples. The CostSaver DNA protocol was tested on seven different samples, including bovine ruminal fluids, cow and mice feces, mice skin, sludge from digesters treating swine waste, and produced water from oil fields, in addition to performing the comparison between the CostSaver DNA protocol and the commercial kit for soil DNA extraction. The yield, purity and quality of the extracted DNA, as well as the quality of the 16S rRNA amplicon sequencing, using the PHRED index, number of Amplicon Sequence Variants (ASVs), and correlation between relative abundances were evaluated. Our findings indicate that the CostSaver DNA protocol offers a favorable balance between cost and benefit, and it is a viable option for obtaining high-quality DNA from diverse samples. The CostSaver DNA protocol can help researchers to perform 16S rRNA amplicon metagenomics in a reliable, consistent, straightforward, and cost-effective manner.

Keywords: Metagenomic DNA extraction, Phred index quality, Complex samples, 16S rRNA metabarcoding, Cost-benefit balance.

1 INTRODUCTION

The evolution in high-throughput DNA sequencing technology has made it possible to more comprehensively understand the microbial community profiles of different hosts and habitats (e.g., air, soil, water, gastrointestinal tract, and skin) (Bharti & Grimm, 2021; Reese & Dunn, 2018; Salihoğlu & Önal-Süzek, 2021), as well as high-complexity samples, such as oil residues and wastewater (Neria-González et al., 2006; Salama et al., 2019). Nevertheless, the sequencing quality is bound to the choice of DNA preservation and isolation methods, increasing the demand for the development of high yield and high-quality DNA extraction techniques.

Molecular biology techniques are extremely important for the analysis of samples from different microbiomes. These techniques require effective DNA extraction methods that ensure adequate quality, essential for the execution of subsequent steps (Akbari et al., 2014), such as DNA amplification (Bustin et al., 2009). The use of polymerase chain reaction (PCR) for DNA amplification may have limitations associated with interfering substances (e.g., detergents, salts, organic solvents, among others) present in the extracted DNA (Bessetti, 2007; Brennecke et al., 2017; Matsuo, 2021). These interferents can negatively impact the sensitivity and specificity of the amplification reactions (Bachoon et al., 2001; Matsuo, 2021). In this context, several methods have been used to isolate DNA from biomass, including samples with complex matrices, with several commercial kits available on the market that use chemical or enzymatic lysis steps, followed by DNA purification on a column (Chen et al., 2011; Dutra, Gomes, et al., 2023; Maghini et al., 2021).

Commercial kits were used for DNA extraction in 62 % of published works that analyzed samples originating in oil fields, most of them used kits for DNA extraction from soil, due to the high level of complexity and impurity present in these environments (Dutra, Gomes, et al., 2023). However, these kits have relatively high costs, which may turn

unfeasible the DNA extraction from large amounts of samples. Thus, the use of homemade DNA extraction protocols has increased (J. D. N. Silva et al., 2021; Stevenson et al., 2011; Yu & Morrison, 2004). In addition, homemade protocols may be more effective in relation to DNA yield, compared to commercial kits, especially in high-complexity samples such as ruminal digest, feces of dairy cows, and samples with high oil content (J. D. N. Silva et al., 2021; Yu & Morrison, 2004). These samples can be challenging due to the presence of PCR inhibitors such as proteins, polyphenolic, and polysaccharide compounds (J. D. N. Silva et al., 2021; Yu & Morrison, 2004).

Studies based on 16S rRNA region sequences obtained by metagenomic amplicon sequencing have allowed to expand our knowledge about microbial communities and is an efficient strategy for understanding the diversity, taxonomic classification and functional potential of microbiomes (Dutra, Garc, et al., 2023; Dutra, Gomes, et al., 2023; Handelsman et al., 1998). For this, it is necessary to obtain DNA of high yield and high-quality, which is uncommon in microbiome samples (Akbari et al., 2014; Bharti & Grimm, 2021).

After PCR amplification of the 16S rRNA region, sequencing is performed (P. L. F. Johnson & Slatkin, 2006), and the PHRED score has been used to assess the quality of these fragments (Malakar et al., 2021). The use of PHRED score can be employed as a tool for careful analysis of these sequences' quality to decide which reads should be maintained for analysis (Wedemeyer et al., 2017). The higher the PHRED score, the greater the confidence in the evaluated nucleotide (Christensen, 2018). Nonetheless, besides having no cutoff point to determine sequencing quality, a PHRED score of 20, which corresponds to a 1.0 % probability of error (Christensen, 2018), is commonly used as a threshold for acceptable sequencing quality (Habibi et al., 2022).

Therefore, the present study proposes an efficient protocol for metagenomic DNA extraction from diverse and complex samples, adapted from Yu and Morrison (2004), called

"CostSaver DNA", which is suitable for metabarcoding analysis. This method combines an enzymatic lysis step with mechanical lysis to extract DNA, requiring only 0.25 g of sample. In addition, we propose to evaluate the performance of the CostSaver DNA protocol compared to the FastDNA Spin Kit for Soil (MP Biomedicals, USA) for extracting metagenomic DNA from produced water obtained from oil field storage tank. This protocol focuses on reducing the cost of DNA extraction while maintaining high-quality DNA with reliable taxonomic representation for metabarcoding sequencing.

2 METHODS

2.1 SAMPLES FROM DIFFERENT MICROBIOMES

The CostSaver DNA protocol was tested to isolate total DNA from samples of seven (7) distinct conditions: bovine ruminal fluid (BRF), biodigester sludge swine farm (BSSF), cow feces (CF), facultative aerobic pond sludge in swine fridge (FAPSSF), mice feces (MF), mice skin (MS), and produced water (PW) from an oil field storage tank (Table 1). The samples were transported in ice and stored in ultrafreezer (-80°C) until the processing and extraction. Sampling was carried out in biological triplicates for BRF, CF, MF, and MS, with each replicate obtained from a different animal. The environmental samples of BSSF, FAPSSF, and PW were collected in separate containers and subsequently homogenized into a single container, generating composite samples. DNA was extracted from this single container, considering these samples as technical replicates. The samples were prepared in accordance with the Table 1.

All animal samples were collected following guidelines of the Brazilian College of Animal Experimentation (COBEA) and approved by the Use of Animals Ethics Committee from UFMG (CEUA – UFMG) under the protocol 67/2021. For BRF collection, cows were restrained using a crush and the liquid was collected by orogastric intubation and deposited in sterile flasks. MS was collected from the dorsal region of mice (1 cm^2) post euthanasia with

anesthetic deepening (300 mg/mL of ketamine and 30 mg/mL of xylazine), samples were later cut into smaller pieces to facilitate homogenization. CF and MF were freshly collected from healthy cows and mice, respectively, and stored in sterile flasks. For the study, we collected samples of granular sludge from an anaerobic reactor and granular sludge from an aerated pond (FAPSSF). The sludge samples were initially collected in 20 L high-density polyurethane bottles and later redistributed into 500 mL containers in the laboratory. Subsequently, the samples underwent the processing steps described in Table 1 and were stored at -80°C until analysis. Samples of produced water (PW) were collected from lateral valves at two different heights (1.00 m and 2.75 m) of a draining tank with a total length of 14.63 m. This tank receives residual water and oil from other storage tanks. Sterile plastic containers with a volume of 5.0 L were used for each sampling point. The samples were immediately transported to the laboratory at room temperature within a maximum of 2 hours. Following transportation, the samples were processed according to the procedures outlined in Table 1 and stored in a freezer at -80°C until further use. In total, three samples were collected for each condition studied.

Table 1. Site of sample collections and pretreatment**.**

Condition	Description	Pretreatment
Bovine ruminal fluid (BRF)	Collected from animals that were subjects of scientific experiments free of pain and suffering, from UFMG.	Not applicable.
Biodigester sludge swine farm (BSSF)	Companies located in Southern Minas Gerais.	The samples were centrifuged at 13.000 × g and the supernatant discarded.
Cow feces (CF)	Collected from animals that were subjects of scientific experiments free of pain and suffering, from UFMG.	Not applicable.
Facultative aerobic pond sludge in swine fridge	Companies located in Southern Minas Gerais.	The samples were centrifuged at 13.000 × g and the supernatant discarded.

(FAPSSF)		
Mice feces (MF)	Collected from animals that were subjects of scientific experiments free of pain and suffering, from UFMG.	Not applicable.
Mice skin (MS)	Collected from animals that were subjects of scientific experiments free of pain and suffering, from UFMG.	The tissue was cut into small pieces with sterilized scissors.
Produced water from oil field storage tank (PW*)	Collected from a tank that receives residual fluid from other tanks located at the Campos Elísios Terminal (Petrobras/RJ - Brazil).	Samples filtered using a 0.22 µm DNA free membrane with a 90 mm of diameter. The membranes were cut into small pieces with sterile scissors.

* Corresponds to the aqueous phase of a multiphasic fluid.

2.2 SAMPLE DNA EXTRACTION PROTOCOL

Total DNA was isolated using the protocol adapted from Yu and Morrison (2004) and it was named "CostSaver DNA". This adapted and optimized homemade protocol was performed in two steps: (1) cell lysis; and (2) DNA precipitation and removal of remaining RNA (Table 2). Unlike the original protocol, the step of protein removal and purification was not performed. Extraction was performed once for each biological triplicate of each condition. The details of the extraction are described in Table 2.

Table 2. Description of the CostSaver DNA protocol.

Phases	**Adapted protocol**
Cell lysis	1. Transfer the frozen sample (~0.25 g) to a sterile microtube of 2.0 mL containing 50 mg glass beads (425-600 µm). Add 1.0 mL of lysis solution buffer: 5 mM NaCl, 0.5 mM Tris-HCl (pH 8.0), plus 0.5 mM EDTA, and 4.0 % m/v sodium dodecyl sulfate (SDS). 2. Homogenize for 1 min at 6,500 rpm with Precellys (Bertin Technologies). 3. Incubate at 70 °C for 15 min, with gentle shaking by hand every 5 min. 4. Centrifuge at room temperature for 5 min at 16,000 × *g* and transfer the

	supernatant to a new tube.
	5. Add 4.0 µL of RNase (10 mg/mL) and incubate for 1 h at 37 °C.
	6. Add 260 µL of ammonium acetate solution (10 M), invert the tubes 5X by hand and incubate on ice for 5 min.
	7. Centrifuge at 4 °C for 10 min at 16,000 × *g*.
Precipitation of nucleic acids and removal of RNA	8. Divide the supernatant into two 2.0 mL microtubes and add 1.0 mL of cold isopropanol and mix by inversion and incubate on ice for 30 min.
	9. Centrifuge at room temperature for 15 min at 16,000 × *g* and discard the supernatant.
	10. Wash the pellet with 300 µL of cold 70 % v/v ethanol and centrifuge for 10 min at 16,000 × *g*. Remove ethanol by aspiration and let it dry for about 15 minutes.
	11. Resuspend the pellet in 20-100 µL of sterile water by incubating at 65 °C for 1 h or overnight at 4 °C.

The PW samples were selected to verify the efficiency of the CostSaver DNA protocol through comparison with the commercial soil extraction kit FastDNA Spin Kit for Soil (MP Biomedicals, USA). This sample was selected due to its high complexity (i.e. difficulty to extract due to high levels of oils, very low levels of microorganisms). The quantification and purity (measured through $A_{260/280}$ and $A_{260/230}$) of the extracted DNA were confirmed using the NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNA integrity was verified by electrophoresis of 200ng of each sample in a 1.0 % m/v agarose gel (30min, 100V, 30A), stained with SaferDye (KASVI, Brazil) and revealed under UV light. The 1 kb ladder (Ludwig Biotec, RS, Brazil) was used as size reference.

2.3 16S RRNA AMPLICON SEQUENCING

The paired-end DNA sequencing and the preparation of the libraries were performed by the company Neoprospectra Microbiome Technologies (Florianópolis, Brazil). The amplification was performed with primers for variable regions V3-V4 of the 16S rRNA gene: 341F 5'-CCTACGGGRSGCAGCAG-3' (Yong Wang & Qian, 2009) and 806R 5'-GGACTACHGGGGTWTCTAAT-3' (Caporaso et al., 2012), using the MiSeq Sequencing System (Illumina Inc., USA), using the paired-end type Kit V2 for 500 cycles (2 × 250 bp).

2.4 ANALYSIS OF READ QUALITY

The quality of reads was evaluated at three different steps: (i) from the raw data; (ii) after merging the forward and reverse reads, performed with the --fastq_mergepairs module of VSEARCH (Rognes et al., 2016); and (iii) after adapter removal with the software Cutadapt (Martin, 2011) and filtering of low-quality read with the --fastq_filter module of VSEARCH (Rognes et al., 2016) (parameters: --fastq_maxee = 1 and --filter_minlen = 350). At each of these steps, the quality of reads was analyzed in two ways: (a) analysis of the mean PHRED qualities of bases per position per sample and (b) analysis of the PHRED quality frequencies of all bases per sample. The ggplot2 package (3.4.0) was used for the two analyses. For a more simplified representation, these analyses are also presented when all sample replicates are combined.

2.5 STATISTICAL ANALYSIS OF AMPLICON SEQUENCE VARIANTS IN THE SAMPLES

The metagenome replicates were statistically compared based on the frequencies of the Amplicon Sequence Variants (ASVs). The ASVs were obtained using the customized pipeline proposed in the repository: https://github.com/LBMCF/pipeline-for-amplicon-analysis. The pipeline is based on the USEARCH-32 bits (Edgar, 2010) and VSEARCH programs, which initially merge all forward and reverse reads, then cut adapters using

Cutadapt (Martin, 2011), and filter reads with low quality. These softwares also generate abundance tables and perform taxonomic classifications of ASVs using the SINTAX algorithm (Edgar, 2010) of USEARCH against the SILVA taxonomic database (Quast et al., 2013). Statistical comparisons of sample replicates were performed using the Friedman test and the Siegel-Castellan *post hoc* test. Additionally, bar graphs of phyla and genera per replicate were generated from ASVs using the ggplot2 package, with a threshold between 0.1 and 1.0 %. Additionally, a Spearman's rho correlation analysis was performed to evaluate the correlation between the ASVs obtained from the CostSaver DNA protocol and those obtained from sequencing produced water samples using the commercial FastDNA Spin Soil kit.

3 RESULTS

3.1 COSTSAVER DNA PROTOCOL

3.1.1 Evaluation of purity and yield of extracted DNA

DNA extracted from the seven samples using the CostSaver DNA protocol was quantified and evaluated for purity in absorbance ratios at 260/280 and 260/230 nm (Table 3).

Table 3. The yield and purity of the metagenomic DNA from seven different samples isolated with the CostSaver DNA protocol.

Sample	**Yield (µg/50µL)**	**$A_{260/280}$**	**$A_{260/230}$**
BRF	74.40 ± 16.46	1.82 ± 0,03	2.34 ± 0.06
BSSF	8.21 ± 1.44	1.81 ± 0.01	1.30 ± 0.02
CF	56.29 ± 0.50	1.84 ± 0.01	2.39 ± 0.01
FAPSSF	12.16 ± 2.83	1.63 ± 0.01	0.85 ± 0.04
MF	3.84 ± 1.81	1.60 ± 0.04	1.86 ± 0.32
MS	0.45 ± 0.06	1.65 ± 0.06	0.29 ± 0.03
PW	10.19 ± 3.73	1.57 ± 0.05	0.80 ± 0.08

* Mean ± standard deviation values in triplicate. Samples: BRF (bovine ruminal fluid); BSSF (biodigester sludge swine farm); CF (cow feces); FAPSSF (facultative aerobic pond sludge in swine fridge); MF (mice feces); MS (mice skin); and PW (produced water from oil field storage tank).

The samples with the highest yield (above 10 µg) were BRF, BSSF, FAPSSF and PW. The $A_{260/280}$ ratio was above 1.80 only in the BRF, BSSF, and CF samples, while the other samples had a $A_{260/280}$ ratio below 1.70. It is important to note that an ideal A260/280 ratio for pure DNA is about 1.80 (Angthong et al., 2020). On the other hand, only the BRF, CF, and MF samples showed a $A_{260/230}$ ratio above 1.80, and the other samples had values lower than 1.30. $A_{260/230}$ values above 2.0 indicate good quality, and values below 1.5 may indicate significant presence of polyphenolic, and polysaccharide compounds which can inhibit PCR or hinder DNA sequencing (Angthong et al., 2020). The quality of DNA samples was analyzed by agarose gel electrophoresis (Figure 1). Although some samples did not exhibit adequate purity ratios $A_{260/280}$ and $A_{260/230}$, all samples, showed high molecular weight DNA, with intact and clear bands, except for PW, which presented a DNA band with a very low signal intensity that was almost imperceptible on the agarose gel.

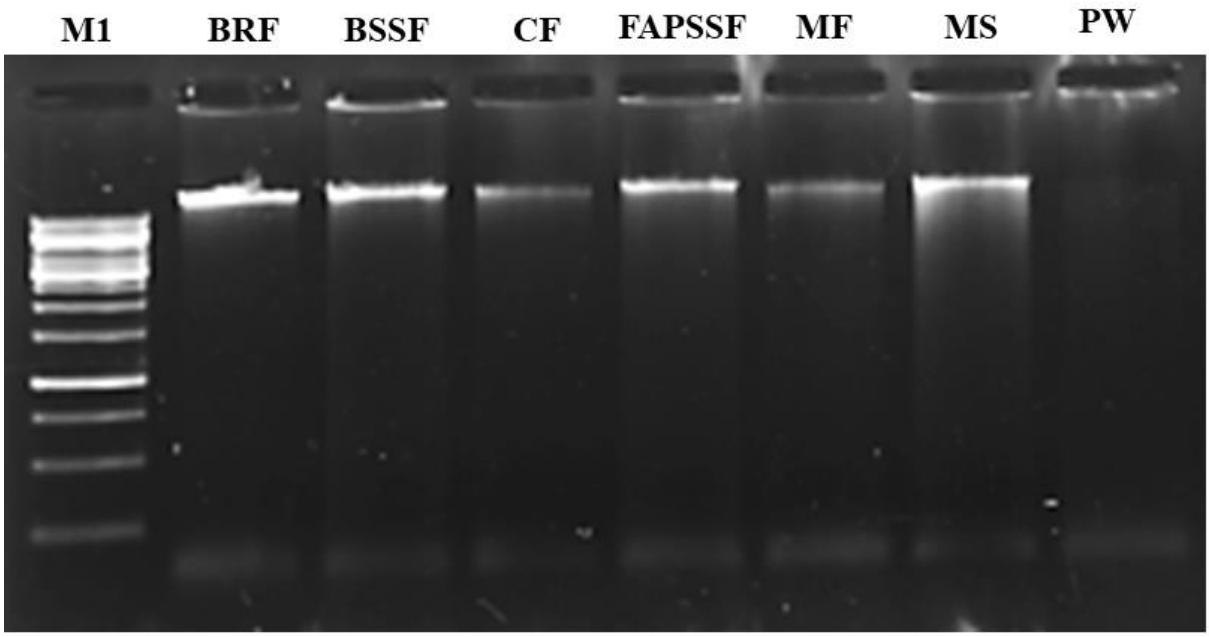

Figure 1. Agarose gel electrophoresis of metagenomic DNA extracted from seven different microbiome samples using the CostSaver DNA protocol.

M1: 1 kb ladder (Ludwig Biotec, RS, Brazil). Samples: BRF (bovine ruminal fluid); BSSF (biodigester sludge swine farm); CF (cow feces); FAPSSF (facultative aerobic pond sludge in swine fridge); MF (mice feces); MS (mice skin); and PW (produced water from oil field storage tank).

3.1.2 Sequencing quality of different samples using the adapted homemade method

16S rRNA amplicon sequencing of metagenomic DNA (metabarcoding) from different samples was performed and the quality of generated reads was assessed through the PHRED score (Figure 2). The PHRED scores according to base pair position is shown in Figure 2A, B and C, while the PHRED scores according to sequence frequencies are presented in Figure 2D, E, and F. Raw forward and reverse sequences are depicted in Figures 2A and 2D. In turn, the raw data with merged sequences are shown in Figures 2B and 2E, and the sequences after removing the data with PHRED scores below 20 are indicated in Figures 2C and 2F.

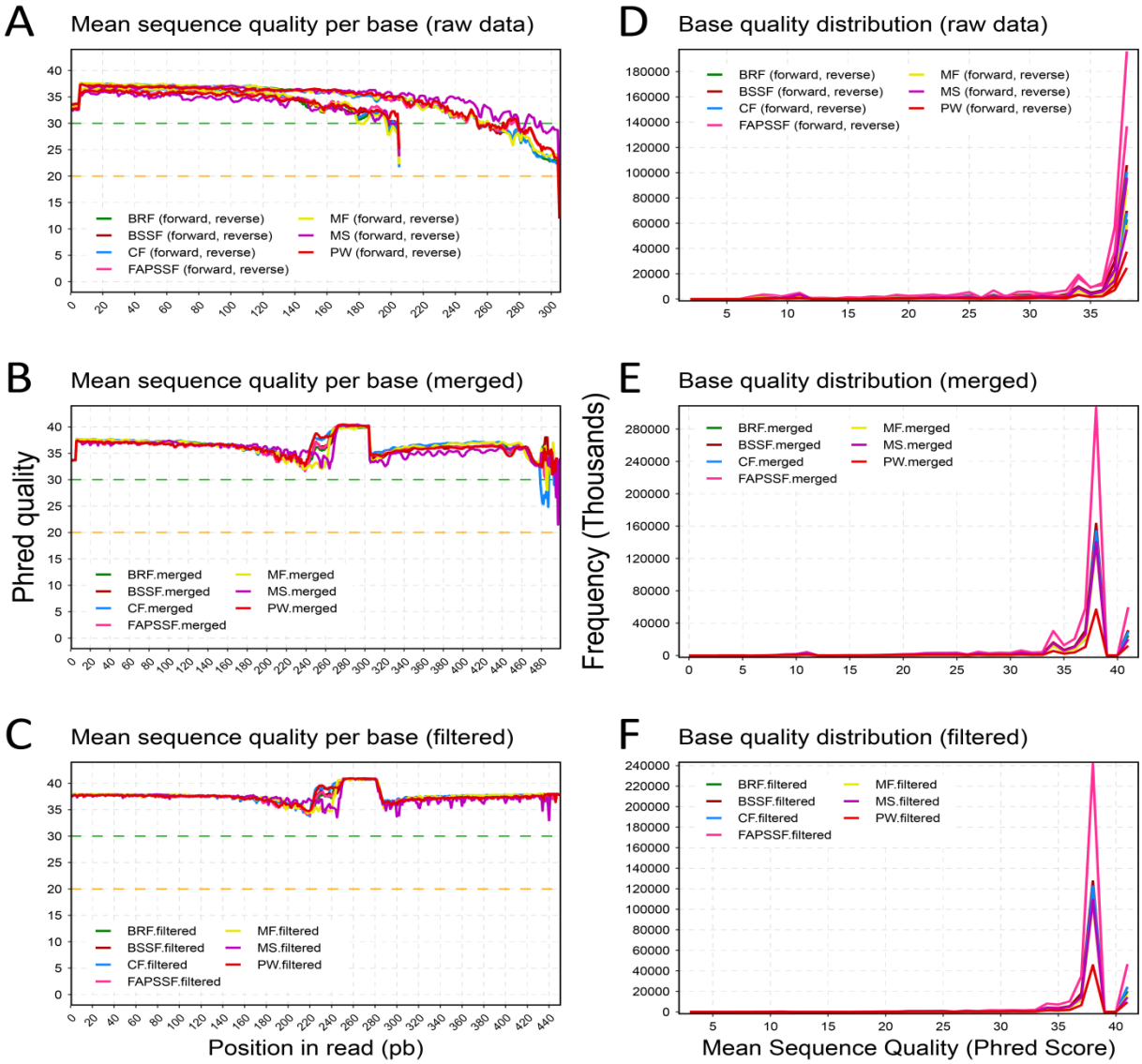

Figure 2. PHRED scores of reads obtained after DNA sequencing from different microbiome samples.
A, B and C: PHRED quality according to base pair position; D, E and F: PHRED quality according to sequence frequencies. Lines: green - BRF (bovine ruminal fluid); dark red - BSSF (biodigester sludge swine farm); blue - CF (cow feces); pink - FAPSSF (facultative aerobic pond sludge in swine fridge); yellow - MF (mice feces); purple - MS (mice skin); and red - PW (produced water from oil field storage tank)

The quality distribution of reads based on the raw PHRED score was optimal in all samples of microbiomes ranging from 11–38 (Figure 2A and 2B). It should be noted that the readings at bases 205 and 305 in the forward and reverse sequences, respectively, showed lower reliability and may contain errors, with PHRED scores below 20 (Figure 2A). In addition, the data presented low frequencies of PHRED scores below 20, and a high frequency of PHRED scores at 38 (Figure 2D).

After merging the forward and reverse sequences, the PHRED score showed a

presented range of 22-41 (Figure 2B and Figure 2E). Notably, base reads in the position range of 275-305 showed higher reliability with a PHRED score of 41, while positions between 480 and 495 had a higher probability of errors, although no position was found to have a PHRED score below 20 (Figure 2B). Additionally, were no frequencies of PHRED scores below 20, and a high frequency of PHRED scores at 38 (Figure 2E).

After performing quality control, sequences with a PHRED score below 20 were removed (Figures 2C and 2F). As a result, the scores ranged from 33-41, with the highest scores were found between positions 250-285 (Figure 2C). Furthermore, the highest frequency also in the PHRED score of 38 (Figure 2F).

3.1.3 The use of the CostSaver DNA protocol maintained the taxonomic pattern between replicates of different samples.

The relative abundance of genera identified in the replicates of different samples is presented in Figure 3. The Freedman test was additionally used to verify the existence of significant differences between replicates (Supplementary Material, Table S1).

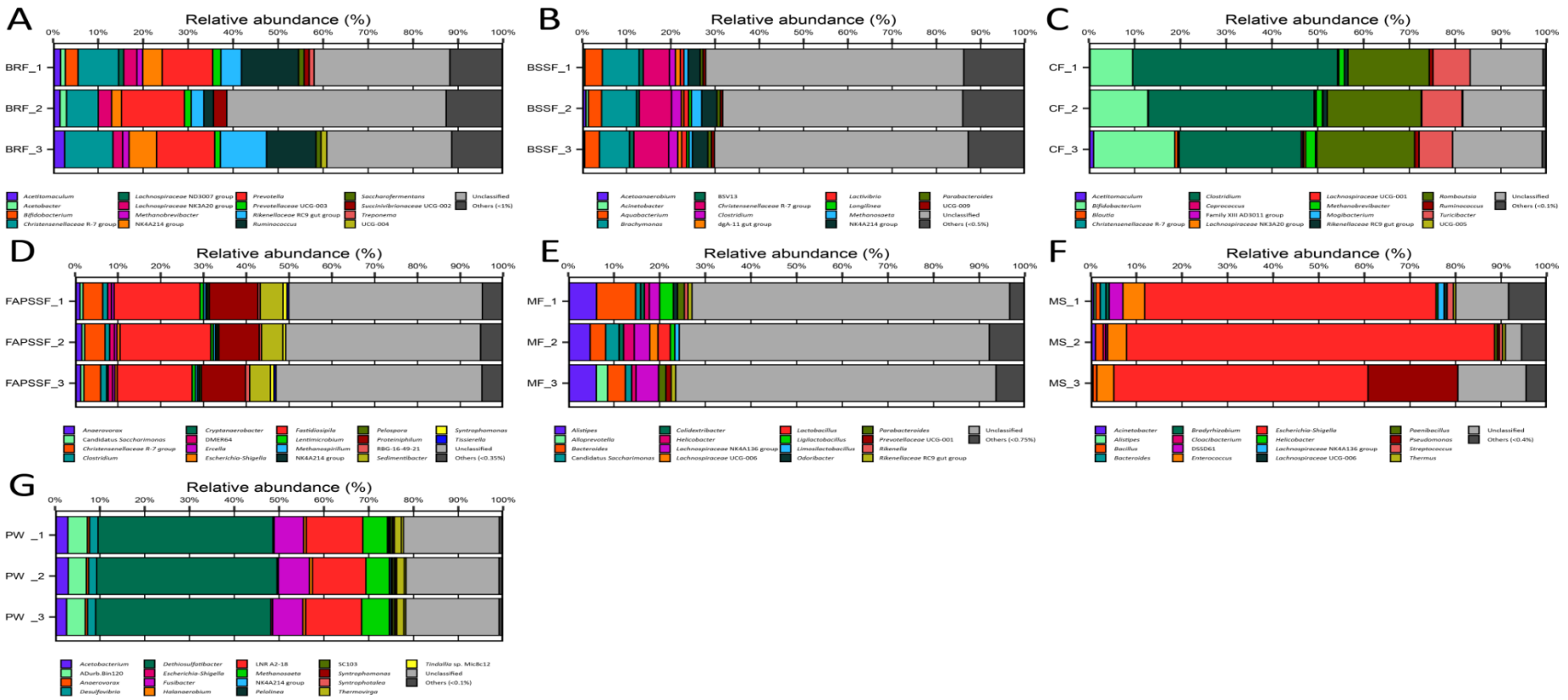

Figure 3. Relative abundance of genera identified in the replicates of different microbiome samples

A) BRF (bovine ruminal fluid); B) BSSF (biodigester sludge swine farm); C) CF (cow feces); D) FAPSSF (facultative aerobic pond sludge in swine fridge); E) MF (mice feces); F) MS (mice skin); and G) PW (produced water from oil field storage tank). Each color in the bar graphs indicates a different genus, and the proportions indicate the abundance of each genus in replicates of the different samples.

It was observed that only the PW samples (Figure 3G) did not show significant differences between technical replicas (Supplementary Material, Table S1). In contrast, replicates of samples from animals (Figures 3A, C, E and F) and effluent treatment system sludge (Figures 3B and D) showed significant differences (Supplementary Material, Table S1). However, when comparing the bar graphs, it was found that the relative proportions of each category were similar to each other (Figure 3).

3.2 COSTSAVER DNA PROTOCOL X COMMERCIAL KIT

3.2.1 Performance comparison of DNA extraction

The performance of the CostSaver DNA protocol and a commercial kit (FastDNA Spin for Soil) were compared in terms of cost, execution time, execution of purification step, yield and purity of extracted DNA (Table 4).

Table 4. Performance of the DNA extraction methods CostSaver DNA protocol and the commercial kit FastDNA Spin for Soil for a produced water sample.

Sample	Cost/ sample ($)	Time/ sample (h)	Purification step*	Yield (µg)	$A_{260/280}$	$A_{260/230}$
PWA	0.54	6	No	10.19 ± 3.73a	1.57 ± 0.05a	0.80 ± 0.08a
PWB	8.22	2	Yes	17.94 ± 6.29a	1.87 ± 0.04b	0.39 ± 0.1b

Produced water (PW) from oil field storage tank. DNA extracted from PW with CostSaver DNA (PWA) and FastDNA Spin Kit for Soil (MP Biomedicals, USA) (PWB). *Execution of column/filter-based purification Different uppercase letters in the columns indicate statistical difference by Tukey's test ($P < 0.05$) in present study.

The commercial kit FastDNA Spin Kit for Soil (PWB) had a shorter execution time since it did not require sample incubation for long periods when compared to the CostSaver DNA protocol (PWA). However, the CostSaver DNA protocol (PWA) was the most cost-effective (Table 4). When comparing the yield and purity of the DNA extracted from PW

using the two methods, it was possible to observe that the commercial kit presented a higher DNA yield and a higher $A_{260/280}$ in comparison to the CostSaver DNA protocol; however, the commercial kit had a lower $A_{260/230}$. It is important to note that the ideal $A_{260/280}$ for pure DNA is 1.80, and the ideal $A_{230/260}$ is 2.00 (Angthong et al., 2020). When evaluating extracted DNA quality through agarose gel electrophoresis, a band with less degraded DNA was observed when using the CostSaver DNA protocol when compared to the commercial kit (Figure 4).

When comparing the yield and purity of the DNA extracted from PW using the two methods, it was possible to observe that the commercial kit presented a higher DNA yield and a higher $A_{260/280}$ in comparison to the CostSaver DNA protocol; however, the commercial kit had a lower $A_{260/230}$. When evaluating extracted DNA quality through agarose gel electrophoresis, a band with less degraded DNA was observed when using the CostSaver DNA protocol when compared to the commercial kit (Figure 4).

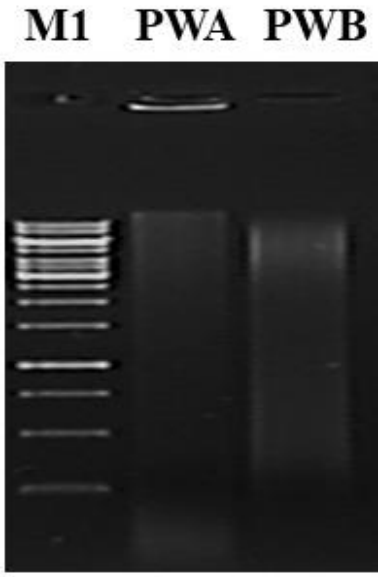

Figure 4. Agarose gel electrophoresis of metagenomic DNA extracted from produced water by two different methods.

PWA: CostSaver DNA protocol; and PWB: commercial kit FastDNA Spin Kit for Soil, MP Biomedicals, USA. M1: 1 kb ladder (Ludwig Biotec, RS, Brazil).

3.2.2 Performance comparison of sequencing quality

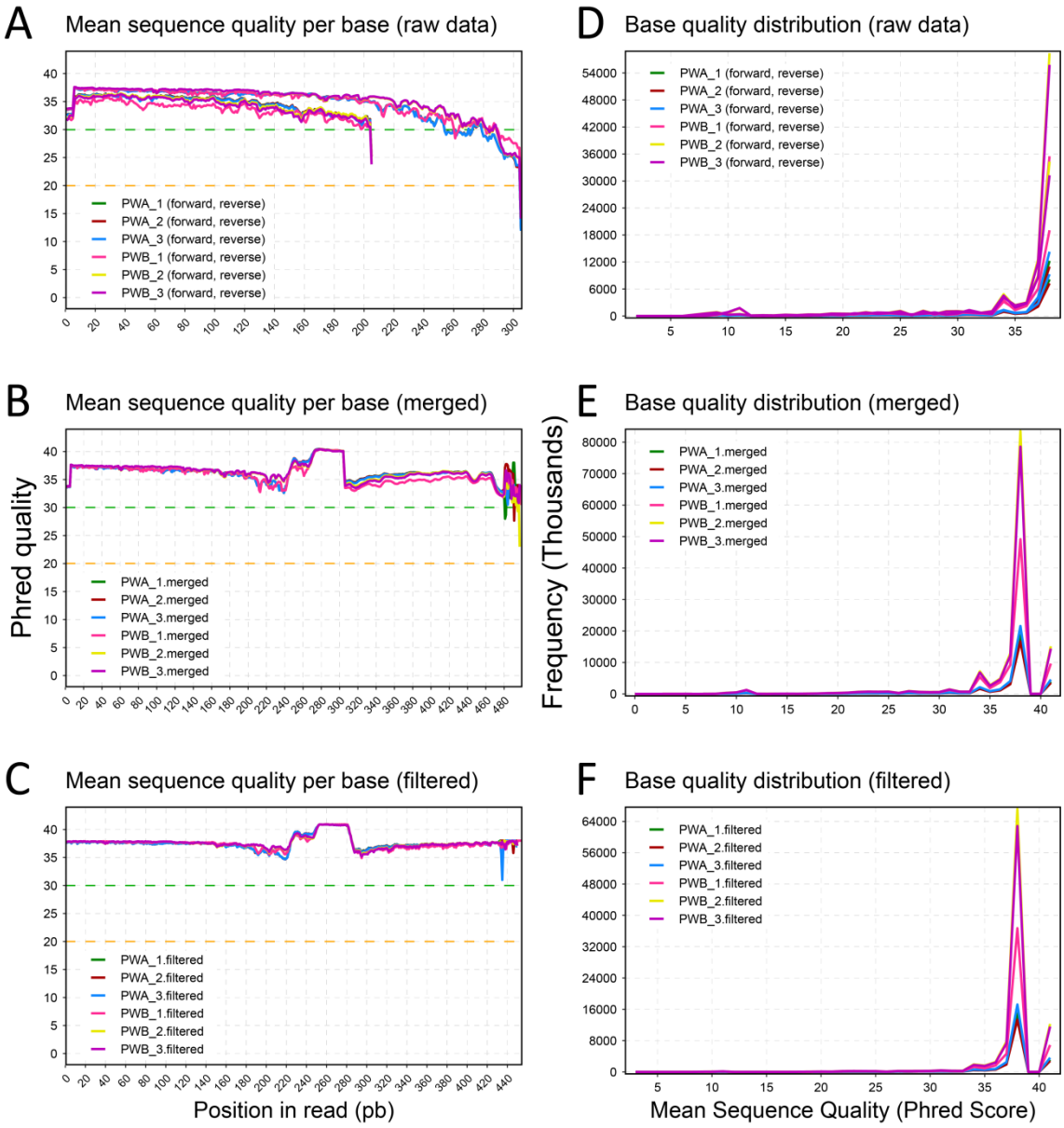

Figure 5. PHRED scores of reads obtained after DNA sequencing from produced water. Samples were extracted with the CostSaver DNA protocol (PWA) and commercial kit FastDNA Spin Kit for Soil, MP Biomedicals, USA (PWB). A, B and C: PHRED quality according to base pair position; D, E and F: PHRED quality according to sequence frequencies.

The quality distribution of reads, based on PHRED scores of the raw forward and reverse sequences, was optima in both the CostSaver DNA protocol and the commercial kit, as evidenced by a broad range of scores ranging from 12-38 (Figure 5A and 5D).). It should be noted that the readings at bases 205 and 305 in the forward and reverse sequences,

respectively, showed lower reliability and may contain errors, with PHRED scores below 20 (Figure 5A). In addition, the data presented low frequencies of PHRED scores below 20, and a high frequency of PHRED scores at 38 (Figure 5D).

After merging the forward and reverse sequences, the PHRED scores ranged between 23-41 (Figure 5B and Figure 5E). Notably, base reads in the position range of 275-305 showed higher reliability with a PHRED score of 41, while positions between 480 and 495 had a higher probability of errors, although no position was found to have a PHRED score below 20 (Figure 5B). Additionally, there were no frequencies of PHRED scores below 20, and a high frequency of PHRED scores at 38 (Figure 2E).

After performing quality control, sequences with a PHRED score below 20 were removed (Figures 5C and 5F). As a result, the scores ranged from 30.5-41, with the highest scores were found between positions 250-285 (Figure 2C). Furthermore, the highest frequency of PHRED scores was 38 (Figure 2F).

3.2.3 The use of the CostSaver DNA protocol and the commercial kit maintained the taxonomic pattern among samples

The relative abundance of genera identified in the PW sample replicates extracted with the CostSaver DNA protocol and a commercial kit is presented in Figure 6. Additionally, the Freedman test was used to evaluate the presence of significant differences between the replicates and samples extracted with the two different protocols (Supplementary Material, Table S2).

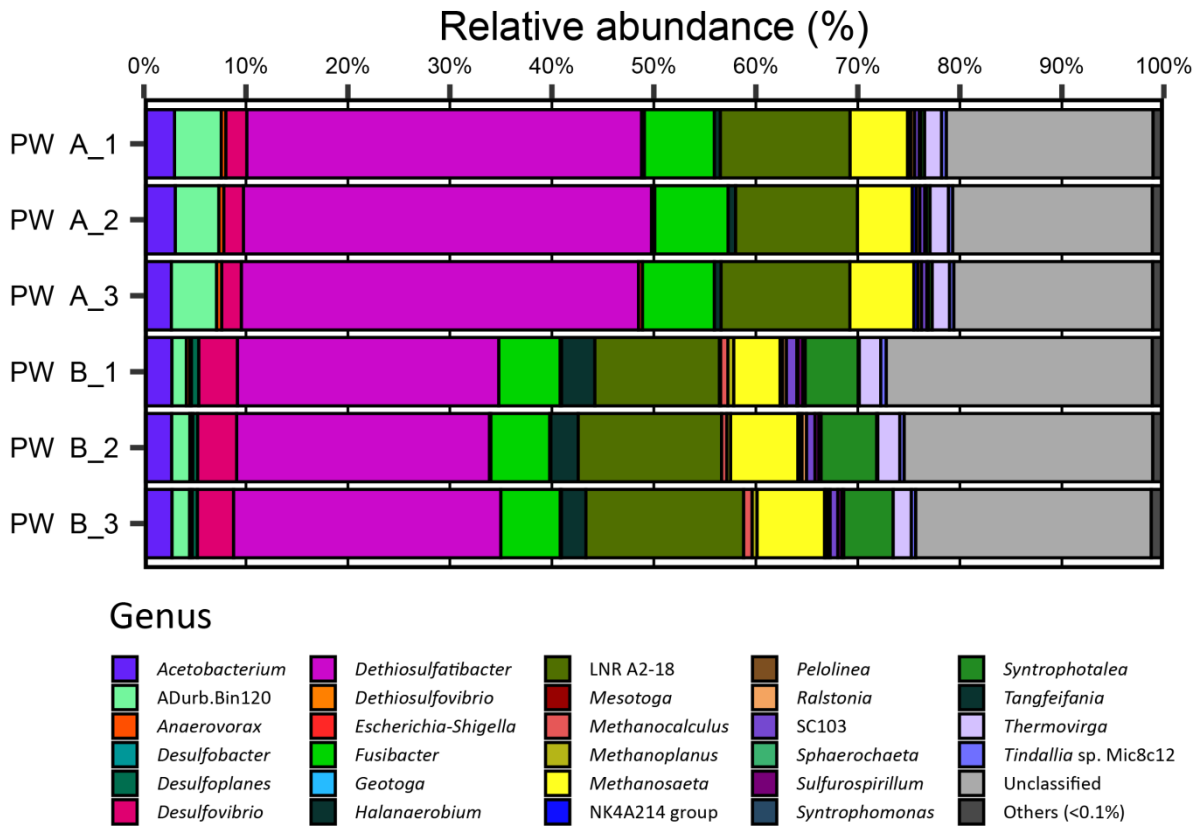

Figure 6. Relative abundance of genera identified in produced water sample replicates extracted by two different methods. PWA: CostSaver DNA protocol; and PWB: commercial kit FastDNA Spin Kit for Soil, MP Biomedicals, USA. Each color in the bar graphs indicates a different genus, and the proportions indicate the abundance of each genus in replicates of the different samples.

Through the Freedman test (Supplementary Material, Table S2), it was observed that there were no significant differences in the number of ASVs between replicates in both samples, PWA (extracted using the CostSaver DNA protocol) and PWB (extracted with the commercial kit) (Figure 6B). However, when comparing the two methods (CostSaver DNA and commercial kit), significant differences were found ($F < 2.2$ e-16) (Supplementary Material, Table S2). This is evidenced by the Syntrophotalea and Halanaerobium genera, which showed the greatest discrepancy between the relative abundances when the two extraction methods were evaluated, below 5.46 % and 3.27 % in samples extracted with the commercial kit and below 0.38 % and 0.73 % in samples extracted with the CostSaver DNA

protocol, respectively.

Despite this fact, there was only a slight variation in the proportions of each genera in the bar graphs (Figure 6), suggesting a relatively uniform distribution of genera among samples. This corroborates the results observed in the correlation analysis, which shows a positive and significant correlation (rho = 0.68, $p \leq 0.05$) between samples extracted using the CostSaver DNA protocol and the FastDNA Spin Kit for Soil.

4 DISCUSSION

CostSaver DNA is a metagenomic DNA extraction protocol applied to different types of samples with low extraction cost and short execution time, obtaining extracted DNA with sufficient quality for 16S rRNA amplicon sequencing. samples.

After performing the extractions with the adapted homemade protocol, $A_{260/280}$ below 1.80 were obtained for the FAPSSF, MF, MS, and PW samples, suggesting inefficiency in protein removal and other contaminants that absorb at 280 nm (Angthong et al., 2020). This was probably due to the complexity of the samples, which makes DNA extraction difficult (Oldham et al., 2012), in addition to containing contaminants that can interfere with cell lysis, nucleic acid capture, or enzyme activity (Wilson, 1997). Additionally, $A_{260/230}$ lower than 2.0 were found in all samples, except BRF and CF, which suggests contamination with, for example, phosphates, salts, phenols, detergents (e.g., EDTA), carbohydrates, lipids, guanidine HCl, and magnetic beads (Díaz et al., 2022; P. F. Liu et al., 2009). Although some environmental samples showed unsatisfactory purity, samples processed with the CostSaver DNA protocol exhibited high molecular mass DNA, which is an important measure for evaluating DNA integrity (Angthong et al., 2020). Furthermore, the high quality of the 16S rRNA amplicon sequences from environmental samples was evidenced by PHRED scores above 20 (Christensen, 2018), observed both before and after the quality control.

Notwithstanding these challenges, the purity ($A_{260/280}$ and $A_{260/230}$) of the extracted

DNA does not significantly affect the alpha and beta diversity of the samples (Van Syoc et al., 2021). In fact, Van Syoc et al. (2021) found that although all four DNA extraction methods they evaluated had suboptimal $A_{260/280}$ and $A_{260/230}$ ratios, the resulting microbial community profiles obtained from sequencing the 16S rRNA gene of the chicken cloacal microbiome were not significantly affected by the different extraction methods. Within this context, sequencing quality is of utmost importance, as it can be a determining factor for the efficient acquisition of microbial diversity in a sample (Kozich et al., 2013).

The significant differences in ASVs displayed among replicates of different samples extracted with the CostSaver DNA protocol, except for PW, may be due to several factors, such genera distribution present in the sample, presence of contamination (outliers), and biological variability (Weiss et al., 2017). Nevertheless, the microbial profile among replicates of different microbiome samples remained proportional, thereby ensuring the reliability of the CostSaver DNA protocol.

When evaluating the $A_{260/280}$ of DNA from PW samples extracted with the commercial kit, a satisfactory ratio above 1.80 is observed (Angthong et al., 2020), which is justified by the purification step, present in the commercial kit, that is absent in the CostSaver DNA protocol. In the present study, the step of protein removal with proteinase K and purification was excluded from the CostSaver DNA protocol after the adaptation of the method by Yu and Morrison (2004) to minimize DNA loss, since environmental samples usually present low DNA yield due to the high presence of contaminants (Akbari et al., 2014). Although the commercial kit showed higher purity regarding the removal of proteins and other contaminants that absorb at 280 nm, the commercial kit presented a lower ratio of A260/230 compared to the CostSaver DNA protocol.

In this case, the kit itself may have been the source of DNA contamination with other contaminants that absorb at 230 nm, such as chaotropic salts present in silica columns that are

commonly used in commercial kits (Tanveer et al., 2016). Furthermore, the DNA extraction using the CostSaver DNA protocol presented a more intact band on the agarose gel, indicating a lower degree of material degradation (Koshy et al., 2017).

The high quality of the 16S rRNA amplicon metagenomics sequences derived from samples PW extracted with the CostSaver DNA protocol was demonstrated by the PHRED scores exceeding 20 (Christensen, 2018). This was also evident in the sequences obtained through amplicon metagenomics sequencing of DNA extracted using the commercial kit which also showed no significant losses, indicating good quality (Habibi et al., 2022).

There were no significant differences between the replicates of PW extracted with the CostSaver DNA protocol and the commercial kit. Although, despite the statistical differences between the number of ASVs in the groups (CostSaver DNA protocol and commercial kit), genera abundances were proportional among the samples, except for the *Syntrophotalea* and *Halanaerobium* genera, with the commercial kit presenting a greater abundance of these genera when compared to CostSaver DNA protocol. Pérez-Brocal et al. (2020) evaluated the recovery of bacterial and fungal DNA by comparing different DNA extraction methods for using in lung microbiome studies: the authors observed that the extraction method, although affecting only a small percentage of the microbial community, influences the detection and abundance of bacterial and fungal taxa detected in the samples. However, in the present study, these genera present only in PW samples extracted with the commercial kit had an abundance below 0.5 %. Furthermore, Lu et al. (2015) claim that the extraction method is not capable of substantially distorting the composition of the microbial community. Thus, the interpretation of the obtained results obtained should be performed according to the ecological context and considering complementary information about it (Dutra, Garc, et al., 2023; Dutra, Gomes, et al., 2023). In addition to exhibiting viable characteristics for application in various microbiomes, the CostSaver DNA protocol also has a lower cost, representing savings of

93.83 % compared to the FastDNA Spin Kit for Soil. (Eagle et al., 2023) conducted an analysis of the cost of DNA extraction per sample using six commercial kits and found that values ranged from US$ 3.54 for the DNeasy Blood and Tissue Kit (Qiagen) to US$ 11.25 for the EZ1 DNA Tissue Kit (Qiagen), which means that even the kit with the lowest cost was still 86.76 % more expensive than the protocol developed and tested in the present study.

4 CONCLUSION

Our results demonstrated that the CostSaver DNA protocol has a good cost-benefit ratio and is feasible to the analyzes of different sample types, since it obtained extracted DNA with good quality at a low cost. Thus, it can assist scientists who wish to use 16S rRNA amplicon sequencing in a robust, reproducible, easy, and economical way, especially with a large number of samples, for which the costs would be too high if using commercial kits.

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CAPÍTULO 3

CAPÍTULO 3 - EFFECTIVE BIOCORROSIVE CONTROL IN OIL INDUSTRY FACILITIES: 16S RRNA GENE METABARCODING FOR MONITORING MICROBIAL COMMUNITIES IN PRODUCED WATER

Controle efetivo da biocorrosão em instalações da indústria do petróleo: metabarcodificação do gene 16s rRNA para o monitoramento de comunidades microbianas na água produzida

Artigo publicado na revista "Microorganisms" em janeiro de 2023.

Nas últimas décadas, diversos estudos têm evidenciado as limitações do monitoramento baseado exclusivamente no Número Mais Provável (MPN) para avaliação de risco associado à corrosão microbiologicamente influenciada (CIM). Esses métodos convencionais podem fornecer resultados inconclusivos e até mesmo incorretos, levando a ações equivocadas por parte dos operadores da indústria petrolífera.

Diante dessa problemática, os métodos moleculares microbiológicos (MMM) têm despertado interesse entre os profissionais do setor. O uso desses métodos tem possibilitado a detecção e identificação abrangente e precisa de grupos microbianos e espécies presentes em substratos complexos, principalmente por meio do uso do gene marcador 16S rRNA, que permite o sequenciamento direcionado de bactérias e arqueas. No entanto, quando se trata de campos de petróleo, surge uma importante questão: será que a abordagem de sequenciamento do gene 16S rRNA a partir de culturas microbianas nos fornece uma visão substancial do microbioma presente?

Para responder a essa pergunta, realizamos um estudo no qual conduzimos o sequenciamento do gene 16S rRNA em amostras de água produzida coletadas diretamente dos tanques de reservatório de resíduos de petróleo. Além disso, analisamos também amostras de água produzida enriquecidas com um meio de cultura específico para promover o crescimento de bactérias redutoras de sulfato, produtoras de ácido e anaeróbias totais, as quais são amplamente reconhecidas na literatura como estando relacionadas à corrosão microbiologicamente influenciada (CIM). Ao comparar as amostras coletadas diretamente do ambiente com as amostras enriquecidas, nosso objetivo foi identificar correlações entre a composição microbiana da água produzida, as características físico-químicas do ambiente e o enriquecimento de espécies de interesse.

Confirmamos que o sequenciamento do gene 16S rRNA a partir do DNA de amostras de água produzida coletadas diretamente do ambiente, juntamente com a análise das

características físico-químicas do ambiente, é uma abordagem mais representativa e abrangente no monitoramento de campos petrolíferos, em comparação com o sequenciamento de amostras enriquecidas. Essa estratégia nos permite obter uma visão mais completa e precisa das comunidades microbianas presentes nos diversos ambientes da indústria petrolífera.

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Article

Effective Biocorrosive Control in Oil Industry Facilities: 16S rRNA Gene Metabarcoding for Monitoring Microbial Communities in Produced Water

Joyce Dutra [1,2], Glen García [3], Rosimeire Gomes [1], Mariana Cardoso [3], Árley Côrtes [2], Tales Silva [2], Luís de Jesus [2], Luciano Rodrigues [4], Andria Freitas [2], Vinicius Waldow [5], Juliana Laguna [2], Gabriela Campos [2], Monique Américo [2], Rubens Akamine [5], Maíra de Sousa [5], Claudia Groposo [5], Henrique Figueiredo [4], Vasco Azevedo [1,2,3] and Aristóteles Góes-Neto [1,3,*]

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Abstract: Microbiologically influenced corrosion (MIC) or biocorrosion is a complex biological and physicochemical process, Strategies for monitoring MIC are frequently based on microbial cultivation methods, while microbiological molecular methods (MMM) are not well-established in the oil industry in Brazil. Thus, there is a high demand for the development of effective protocols for monitoring biocorrosion with MMM. The main aim of our study was to analyze the physico-chemi- cal features of microbial communities occurring in produced water (PW) and in enrichment cultures in oil pipelines of the petroleum industry. In order to obtain strictly comparable results, the same samples were used for both culturing and metabarcoding. PW samples displayed higher phylogenetic diversity of bacteria and archaea whereas PW enrichments cultures showed higher dominance of bacterial MIC-associated genera. All samples had a core community composed of 19 distinct genera, with MIC-associated *Desulfovibrio* as the dominant genus. We observed significant associations between the PW and cultured PW samples, with a greater number of associations found between the cultured sulfate-reducing bacteria (SRB) samples and the uncultured PW samples. When evaluating the correlation between the physicochemical characteristics of the environment and the microbiota of the uncultivated samples, we suggest that the occurrence of anaerobic digestion metabolism can be characterized by well-defined phases. Therefore, the detection of microorganisms in uncultured PW by metabarcoding, along with physi-cochemical characterization, can be a more efficient method compared to the culturing method, as it is a less laborious and cost-effective method for monitoring MIC microbial agents in oil industry facilities.

Keywords: petroleum; produced water; microbiologically influenced corrosion; oil industry; metabarcoding

1. Introduction

Microbiologically influenced corrosion (MIC) or biocorrosion is a process the leads to the deterioration of pipelines, tanks, and other facilities in the oil industry due to the metabolic activities of microorganisms [1,2]. These microorganisms mainly include Archaea and Bacteria, but also eukaryotes, such as Fungi [3–5]. Fungal community and aerobic prokaryotes consume molecular oxygen (O_2), contributing to the establishment and survival of anaerobic microorganisms, which may have mechanisms that trigger biocorrosion, such as the reduction of Fe(0) through extracellular electron transfer (EET-MIC) [6].

In addition, some sulfate-reducing archaea (SRA), such as *Archaeoglobus*, contributes to a significant increase in corrosion due to the reduction of sulfate (SO_4^{2-}) to hy-drogen sulfide (H_2S) [7]. Nonetheless, bacteria are usually the most studied microorganisms associated with the process of metallic biocorrosion in the oil industry [8]. Sulfate-reducing bacteria (SRB) and acid-producing bacteria (APB) produce H_2S and organic acids, which are highly corrosive products, as an integral part of their metabolism [4,8]. Both ecological groups are classic examples of microorganisms that contribute to MIC in oil industries [4,8–10]. Other bacterial groups that deserve attention in biocorrosion processes are elemental sulfur-reducing bacteria (S_0RB) [11], iron-reducing bacteria (IRB) [12], nitrate-reducing bacteria (NRB) [13], and thiosulfate-reducing bacteria (TRB) [14].

MIC monitoring is a complex task due to the heterogeneity of biofilms composition and distribution inside pipelines, as well as the localized nature of biocorrosion [15,16]. In oil industry facilities, microorganisms are distributed in different environments [17], either as biofilm components adhered to the surfaces of pipelines and tanks [1,18], in water microdroplets dispersed in the oil phase [17], or suspended in the aqueous phase [19]. The aqueous phase can be composed of formation water (FW), originally present in the reservoir, injection water (IW), used for enhanced oil recovery [19], and produced water (PW), that can be a mixture of the FW and IW [20].

Metallic installations in the oil industry are prone to failures [2], and these failures are often associated with corrosion processes: about USD 2.5 trillion is spent yearly on repairs and mitigation of its consequences [21]. Among that total, it is estimated that 10 to 20% of those cases involve MIC [21–23]. The culture-dependent most probable number (MPN) method based on serial dilution in culture media is the most widely used method for the detection and quantification of microorganisms in the oil industry [24], and it is commonly used to monitor SRB, APB, and general anaerobic heterotrophic bacteria (GANB) [25].

In recent decades, some studies have shown that monitoring using MPN for the purposes of MIC risk assessment can provide inconclusive and incorrect results, leading to erroneous actions [26,27]. This is because, usually, no more than 1% of microbial species present in environmental samples are able to grow in a specific culture medium [26–28].

Culture-independent techniques such as microbiological molecular methods (MMM) have been attracting the interest of oil industry operators [21,29,30]. The application of MMM has made it possible to more comprehensively and accurately detect and identify microbial groups and species occurring in complex substrates [31]. Those methods rely on biomarkers such as adenosine triphosphate (ATP), ribonucleic acid (RNA), deoxyribonucleic acid (DNA), proteins, and metabolites [32,33].

Metagenomics is based on the large-scale sequencing of DNA molecules directly extracted from environmental samples [34], which allows for a genomic investigation of microbial communities without the need for prior cultivation in the laboratory [35]. Thus, Next-Generation Sequencing (NGS) technology associated with the development of massively parallelized sequencing platforms and bioinformatic programs to analyze generated data have promoted an exponential increase in the number of genomes and metagenomes sequenced at low cost, contributing to the dissemination and improvement of metagenomics [10,36,37]. Furthermore, metagenomics can employ a pre-amplification step followed by large-scale sequencing of genomic regions of biomarkers, an approach called amplicon metagenomics or metabarcoding [21].

In this study, we performed metabarcoding to analyze the spatial variation in the structure of bacterial and archaeal communities in PW from storage tanks and enrichments obtained on the culture media for SRB, APB, and GANB, and their correlation with physicochemical features of PW.

2. Materials and Methods

2.1. Sampling of Produced Water (PW)

The collection of PW samples was performed in an oil storage and transfer terminal, located at Duque de Caxias-RJ, Brazil. PW samples were collected from side valves connected at two heights (1.00 m and 2.75 m) of a drainer tank, a total of 14.63 m, which receives the residual volume of water and oil from other storage tanks. Sampling was performed in triplicate using three sterile plastic containers of 5.0 L per sampling point. The samples were immediately transported at room temperature, for a maximum of 2 h, to the laboratory, where all the samples were subjected to three distinct processes: (i) physicochemical characterization (lactate, acetate, propionate, formate, butyrate, sulfate (SO_4^{2-}), soluble sulfides (S^{2-}), and chloride (Cl^-) (ii) culturing by inoculation in distinct flasks containing specific culture media for the growth of SRB, APB, and GANB; (iii) filtering with the use of membranes (Kasvi, Curitiba, Brazil) with 0.22 µm diameter mesh using vacuum pumps for microbial cell retention and subsequent extraction of metagenomic DNA.

The fatty acid and ions analysis was perfomed using a Dionex-2500 ion chromatograph (Dionex Corporation, Sunnyvale, CA, USA) with an AS18 (4 × 250 mm) column at 30 °C with a flow rate of 1.0 mL/min for 30 min per sample eluted in a 2–40 mmol/L gradient of KOH [38,39]. The alkalinity was analyzed by potentiometric titration [40]. The Agilent SpectrAA 280 atomic absorption spectrometer was used for the quantification of Fe. The flame used was air acetylene, with a wavelength of 248.3 nm and a slit width of 0.2 nm. The calibration curve was prepared from a 1000 mg/L iron standard solution. In this protocol it is not possible to distinguish between Fe^{2+} and Fe^{3+}. The pH and temperature were analyzed using a Kasvi pH-meter (K39-1014B).

2.2. Produced Water (PW)

Microbial cells from PW samples from collection points at 1.00 m and 2.75 m were concentrated using disposable filtration systems with polyethersulfone (PES) membrane filter (91 mm diameter and 0.22 µm pore size). From each sampling point, approximately 450 mL of PW were filtered. Filters (Kasvi, Curitiba, Brazil) with retained microbial cells were placed in sterile 50 mL tubes and stored in an ultrafreezer (−80 °C) for subsequent extraction of DNA.

2.3. SRB, APB, and GANB Enrichment Cultures Obtained from PW

Detection and quantification of microorganisms of major physiological groups (SRB, APB, and GANB) were performed using the MPN method [41]. The MPNs of acid-producing bacteria (APB), sulfate-reducing bacteria (SRB), and general anaerobic heterotrophic bacteria (GANB) were determined from ten-fold serial dilutions ranging from 10^{-0} to 10^{-8} using 1 mL of PW samples (of 1.00 m and 2.75 m) inoculated into 10 mL flasks containing 9 mL specific medium for each group (APB, SRB, and GANB), and the time between collection and testing did not exceed 48 h. The assay was performed in triplicate. The SRB culture medium composition was: 0.5 g/L KH_2PO_4, 1 g/L NH_4Cl, 1 g/L Na_2SO_4, 1 g/L $CaCl_2 \cdot 6H_2O$, 1.83 g/L $MgCl_2 \cdot 6H_2O$, 1 g/L yeast extract, 7 ml/L sodium lactate, 0.5 g/L $FeSO_4 \cdot 7H_2O$, 1.9 g/L agar-agar, 4 mL/L rezasurin, and 0.12 g/L sodium thioglycolate, with a pH of 7.6 [42]. For the culturing of APB, the culture medium TSI (Agar Triple Sugar Iron) was used composition: 3 g/L meat extract, 3.g/L yeast extract, 15 g/Lcasein pancreatic digest, 5 g/L protease peptone, 1 g/L dextrose, 10 g/L lactose, 10 g/L sucrose, 0.2 g/L ferrous sulfate, 5 g/L sodium chloride, 0.3 g/L sodium thiosulfate, 12 g/L agar, and 24 mg phenol red [43]. In turn, for the culturing of GANB, the medium with the following

composition was used: 5 g/L glucose, 4 g/L universal peptone, 1 g/Lyeast extract and 4.0 mL of rezasurin solution 0.025% *m/v* [44].

The flasks containing the cultures were incubated at a temperature of 30 ± 2 °C (similar to in situ temperature), for a total of 28 days. After incubation, the first flask inoculated with PW was selected for further DNA extraction. Cultures were centrifuged at 11,000× *g* for 1 min at room temperature. For quantification, mathematical equations were used, based on Poisson's law, as shown in the Supplementary Materials. Cell pellets were stored in an ultrafreezer (−80 °C) for later extraction of DNA. The first flask inoculated with PW was selected for further DNA extraction.

2.4. DNA Extraction

The DNA from PW was extracted from membrane filters with 100 and 250 mg retained microbial cells from PW (p_1.00 and p_2.75 m) and from 0.1 g of pellet from SRB, APB, and GANB enrichment cultures obtained from PW (from points 1.00 and 2.75 m). DNA extraction was performed in triplicate using the FastDNA™ Spin Kit for Soil (MP Biomedicals, Jacksonville, USA), following the manufacturer's instructions. Quantification and purity (evaluated through A260/280 and A260/230) were confirmed in the NanoDrop (Thermo Fisher Scientific, Waltman, USA). DNA integrity was verified through visualization of 1.0% (*m/v*) agarose gel electrophoresis.

2.5. 16S rRNA Gene Amplicon Metagenomic Sequencing

Amplification was performed with primers for the V_3–V_4 region of the 16S rRNA gene: 341F (5′-CCTACGGGRSGCAGCAG-3′) [45] and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [46]. Library preparation and sequencing were performed in the MiSeq sequencing platform (Illumina Inc., USA), using the paired-end type Kit V2 for 500 cycles (2 × 250 bp).

2.6. Bioinformatic Analyses

Metabarcoding data were analyzed using the Amplicon Sequence Variant (ASV) approach and utilizing a customized pipeline developed in Python 3. The pipeline source code is deposited on GitHub (https://github.com/LBMCF/pipeline-for-amplicon-analysis), accessed on 14 January 2022.

This pipeline uses USEARCH [47] of 32-bits, VSEARCH [48], Cutadapt [49], and FastQC [50]. Initially, the quality control of the reads was evaluated by FastQC. Then, all reads were merged with USEARCH using the —fastq_merge pairs option, and sequencing adapters were cut with Cutadapt. Quality control was performed with VSEARCH using the —fastq_filter and —fastq_maxee = 0.8 options. For the elimination of duplicates, VSEARCH was also used with the option —derep_fulllength. The ASV table was generated with USEARCH with the —unoise3 option. The removal of chimeras and the generation of the table of ASVs were performed using the USEARCH unoise3 algorithm (option —unoise3). Additionally, the generation of the absolute abundances table was performed with the usearch_global and id = 0.99 VSEARCH options. The taxonomic identification and classification of ASVs were performed using SINTAX [51] in USEARCH with the database SILVA SSU NR 138.1 [52] as a reference. Finally, the consolidation of the abundance table with the taxonomic identifications was carried out with the customized script get_abundances_table_asv.py, included in the source code. For ecological analyses, the taxonomic level of genus was used.

The quality of the sequencing of the third replicate of the APB_1.00 sample was not satisfactory. Therefore, a statistical inference strategy was used, in which the ASVs of the third replicate of the variable (APB_1.00) were inferred from the first and second replicate. This technique is called the conditional mean algorithm. This technique was used to maintain the standard of three replicates per sample. The rules used are presented below:

- If x = 0 and y = 0, then the conditional mean is 0;
- If x = 5 and y = 0, then the conditional mean is 5;
- If x = 0 and y = 8, then the conditional mean is 8;

- If x = 3 and y = 4, then the conditional mean is 3.5 (or 3, since the integer part is used);
- If x = 6 and y = 9, then the conditional mean is 7.5 (or 7, since the integer part is used).

2.7. Integrating Metabarcoding and of PW Samples and Enrichments

Relative abundances (%) were calculated from the ASVs and analyses were performed at the phylum and genus levels. These relative abundances were calculated using the dplyr package (v1.1.0) in R [53]. Comparative analyses of PW metabarcoding data and its corresponding enrichment cultures (SRB, APB, and GANB) were verified at the distinct sampling points (beta diversity). For this, alpha diversity data was obtained using PAST 4.08 [54]. These data were correlated with the relative abundances of the genera in each sample, also using the same program, through canonical correspondence analysis (CCA). The Shannon index (Shannon_H) was used to determine the attribute of diversity (richness and abundance), the Simpson index (Dominance_D) was used to assess dominance, and the Equitability Index was utilized to measure the uniformity of distribution of ASVs between groups [55]. In beta diversity, the correlations of information related to the distribution patterns of microbial genera within the ecosystem were evaluated based on Shannon diversity, dominance, and equitability indices.

For the analysis of shareability and uniqueness of microbial genera among the samples, the UpSetR package (version 1.4.0) in R was used [56]. To plot the bar graph by phylum, a threshold of 0.5% was used, and those below 0.5% were merged into a same category named "Others". For the genus level, a threshold greater than or equal to 1.0% was also used, and all genera below 1.0% were described as "Others".

A statistical analysis of the association between genera and samples was performed (PW_1.00 m, PW_2.75 m, SRB_1.00 m, SRB_2.75 m, APB_1.00 m, APB_2.75 m, GANB_1.00 m, and GANB_2.75 m), represented in a network, so that the network shows only strong associations based on absolute abundances. The analysis was calculated using the indicspecies package version 1.7.6 [57] with the multipatt and the point biserial correlation coefficient (r.g.) as a function for calculating the strength of association (SA) between genera and samples. The significance cut-off point for the phi coefficient was set at $p < 0.05$. The association network was built in Cytoscape, version 3.5.1 [58] using the "edge-weighted spring embedded" layout. The size of the nodes (node degree) represents the logarithm of the absolute abundance of the genera. The length of the edges represents the strength of association: the shorter the edge length, the stronger the association between the genera and the samples.

Principal Component Analysis (PCA) was used to explore all data in an integrative way. PCA summarizes information from a very high number of original variables into a small set of statistical variables. In our study, the inter-relationships between sets of microbial genera were analyzed using the first two principal components (PC1 and PC2). The data were initially submitted to the Hellinger transformation and, later, analyzed using PCA and visualized using two-dimensional scatter plots using the packages ggplot2 v3.3.5 and factoextra v1.0.7 R [59,60]. Through the distance between sampling points (tank height and cultures from environmental inocula), it was inferred whether the characteristics were similar or divergent. The length of the vectors indicates the region that has more (or less) microbial genera influencing the variability of the data, and the angle formed between the variables indicates the correlation between them. Exploratory analyses were also performed through clustering methods. Cluster analysis were performed and associated with a color matrix for better visualization of the results (only those with an abundance of 0.5% or more were displayed), using the R Heatmaply package [61,62].

2.8. Correlations of the Composition of Microbial Communities to the Physicochemical Parameters of PW

In order to infer the potential metabolic functions of the microbial genera detected by metabarcoding, physicochemical characterization of the PW samples was initially performed. For this, the biocorrosion diagnostic protocols for the examination of water and

wastewater were used [63]. The analyses included pH, organic acids (lactate, acetate, propionate, formate, and butyrate), sulfate, soluble sulfides, iron, chlorides, salinity and thermal conductivity, and all these analyses were adapted according to [38,39], along with alkalinity, modified by Petrobras [40]. Subsequently, physicochemical data were analyzed, in an integrative way, with the abundances of microbial genera, using the canonical correspondence analysis (CCA).

3. Results

3.1. MPN Methods

Enrichment cultures from sampling points p_1.00 m and p_2.75 m showed microbial quantification of 10^5 MPN/mL of SRB and GANB, with slightly higher growth of APB, 10^6 MPN/mL (Supplementary Materials). Regardless of the results of MPN quantification, all culture flasks that showed positive growth were processed, and their DNA was extracted.

3.2. Shareability and Uniqueness Patterns and Relative Abundance of Phyla and Genera Revealed by Metabarcoding in PW and Enrichments

The main patterns of the communities of both produced water (PW_1.00 m and PW_2.75 m) and enrichments (SRB_1.00 m and SRB_2.75 m; APB_1.00 m and APB_2.75 m; and GANB_1.00 m and GANB_2.75 m) are depicted Figure 1.

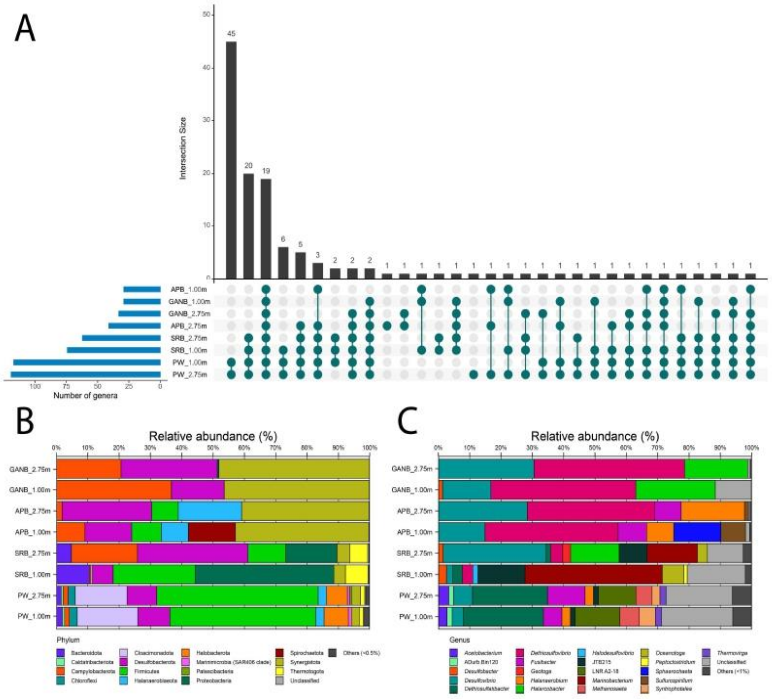

Figure 1. Shareability, uniqueness, and relative abundance (%) of identified microbial groups in metabarcoding PW samples (PW) at heights 1.00 m and 2.75 m and in, SRB, APB, and from a drainage tank. (**A**) Black vertical bars indicate the number of shared and unique genera among samples marked with green balls. Blue horizontal bars indicate the number of genera in each sample. (**B**) Bar graph. Each color indicates a different phylum, and the proportions indicate the abundance of each phylum in the different samples, at different heights. (**C**) Bar graph. Each color indicates a different genus, and the proportions indicate the abundance of each genus in the different samples, at different heights.

PW_1.00 m and PW_2.75 m samples showed the highest number of unique gener compared to the other samples, with 45. Amongst the enrichment cultures, the SRB group was the most diverse, displaying 20 genera shared between them (SRB1.00 and SRB_2.75) and with the PW samples (PW_1.00 m and PW_2.75 m) (Figure 1A and Supplementary Materials).

We observed that the phyla Halobacterota (6.68–7.72%) and Cloacimonadota (16.51–19.31%) were identified with a relative abundance value higher than 0.5% only in samples PW_1.00 m and PW_2.75 m (Figure 1B). Phylum Halobacterota was represented by methanogenic archaea, with a higher abundance of the genus *Methanothrix* (Figure 1C). In contrast, the phylum Cloacimonadota was represented by only one taxon described as Cloacimonadaceae LNR A2-18 (Figure 1C). The phylum Proteobacteria was significant only in samples of SRB_1.00 m and 2.75 m (Figure 1B), with a higher abundance of the genus *Marinobacterium* (16.04–43.82%). In contrast, unclassified organisms were more abundant in samples PW_1.00 m (22.70%), PW_2.75 m (21.18%), SRB_1.00 m (18.50%), SRB_2.75 m (11.39%), and GANB_1.00 m (11.47%).

There were 19 genera shared by metabarcoding in PW and the corresponding enrichments (Figure 1A). The phylum Desulfobacterota was highlighted (Figure 1B), and the genus *Desulfovibrio* displayed relative abundances above 1.9% in all samples (Figure 1C). It is worth noting that the enrichment SRB_2.75 m showed a higher relative abundance of *Desulfovibrio* (32.84%) compared to the other samples. However, in SRB_1.00 m, the relative abundance of *Desulfovibrio* was notably lower (1.9%) compared to other samples.

3.3. Microbial Diversity of PW and Enrichments

Alpha diversity indices (diversity, dominance, and equitability indices) and Canonical Correspondence Analysis (CCA) are depicted in Figure 2A,B, respectively.

The highest microbial diversity corresponded to PW samples (PW_1.00 m and PW_2.75 m) (Figure 2A,B). A significant pattern was detected in both PW_1.00 m and PW_2.75 m samples, which showed a higher Shannon diversity index and equitability.compared to cultured samples (Figure 2A), except for the SRB_1.00 m and SRB_2.75 m samples, which did not present statistical differences with the PW_1.00 m and PW_2.75 m samples regarding equitability, (Supplementary materials). On the other hand, the dominance was low in PW samples (PW_1.00 m and PW_2.75 m). Moreover, GANB (1.00 m and 2.75 m) enrichments exhibited a higher dominance, compared to PW samples (Supplementary materials).

CCA analysis of the PW and enrichments explained 100% of the cumulative variance of the correlation between the diversity indices and the distribution of communities of the different sample types at distinct heights (Figure 2B). The vectors referring to diversity (Shannon index) and equitability indices share the same quadrant (a) and are close. These samples contained a group of methanogenic archaea that were not identified in the enrichments. The vector referring to dominance is located in the (c) quadrant, opposite to the diversity and equitability indices, with a higher dominance in GANB samples, especially of *Dethiosulfovibrio* (46.25–48.04%).

3.3.1. Statistical Evaluation of Patterns among Microbial Communities Directly Retrieved from PW and Enrichment Cultures

Statistical analyses describing patterns of microbial communities obtained from both PW (PW_1.00 and PW_2.75) and corresponding cultures (SRB, APB and GANB) from PW samples, at heights of 1.00 m and 2.75 m, are depicted in Figure 3A,B. Specifically, in Figure 3A, the association analysis between microbial genera detected in produced water (PW) and cultures (SRB, APB, and GANB is displayed. The interaction network exhibited in Figure 3A comprises four distinct groups of samples: (1) PW (green), (2) SRB (pink), (3) APB (yellow), and (4) GANB (blue). Group 1 (PW) presentes two well-defined branches, representing the largest group of microorganisms. Groups 2 (SRB) and 3 (APB) exhibit three branches, and group 4 (GANB) displays two distinct branches (Figure 3A). Several microbial genera were significantly and exclusively associated with to both produced water and cultured samples. The highest number of exclusively associated genera (n = 65) was

observed in the PW samples, followed by the cultured sample (SRB n = 15).APB samples showed only two genera statistically and exclusively associated, while GANB samples did not display any significant or exclusive associated genera (Figure 3A).

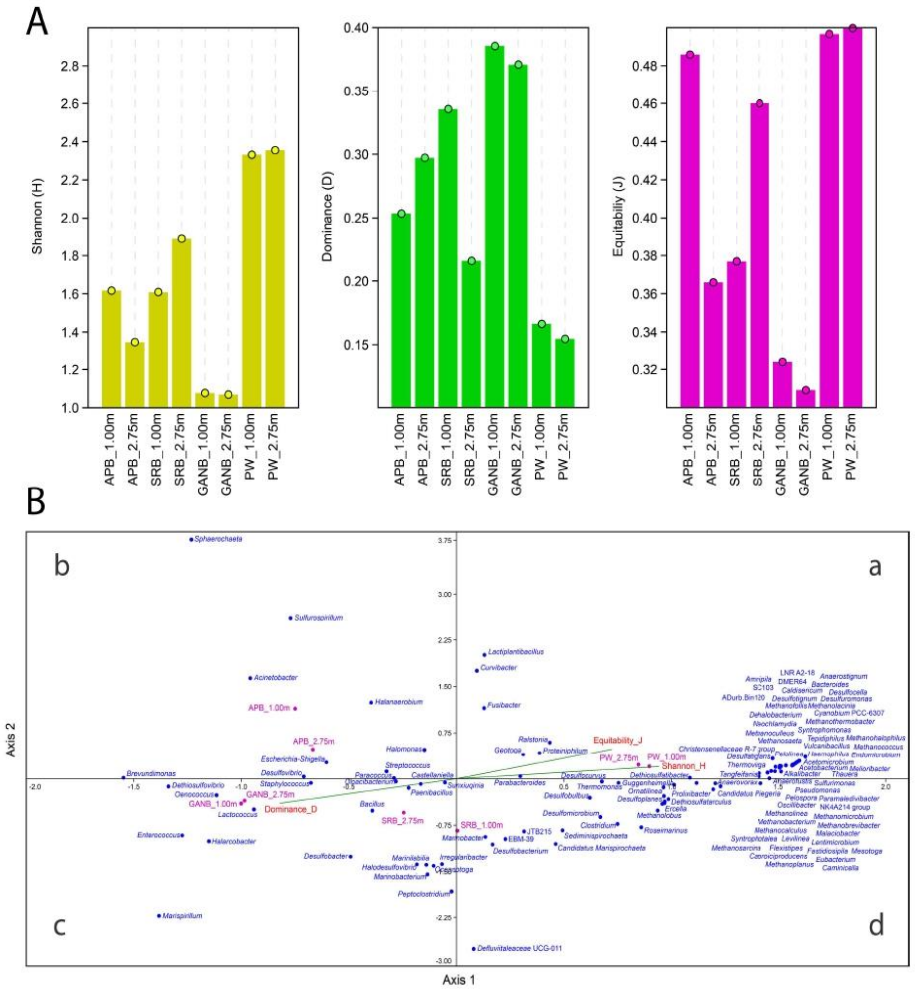

Figure 2. Alpha diversity indices and Canonical Correspondence Analysis (CCA) of microorganisms identified by metabarcoding in PW samples (PW) at heights 1.00 m and 2.75 m and, SRB, APB, and GANB enrichments at heights of 1.00 m and 2.75 m of a drainage tank (**A**) Vertical axis indicates the values of diversity indices, and the x axis indicates the analyzed samples (**B**) CCA: Pink circles indicate the sample type. The elements in red indicate the analyzed community ecological indices. Blue circles indicate the microbial genera that were detected in PW and enrichment cultures at different heights. Green vectors indicate the direction of each variable associated with the microbial genera of each sample type (a) quadrant I (b) quadrant II (c) quadrante III (d) quadrante IV.

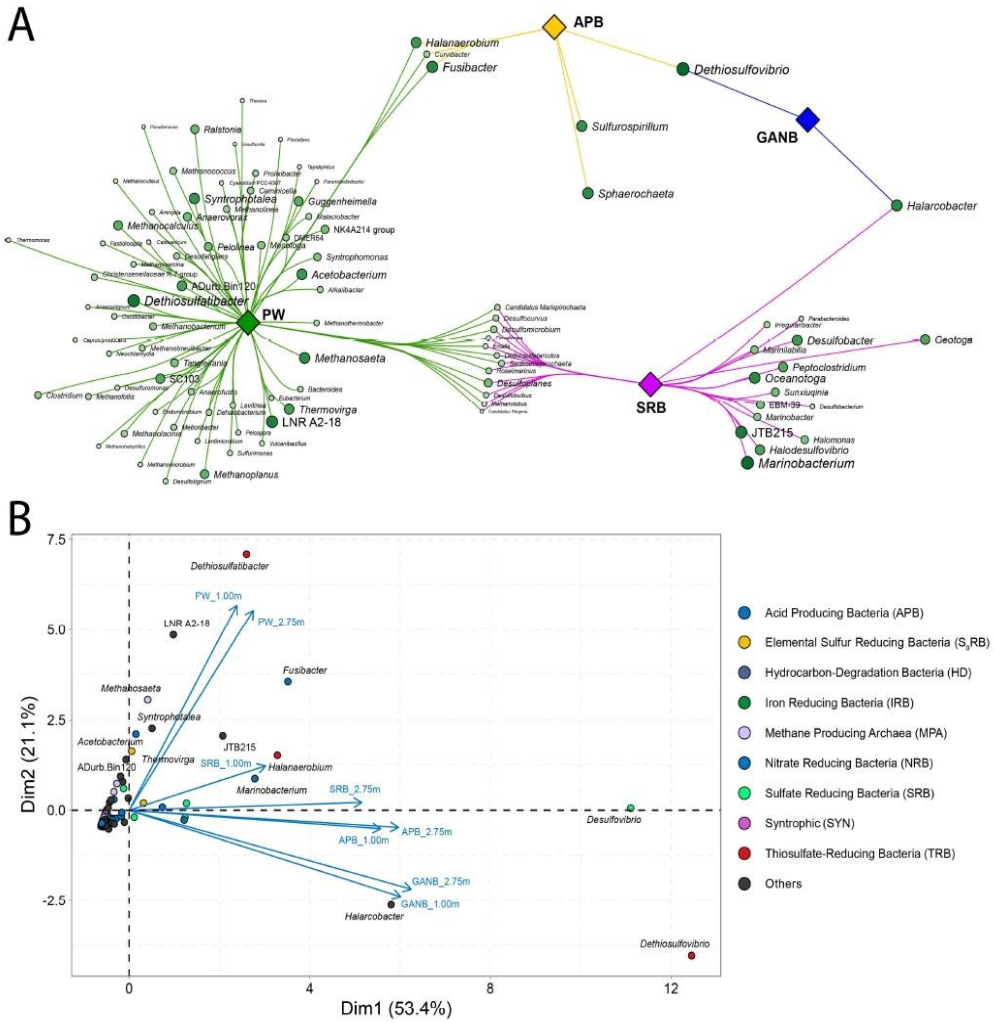

Figure 3. Association network and principal component analysis (PCA) of microorganisms identified by metabarcoding from PW samples (PW) at heights 1.00 m and 2.75 m and in enrichment cultures from PW samples, SRB, APB, and GANB at heights of 1.00 m and 2.75 m of a drainage tank (**A**) refers to association network by sample type and (**B**) refers to PCA analysis. In A, the diamonds indicate the nodes referring to the samples, which are differentiated by colors. The edges indicate the associations also represented in different colors depending on the sample type. The circles indicate the genera, with a longer diameter and higher color intensity indicating greater relative abundance. In B, the circles indicate the genus of microorganisms, and each color high lights a different group. Vectors refer to samples at different heights. PW: metabarcoding PW samples at heights 1.00 m and 2.75 m; and enrichment cultures from PW: SRB, APB and GANB at heights of 1.00 m and 2.75 m.

PW samples were associated with SRB and APB groups (Figure 3A). The interaction with the SRB group was performed through 12 nodes, with emphasis on *Desulfoplanes* (SA = 0.81 and $p = 0.0002$). In turn, the interaction with the APB group was through three genera of similar intensities: *Fusibacter* (SA = 0.68 and $p = 0.0031$), *Halanaerobium* (SA = 0.61

$p = 0.012$), and *Curvibacter* (SA = 0.69 $p = 0.0047$). The association between the APB and GANB groups was represented only by *Dethiosulfovibrio* (SA = 0.76 $p = 0.0007$), while *Halarcobacter* represented the association between the GANB and SRB groups (SA = 0.7, $p = 0.0027$).

Principal component analysis (PC1 and PC2) was performed to explore the interrelationships between the variables and explain these variables in terms of dimensions (Figure 3B). PC1 and PC2 explained 74.50% of data variability: PC1 explained 53.40% and PC2 explained 21.10% of the data variability. The PW_1.00 m and PW_2.75 m samples were closer to each other, and the group variability encountered in those was similar and better explained by PC2 (Figure 3B). Similarly, the GANB 1.00 m and GANB_2.75 m cultures were also more related to each other, and the variability of microbial groups found in these samples was better explained by PC1, when compared to the other samples. APB samples also exhibited a similar behavior, with overlapped vectors. The different sample heights (1.00 m and 2.75 m) were not significant to explain the data variability.

3.3.2. Cluster Analysis in Q and R Modes

In the cluster analysis visualized through a heat map (occurrence and abundance), the samples were separated into two main clusters (Figure 4). Cluster A was divided into two subclusters: A1 composed of samples GANB_1.00 m and GANB_2.75 m, and A2, composed of samples APB_1.00 m and APB_2.75 m. Cluster B was divided into two subclusters (B1 and B2). Subcluster B1 was composed of samples PW_1.00 m and PW_2.75 m. In turn, subcluster B2 was subdivided into two less inclusive subclusters, B2.1 and B2.2. Subcluster B2.1 was constituted by the sample SRB_1.00 m and subcluster B2.2 by the sample SRB_2.75 m (Figure 4).

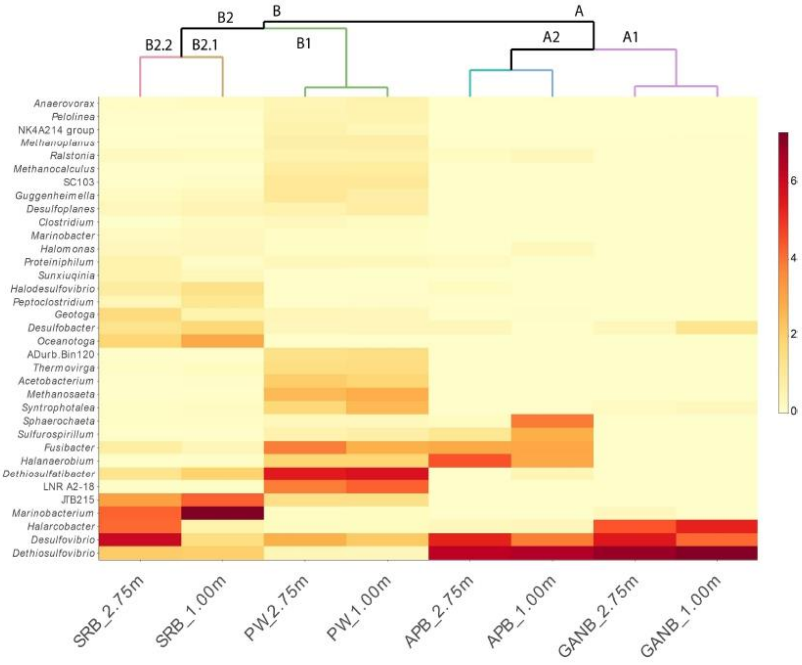

Figure 4. Dendrogram and color matrix of the abundances of microbial genera present in PW and enrichment cultures from a drainage tank. The columns indicate the formation of clusters and subclusters by sample, and the lines indicate the genera. Relative abundances are measured by the intensity of the color: the darker the color, the greater the relative abundance of a given genus.

The groups were formed according to sample type: cluster A (GANB_1.00 m and GANB_2.75 m), and (APB_1.00 m and APB_2.75 m); and cluster B (PW_1.00 m and PW_2.75 m) and (SRB_1.00 m and SRB_2.75 m). In cluster A, dominance of the sulfate and thiosulfate-reducing genera, *Desulfovibrio* and *Dethiosulfovibrio*, was observed. The difference between these subclusters is directly related to the genus *Halarcobacter* (GANB_1.00 m and GANB_2.75 m) and the APB *Halanaerobium* and *Fusibacter* (APB_1.00 m and APB_2.75 m. In cluster B, the produced water samples PW_1.00 m and PW_2.75 m) showed a very similar profile, with high abundance of the thiosulfate-reducing genus *Dethiosufatibacter*, with relative abundances of 25.49% and 23.95%, respectively. In general, the PW samples exhibited a more homogeneous profile of genera and relative abundances above 0.5% (Figure 4).

However, the SRB cultures displayed less similar the microbial profiles between SRB_1.00 m and SRB_2.75 m when compared to other sample groups. A great discrepancy was observed in the relative abundances of the SRB *Desulfovibrio*, with 32.84% in the SRB_2.75 m sample and 1.98% in the SRB_1.00 m sample. Another contrasting pattern between distinct SRB samples by height occurred for *Marinobacterium*, with 43.82% in the SRB_1.00 m sample, and 16.04% in the SRB_2.75 m sample, as well as for the genus *Halarcobacter*, with 15.50% in the SRB_2.75 m sample, and 0.36% in the SRB_1.00 m sample (Figure 4).

3.4. Physicochemical Characterization

3.4.1. Physicochemical Features of PW

Physicochemical conditions displayed different patterns when the evaluation was performed on samples collected at different heights in the tank (Table 1). Point p_2.75 showed a lower pH than point p_1.00. Conversely, butyrate and acetate concentrations were higher at p_2.75. The short chain organic acids (lactate, propionate, and formate) exhibited values below the detection limit. Moreover, anion analyses indicated similar results at both points, except for sulfate, which showed a higher concentration at 1.00 m compared to 2.75 m.

Table 1. Physicochemical characterization of produced water (PW) samples collected from drainage tank at heights of 1.00 m and 2.75 m.

Feature	Unit	p_1.00	p_2.75
pH	-	7.00	6.00
Lactate	mg/L	n.d	n.d
Acetate	mg/L	970.00	1500.00
Propionate	mg/L	n.d	n.d
Formate	mg/L	n.d	n.d
Butyrate	mg/L	29.00	67.00
Sulfate (SO_4^{2-})	mg/L	310.00	170.00
Soluble sulfides (S^{2-})	mg/L	57.80	54.90
Chloride (Cl^-)	mg/L	2.60	2.80
Iron	mg/L	0.48	0.59
Alkalinity	meqs/L	25.50	28.70
Salinity	PPT	37.50	36.40
Electrical conductivity (EC)	Ms	65.00	60.71
Dissolved solids (DS)	PPT	31.55	30.25
Turbidity (Turb)	NTU	90.125	106.25

Sampling points: p_1.00 (1.00 m) e p_2.75 (2.75 m).

3.4.2. Associations between the Physicochemical Features and the Microorganisms Present in PW Environmental Samples

Associations were evaluated between the microbial genera belonging to physiological groups, such as APB, SRB, NRB, TRB, S0RB, IRB, MPA (methane producing archaea), hydrocarbon degradation bacteria (HD), and SYN (syntrophic) and the physicochemical

features, such as acetate (Ac), alkalinity (Alc), iron (Fe), chloride (Cl^-), butyrate (But), turbidity (Turb), soluble sulfides (S^-), salinity (Sal), pH, sulfate (SO_4^{2-}), dissolved solids (DS),and electrical conductivity (EC) of PW samples (p_1.00 and p_2.75) (Figure 5). CCA analysis of environmental PW samples (PW 1.00 and PW 2.75) explained 100% of the cumulative variance between environmental factors and the distribution of microbial communities' present at both heights (p_1.00 and p_2.75).

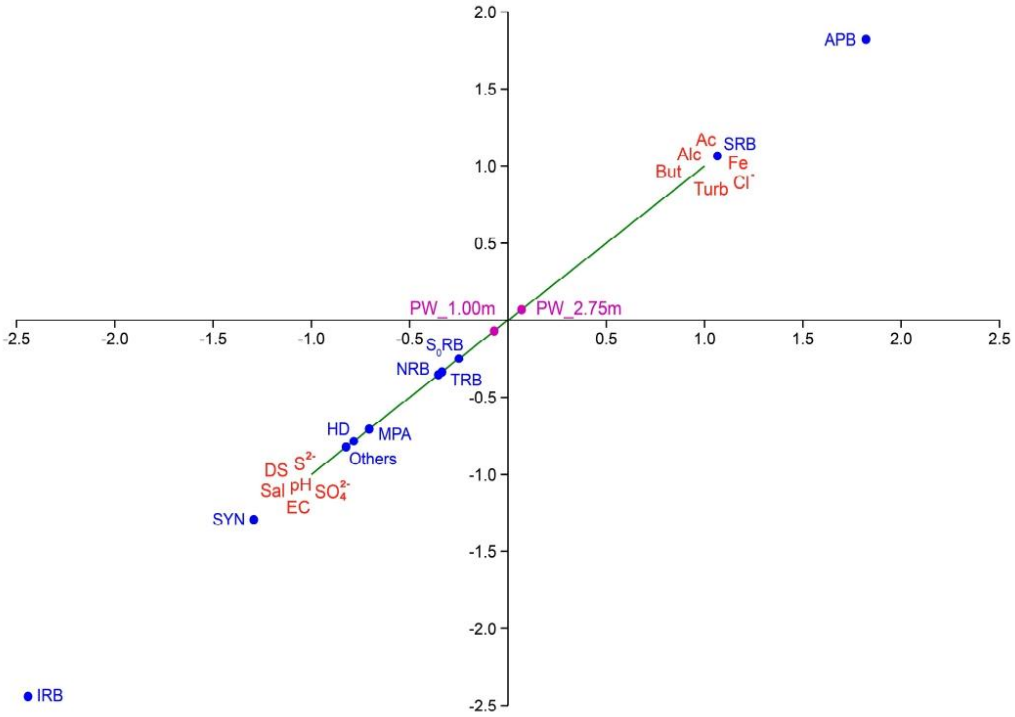

Figure 5. Canonical correspondence analysis (CCA) of the physicochemical features and abundance of microorganisms present in produced water (PW) samples from a drainage tank. The pink circles, indicate the sample type, the red abbreviations are the ions, organic acids, and physicochemical features of produced water (Ac = acetate; Alc = alkalinity; Fe = iron; Turb = turbidity; Cl^- = chloride; But = butyrate; S^{2+} = soluble sulfides; SO_4^{2-} = sulfate; pH = hydrogen potential; EC = electrical conductivity; Sal = salinity and DS = dissolved solids). The blue circles indicate the ecological groups of microorganisms found in the environment, and the green vectors indicate the direction of each variable associated with the ecological groups of each sample.

At the height of 1.00 m, a higher abundance of MPA, HD, IRB, and synthophic (SYN) prokaryotes was retrieved (Figure 5), with a joint location of groups S_0RB, TRB and NRB, in the center of the graph. At the height of 2.75 m, a different microbial profile was observed with a higher amount of fermentative and sulfidogenic organisms (APB and SRB groups) (Figure 5).

4. Discussion

Our study evaluated the microbiota of environmental PW samples (PW) and the corresponding enrichment culture obtained in media for specific microbial groups (SRB, APB, and GANB). These samples were collected from two heights (1.00 and 2.75 m) of a drainage tank that receives fluids from other tanks located in a distribution terminal.

Diversity (taxonomic composition, richness, abundance, evenness, and dominance patterns) as well as multivariate ordination and cluster analyses were performed using 16S rRNA metabarcoding both original produced water and enrichment cultures. Additionally, the physicochemical features of PW were also evaluated in order to associate them with the richness and abundance of the genera present in this environment and, thus, infer the putative metabolism of the studied microbial groups.

In this study, we observed that the growth of GANB was similar to those in SRB and APB cultures. Cultivation of GANB includes the growth of anaerobic organisms that use organic compounds as a carbon and energy source. This group can be considered broad and heterogeneous [64], and it is used as an index of the total contamination by anaerobic microbes in a system since they can promote the establishment of corrosive groups such as SRB to the metal surface [65]. On the other hand, diversity analysis indicated that the culturing of GANB exhibited a higher dominance index, compared to PW and other cultured samples (SRB and APB). Regardless of the culture media used, there was an evident strong selection process caused by the specificity of the culture medium and/or by the incubation conditions, such as temperature and anoxia [66].

High diversity observed in the PW samples (1.00 and 2.75 m) can be attributed to different microbial groups, which do not necessarily contribute to MIC since the unique genera identified from environmental samples displayed an abundance lower than 0.2% and are, therefore, quite rare. Nonetheless, this high diversity in environmental samples can be attributed to the more abundant organisms in the PW samples, such as the methanogenic archaea (6.23%), some of which are capable of causing biocorrosion [67], as well as unclassified microorganisms (22.70%). Conlette et al. [67] evaluated the effects of temperature and pH on corrosion rates and methane production using metal coupons. These authors reported that methane production was strongly correlated with corrosion rates and moderately correlated with pH. Despite not being properly characterized, these microorganisms have already been described in the literature, such as *Cloacimonadaceae* LNR A2-18, which is not culturable and about which there is no available information besides its 16S rRNA gene sequence [68]. According to Lu et al. [69], the non-culturable *Cloacimonadaceae* LNR A2-18 may play important roles in sites where anaerobiosis predominates.

Only five unique genera were detected in cultured samples, which are not known to be associated with MIC (*Brevundimonas*, *Enterococcus*, *Irregularibacter*, *Lactococcus*, and *Oenococcus*) with abundances lower than 0.02%. In contrast, environmental PW samples exhibited almost 10 times more unique genera, totaling 46, with relative abundances lower than 0.15%, besides unclassified organisms (Supplementay materials). This pattern is directly linked to a higher dominance of some microbial groups in cultures due to the strong selection caused by the culture media [66]. Additionally, the physicochemical and nutritional conditions favored the selection of organisms of interest [66,70]. Therefore, microorganisms less adapted to those specific conditions are excluded [66].

Only two genera, *Dethiosulfovibrio* and *Halarcobacter*, occurred in GANB medium, and they were significantly correlated with APB and SRB, but with no significant correlation with environmental PW samples. This may indicate that the GANB culture medium is not efficient at detecting anaerobic microbial diversity, as it was designed to do, probably due to the nutrient deficiencies in the culture medium of the present study, which lacked vitamins and residual metals. Sakamoto et al. [25] compared two culture media with different carbon/sulfate ratios for developing microbial groups present in UASB (Upflow Anaerobic Sludge Blanket) reactor sludge. These authors concluded that the nutritional conditions of the medium influenced the development of the groups and that the presence of vitamins and residual metal solutions favored the development of the group of general anaerobic heterotrophic bacteria (GANB) [25]. In our study, we observed significant and positive associations between uncultured PW samples and enrichment cultures. Nevertheless, Sterflinger et al. [71] question the range of organisms detected in uncultured samples. We emphasize that, in our study, it was possible to identify the presence of microorganisms previously characterized as involved in biocorrosion processes that were not identified

in cultured samples, such as methanogenic archaea. However, it is worth noting that the 16S rRNA gene-based metabarcoding approach only provides insights into the taxonomic composition and community structure, not the functionality of the community [72]. While there are bioinformatics techniques that can be used to estimate metabolic activity, these are only predicted based on physicochemical characterization of PW samples and may not represent a true indication of activity [10].

Desulfovibrio was identified in all PW and enrichment cultures samples and displays an important function in the environment since all the species are SRB, which are reference microorganisms in biocorrosion monitoring in the oil industry [70,73]. However, a high abundance of methanogenic archaea was also observed in environmental PW samples, which may characterize a syntrophic behavior between these two microbial groups [74].

The relative abundance of the genus *Desulfovibrio* in enrichment cultures obtained from PW was increased in this group, since the culture media maximize growth. This suggests that a specific medium does not exclude the development of other groups [75] since, in our study, APB exhibited high abundances (15.75–28.52%) of *Desulfovibrio*, which is a genus of SRB. The strategy that can be used in this situation is the dilution of the inoculum, which eliminates "undesirable" fast-growing species in small quantities, allowing the development of slow-growing species, which are the most abundant organisms in the community [75]. On the other hand, a relative abundance of *Desulfovibrio* of only 1.98% was observed in the SRB_1.00 m, which is an abundance value lower than that of PW samples. Hence, selective media do not guarantee the full development of genera of the same microbial group of interest [75].

Sample features can also significantly influence the abundance of diagnostic genera [76]. For instance, in the our study, in PW collected at 1.00 m (near the bottom of the tank) and subsequently cultured in SRB medium, *Desulfovibrio* showed an abundance of 1.98%, whereas in PW collected at 2.75 m (near the oil/water interface of the tank) also cultured in SRB medium, *Desulfovibrio* abundance was more than 16 times higher (32.84%). CCA analysis retrieved the associations between the physicochemical features and the microbial genera present in the uncultured PW samples. Altogether, these retrieved patterns suggests that anaerobic digestion occurred, with distinct phases at heights of 2.75 m and 1.00 m.

The presence of APB, in conjunction with the lower pH and the higher concentrations of organic acids (acetate and butyrate) at 2.75 m, when compared to 1.00 m, indicates acidogenesis. APB metabolize the products generated in hydrolysis (the first step of the anaerobic digestion process) and secrete compounds such as organic acids, acetate, short-chain volatile fatty acids (SCFA), alcohols and ketones [77–79]. In acetogenesis, most acids, such as butyric acid, propionic acid, and ethanol, are oxidized into acetate, which can be used in methanogenesis and sulfidogenesis [77,80].

PW samples (PW_1.00 m) were collected closer to the bottom of the tank, suggesting lower nutrient availability in comparison with PW samples (PW_2.75 m) collected closer to the oil/water interface. In the PW_1.00 m samples, a dominance of syntrophic, methanogens and hydrocarbon degraders bacteria was detected, where a neutral pH (7.0) was observed, a higher value compared to the lower acidic pH (6.0) detected in the PW_2.75 m samples. This is, consistent with the higher concentration of organic acids and the higher abundance of methanogens, as they are sensitive to lower pH. The availability of high concentrations of sulfate can promote another anaerobic process, the sulfidogenesis [81]. In this stage, sulfate is reduced to H_2S by SRB and SRA [77,82,83].

Besides the fermentative metabolism observed in our study, the abundance of the SRB group may suggest a syntrophic relationship between the aforementioned microbial groups. As APB partially metabolize organic compounds, SRB use fermentation products as electron donors, especially in the case of acetate and butyrate [84]. These volatile acids and propionate are the most important components in the process [84]. Nevertheless, the presence of sulfate in the SRB medium suggests that the preference is for sulfate rather than biological electron acceptors such as methanogens. Nonetheless, the high abundance

of methanogenic archaea implies that the SRB are engaged in syntrophic metabolism, indicating a possible occurrence of methanogenesis at the depth of 1.00 m. Certain genera of sulfate reducers are capable of syntrophically breaking down partially oxidized organic substances, such as lactate, ethanol, and propionate, through hydrogen (electron) transfer between species, in association with hydrogenotrophic methanogens [74].

The TRB were the most abundant group, with a dominance of the genus *Dethiosulfatibacter* (23.95–25.49%), which was not influenced by physicochemical features, since it displayed equivalent abundances in both PW samples collected at heights of 1.00 m and 2.75 m. In our study, we did not confirm if the abundance of thiosulfate reducers is related to the presence of thiosulfate in the medium because this analysis was not performed. Nevertheless, we could infer that must have significant concentrations of thiosulfate at both heights, which may be capable of causing biocorrosion in the medium [85]. According to Atkinson and Richards [85], thiosulfate can be produced by the chemical oxidation of H_2S with O_2. The authors demonstrated that thiosulfate is produced through the electrochemical dissolution of MnS in stainless steel during the onset of pitting corrosion.

The presence of anaerobic digestion, jointly with a high abundance of methanogenic archaea, suggest the occurrence of methanogenesis as well as there may be a positive and significant correlation between methane production and metallic corrosion [67].

5. Conclusions

Our findings showed that the metabarcoding study of PW samples, combined with physicochemical characterization of those samples is more representative in monitoring microbial groups in oil industry facilities, as compared to the MPN method, which is based on culturing. This makes it possible to obtain a much more comprehensive and accurate picture of the microbial communities occurring in the different environments of the oil industry. Therefore, the use of metabarcoding coupled with physicochemical characterization may promote more adequate and efficient microbiological monitoring parameters to control biocorrosive processes in petroleum industry facilities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11040846/s1; Table S1: Quantification of Acid-Producing Bacteria (APB), Sulfate-Reducing Bacteria (SRB), and General Anaerobic Heterotrophic Bacteria (GANB) in Produced Water (PW) samples; Table S2: Relative abundance of phylum by sample type; Table S3: Relative abundance of genus by sample type; Table S4: Values of the Shannon (H), Dominance (D) and Equitability (J) diversity indices of the replicates of the APB, SRB, GANB and PW samples; Table S5: Normality test of alpha diversity indices by type of sample; Table S6: ANOVA and Levene test of alpha-diversity indices by type of sample; Table S7: Tukey Post-hoc Test; Figure S1: Box plot of the Shannon indices for the APB, SRB, GANB and PW samples; Figure S2: Box plot of the Dominance indices for the APB, SRB, GANB and PW samples and Figure S3: Box plot of the Equitability indices for the APB, SRB, GANB and PW samples.

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CAPÍTULO 4

CAPÍTULO 4 - INVESTIGATING THE CAUSE OF BIOCORROSION IN PETROLEUM PIPELINES THROUGH TAXONOMIC AND FUNCTIONAL ANALYSIS OF THE 16S RRNA GENE IN BIOFILMS

Investigando a causa da biocorrosão em dutos de petróleo por meio da análise taxonômica e funcional do gene 16s rrna em biofilmes

A corrosão influenciada por microrganismos (CIM) pode se manifestar de diferentes formas, incluindo a corrosão uniforme e a corrosão localizada por pites. A corrosão por pites é especialmente preocupante, pois causa o desenvolvimento de pequenas cavidades e covas na superfície do metal, resultando em um desgaste heterogêneo e representando um alto risco para a integridade estrutural do material. A previsão desse tipo de corrosão é desafiadora, especialmente em campos de petróleo.

Para compreender melhor os processos corrosivos relacionados à CIM localizada, é essencial realizar uma comparação sistemática da composição microbiana e das atividades metabólicas entre as comunidades sésseis, presentes em biofilmes, e as comunidades planctônicas em diferentes condições ambientais. Essa abordagem oferece informações valiosas sobre a variação da composição microbiana, diversidade e atividades metabólicas associadas aos processos corrosivos.

Dentro desse contexto, o objetivo do presente estudo foi avaliar o tipo de corrosão observada nas paredes dos dutos de transporte de produtos e resíduos de petróleo. Além disso, busca-se comparar a taxonomia e as funções metabólicas da comunidade de biofilme residente no duto com maior taxa de corrosão por pites com a comunidade planctônica presente em um tanque de armazenamento que contém o mesmo tipo de resíduo circulante. Essa análise permitirá identificar diferenças na composição e atividades microbianas entre os dois ambientes, contribuindo para um melhor entendimento dos mecanismos envolvidos na corrosão microbiologicamente influenciada em campos de petróleo.

ABSTRACT

Biocorrosion, also known as microbiologically influenced corrosion (MIC), poses a significant threat to the integrity of materials and incurs in considerable costs in petroleum production and distribution systems. The heterogeneous distribution and composition of biofilms make it challenging to predict and monitor MIC in pipelines. Therefore, comprehending the morphological and compositional changes in metallic structures, the microbial profile of biofilms, and its relationship with the planktonic community is crucial for developing control and prevention strategies against biocorrosion. In this study, corrosion coupons were sampled in pipelines used for transporting various petroleum products (Crude, Stagnant and Slop) and compared the microbial community of biofilms with the planktonic community present in the produced water. These coupons were photographed and observed with scanning electron microscopy, and DNA extracted from biofilms were subjected to genetic analysis and metabolic inference. The highest corrosion rate was observed in the Slop Slop pipeline, primarily due to pitting. Approximately 33.8% of the microbial lineages were found in both corrosive biofilms and the produced water, with the majority of these organisms (92.72%) exhibiting strong ecological resemblance. The genus *Acetobacterium* of acid-producing bacterium (APB) dominated the biofilm and seemed to be involved in the metabolic degradation of 1,2-propanediol, potentially utilizing it as the primary energy source for acetate and/or propanol production. These findings contribute to improving practices for biocorrosion prevention and mitigation, thereby reducing costs and extending the lifespan of equipments.

Keywords: Sessile Communities, BPAs, Planktonic communities; SEM-EDS, Metagenomics; Localized corrosion; Corrosive metabolism.

1. INTRODUCTION

The petroleum industry plays a vital role in global energy supply and the world economy (Lisboa, 2021). This industry encompasses all stages of the production chain, including exploration and production (upstream), transportation (midstream), refining and distribution of petroleum derivatives (downstream) (Hassani et al., 2020; Sahebi et al., 2014). Additionally, the oil industry provides direct and indirect employment opportunities in various areas, including infrastructure construction and maintenance, as well as research and technology (Garcia, 2022).

According to the *Energy Information Administration*, global energy consumption by both OECD (Organization for Economic Cooperation and Development) and non-OECD countries is expected to increase from 15.5% to 73% between 2006 and 2030, with the use of oil-based energy resources. This highlights the increasing importance of the oil industry worldwide (Bachmann et al., 2014). However, producing oil with reduced environmental and financial impacts remains a challenge, as operations monitoring and equipment maintenance are costly. One problem affecting the efficiency and safety of oil operations is the corrosion of pipelines, which can lead to damages, leaks and other failures (Dutra, Gomes, et al., 2023)

Corrosion of pipelines and industrial equipment used in the production and transportation of oil and gas accounts for approximately US$ 2.5 trillion in annual expenses worldwide. It is estimated that between 10% and 20% of these corrosion cases are related to biocorrosion or microbiologically influenced corrosion (MIC) (Machuca & Salgar-Chaparro, 2019). MIC is an electrochemical corrosion process initiated or potentiated by the presence, activity or both of microorganisms, and it is a phenomenon difficult to predict and monitor in the oil industry assets (Skovhus et al., 2017; Videla, 1996).

Metal corrosion can exhibit diverse patterns, but when it comes to Microbiologically Influenced Corrosion (MIC), it is common to observe a combination of uniform corrosion and localized pitting corrosion (Beech, 2004; Miller et al., 2018). Uniform or general corrosion is the result of a continual shifting of anode and cathode regions on a metal surface, leading to nearly uniform corrosive attack on the entire surface, and it can be more easily monitored, predicted and managed. On the other hand, MIC is a type of localized corrosion that leads to the formation of pits, tubercles or crevices on the metal surface, causing heterogeneous wear and posing a higher risk to the material's structural integrity (G. Li et al., 2023; Miller et al., 2018; Yafei Wang et al., 2016).

In the oil industry, MIC is challenging to predict and monitor due to the heterogeneous

distribution and composition of biofilms throughout industrial systems (Little & Lee, 2007; Roche, 2007). Biofilms are established heterogeneously along surfaces, and this condition is related to surface heterogeneity, cell adhesion, ecological relationships between microbial lineages, and physiological changes in microbial cells, including alterations in gene expression associated with motility, amino acid metabolism, energy conservation, stress response, signal transduction, and protein synthesis (Clark et al., 2012; Santos et al., 2022).

However, understanding surface colonization and the microbial structure of biofilms requires comparing microbes in biofilms with those in the planktonic community. Since sessile and planktonic microbial communities consist of different types of microorganisms and may exhibit different responses to environmental conditions, nutrient availability, and contaminants, this information can be useful for developing control and prevention strategies for corrosion in petroleum transport pipelines (Santos et al., 2022).

In this context, the present study aims to assess the type of corrosion occurring on the internal surfaces of pipelines transporting petroleum products and residues, and compare the taxonomy and metabolic functions of the biofilm community residing in the pipeline with the planktonic community in a storage tank containing the same type of circulating residue.

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL PROCEDURE

The field samples were collected from operational industrial assets at Ilha d'Água, situated within the Aquatic Terminals of Guanabara Bay (TABG) in Rio de Janeiro, Brazil. The AISI 1018 carbon steel coupons, featuring a circular design and a collective surface area of 15.62 cm2, were utilized for the analysis.

Coupons were exposed to the internal conditions of three specific pipelines: (1) “Crude” pipeline, used for crude oil transportation, (2) “Stagnant” pipeline, containing stagnant saltwater, and (3) “Slop” pipeline, transporting various residual fluids from equipment washing, rainwater, and others. These pipelines were selected from a set of pipelines based on the criteria of higher risk of MIC. The exposure period for the coupons in the Crude pipeline was 189 days, while the coupons in the Stagnant and Slop pipelines were exposed for 103 days. Prior to the exposure, the coupons were treated according to the NACE SP0775-2018 standard.

After the exposure period, the coupons were removed to collect the formed biofilm and conduct subsequent analyses.

The residues deposited on the coupons were collected using swabs: one end of a cotton swab was rubbed across the entire surface of the coupon, and the amount of deposited material determined the number of replicas collected (two replicas per coupon). The swabs were placed in an Eppendorf tube, and no preservative was used. They were transported in dry ice and subsequently stored at -80°C.The collection of produced water (PW) samples took place at an oil storage and transfer terminal located in Duque de Caxias-RJ, Brazil. PWsamples was collected from side valves connected at 2.75 m of a tank containing residues from different sources in the terminal, and by the time of sampling it had a total fluid column height of 14.63 meters. Sampling was performed in triplicate using three sterile plastic containers of 5.0 liters per sampling point, with an aim to maximize the filling of the containers to prevent the presence of headspace. Samples were transported at room temperature within 2 hours to the laboratory. After collection, filtration was performed using vacuum pumps and membrane filters with a pore diameter of 0.22 µm (Kasvi, Curitiba, Brazil) to retain microbial cells. Approximately 450 mL of PW were filtered from each sampling point. The filters with retained microbial cells were placed in sterile 50 mL tubes and stored in ultrafreezers at -80 °C for subsequent DNA extraction.

After collection, filtration was performed using vacuum pumps and membranes (Kasvi, Curitiba, Brazil) with a pore diameter of 0.22 µm to retain microorganisms. Approximately 450 mL of PW were filtered from each sampling point. The filters with retained microbial cells were placed in sterile 50 mL tubes and stored in ultra-freezers at a temperature of -80 °C for subsequent DNA extraction.

2.2 PHYSICO-CHEMICAL ANALYSIS

Visual observation of the fluids present inside the three pipelines (Crude, Stagnant, and Slop) indicated that they were predominantly composed of oily substances (even in the Stagnant pipeline, supposed to be filled with saltwater). Therefore, the Petrobras_N-1380, 2023 standard protocol for oily samples was employed, and physico-chemical analyses were conducted to determine hydrogen sulfide (H_2S, mg/kg), thiols (R-SH, mg/kg), and water content (H_2O, mg/kg) in the oily fluids from each coupon holder.

2.3 MASS LOSS

To evaluate the mass loss of the metallic coupons, they were subjected to treatment according to the NACE SP0775-2018 standard and individually weighed before and after the exposure period. The corresponding uniform corrosion rate was calculated considering the

mass loss (g), coupon area (mm^2), exposure time (days), and coupon density (g/cm^2), following the (NACE SP0775, 2018) standard.

2.4 SCANNING ELECTRON MICROSCOPY (SEM) AND ENERGY-DISPERSIVE SPECTROSCOPY (EDS)

For scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS) analysis, coupons were fixed onto metal supports (stubs) using a specific carbon type, which was employed to enhance stability and facilitate the examination process. An unexposed coupon (control) was used as a reference during the analyses. All analyses were conducted using a Tescan MIRA3 scanning electron microscope with an Oxford X-act EDS detector with a 10 mm² window and Oxford AZtecOne software.

2.5 METAGENOMIC ANALYSIS OF AMPICONS

2.5.1 DNA Extraction

Metagenomic DNA was extracted from swabs and from the membrane filters that were used to filter AP samples. For the filters, 100 and 250 mg were used for DNA extraction, while for the swabs, extraction was performed directly on whole swabs. DNA extraction from both sample types was conducted in duplicate using the FastDNA™ Spin Kit for Soil (MP Biomedicals, Jacksonville, USA) following the manufacturer's instructions.

After extraction, DNA quantification and purity were assessed using the A260/280 and A260/230 absorbance ratios, measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltman, USA). DNA integrity was verified by agarose gel electrophoresis at 1.0% (w/v).

2.5.2 16S rRNA Gene Metabarcoding Sequencing

The amplification of the 16S rRNA gene was performed using primers targeting the V3-V4 regions: 341F (5'-CCTACGGGRSGCAGCAG-3') (Yong Wang & Qian, 2009) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) (Caporaso et al., 2010). Library preparation and sequencing were carried out on the MiSeq sequencing platform (Illumina Inc., USA) using the V2 paired-end kit for 500 cycles (2 × 250 bp).

2.5.3 Bioinformatic Analysis

The metabarcoding data were analyzed using the Amplicon Sequence Variant (ASV) approach and a custom pipeline developed in Python. The source code of the pipeline is available in the GitHub repository (https://github.com/LBMCF/pipeline-for-amplicon-

analysis), accessed on January 14, 2022. The pipeline utilizes the following tools: USEARCH (Rognes et al., 2016), Cutadapt (Martin, 2011)e FastQC (Andrews, 2010).

First, the reads underwent quality control using FastQC. Then, all sequences were merged with USEARCH using the "--fastq_merge pairs" option, and sequencing adapters were removed with Cutadapt. Quality control was performed with VSEARCH using the "--fastq_filter" and "--fastq_maxee=0.8" options. To eliminate duplicates, the "--derep_fulllength" option of VSEARCH was employed.

Chimeras were removed, and the ASV table was generated using the USEARCH unoise3 algorithm ("--unoise3" option). Additionally, the absolute abundance table was generated using the "usearch_global" and "id=0.99" options of VSEARCH. Taxonomic identification and classification of ASVs were performed using SINTAX (Edgar, 2016) in USEARCH with the SILVA SSU NR 138.1 database (Quast et al., 2013) as a reference. Finally, the abundance table with taxonomic identifications was consolidated using the custom script "get_abundances_table_asv.py" included in the source code. For ecological analyses, the genus-level taxonomic rank was used.

2.5.4 Integrative Analyses

Relative abundances (%) were determined based on ASVs, and the analyses were performed at the genus-level taxonomic rank. Relative abundances were calculated using the dplyr library (v1.1.0) in the R environment (Package, 2023).

For the analysis of microbial genus sharing and exclusivity (Venn diagram) between PW and Coupon samples, the online tool Venny (version 2.1) (Oliveros, 2021)was used.

Alpha diversity metrics, including Shannon index (Shannon_H), Simpson index (Dominance_D), and Equitability index (Equitability_J), were obtained by comparing PW and Coupon metabarcoding data using the PAST 4.08 software (Hammer et al., 2001). To test for statistical differences between PW and Coupon samples regarding alpha diversity indices, an ANOVA followed by the Tukey's test was performed ($p \leq 0.05$).

To plot the bar chart by phylum and genus, a cutoff of 0.5% was set. All values below this threshold were grouped in the "Others" category. Principal Coordinates Analysis (PCoA) was employed as a methodology to perform an integrative analysis encompassing PW and Coupon samples with a threshold of 0.1%. Values below this threshold were grouped in the "Others" category. In this study, we investigated the interrelationships among sets of microbial genera through PCoA, using the first two principal coordinates (PC1 and PC2). The data were initially subjected to Hellinger transformation to meet the requirements of the

analysis. For visualization of the results, two-dimensional scatter plots were generated using Past 4.08 software (Hammer et al., 2001), utilizing Euclidean distance as a measure. These plots allowed the representation of samples in a multidimensional space, with the relative position of the samples indicating their similarities or dissimilarities based on the composition of microbial genera.

2.6 FUNCTIONAL PREDICTION ANALYSIS USING THE 16S RRNA GENE AMPLICON APPROACH

Functional prediction analyses were performed on the 16S rRNA gene amplicon metagenomes from PW and Coupon samples. Functional inference of metabolic pathways based on the MetaCyc database (Caspi et al., 2018) using ASVs was conducted using the standard pipeline of PICRUSt2 software (Douglas et al., 2020). The STAMP v2.1.3 software (Parks et al., 2014) was used to infer statistically significant differences in functional composition among all samples. Differences in functional composition were analyzed using the Welch's t-test, with the Benjamini-Hochberg FDR correction method for multiple test adjustment. A p-value > 0.05 was used as a threshold to discard statistically non-significant correlations between metabolic pathways.

3 RESULTS

3.1 PHYSICO-CHEMICAL CHARACTERIZATION

Physic-chemical analyses were performed exclusively on the fluids collected from the coupon takers for the present experiment, as the physic-chemical characteristics of the produced water is described elsewhere (Dutra, Garc, et al., 2023). A low water concentration was observed in the Crude pipeline, representing only 0.014% of the total content compared to the samples from the Stagnant and Slop pipelines, which had water contents of 39.54% and 60.44%, respectively. This difference was expected since the Crude pipeline is specifically designed for the transportation of crude oil, while the other two pipelines contained saltwater and diverse waste fluids, respectively. The presence of water, even in low concentration, is possible due to the existence of dispersed water microdroplets in the oil phase (Lele et al., 2018).

Thiols content ranging from 24 to 40 mg/kg was observed in the pipeline fluids. Despite this, the analysis results indicated that all three pipelines had an H_2S content below the established detection limit (< 1.0 mg/kg). Table 1 shows the detailed results obtained.

Table 1 Physical-chemical results for oily fluids from the three pipelines on Ilha d'Água

(TABG)

Parameter	Crude	Stagnant	Slop
H_2O (mg/kg)	0,12	331	506
R-SH (mg/kg)	40	24	32
H_2S (mg/kg)	<1,0	<1,0	<1,0

3.2 CORROSION RATE OF COUPONS EXPOSED TO DIFFERENT OILFIELD PIPELINES

Corrosion rate calculations were performed based on the mass loss of carbon steel coupons exposed to the interior of the pipelines. In the Slop pipeline, moderate corrosion was observed according to the NACE RP0775 standard and low corrosion according to the NACE SP0775 standard. The highest recorded corrosion rate was 0.038 mm/year, which corresponds to 61.81% of the total corrosion rate when considering all measurements made on the coupons.

In contrast, the other samples, Crude and Stagnant, exhibited lower corrosion rates of 0.0008 mm/year and 0.0227 mm/year, respectively, which were considered low according to both NACE standards (NACE standard SP0775, 2005).

3.3. MORPHOLOGY AND ELEMENTAL COMPOSITION OF COUPONS

The results of the characterization by Scanning Electron Microscopy coupled with Energy Dispersive Spectroscopy (SEM-EDS) of the control coupons, Crude, Stagnant , and Slop, are presented in Figure 2.

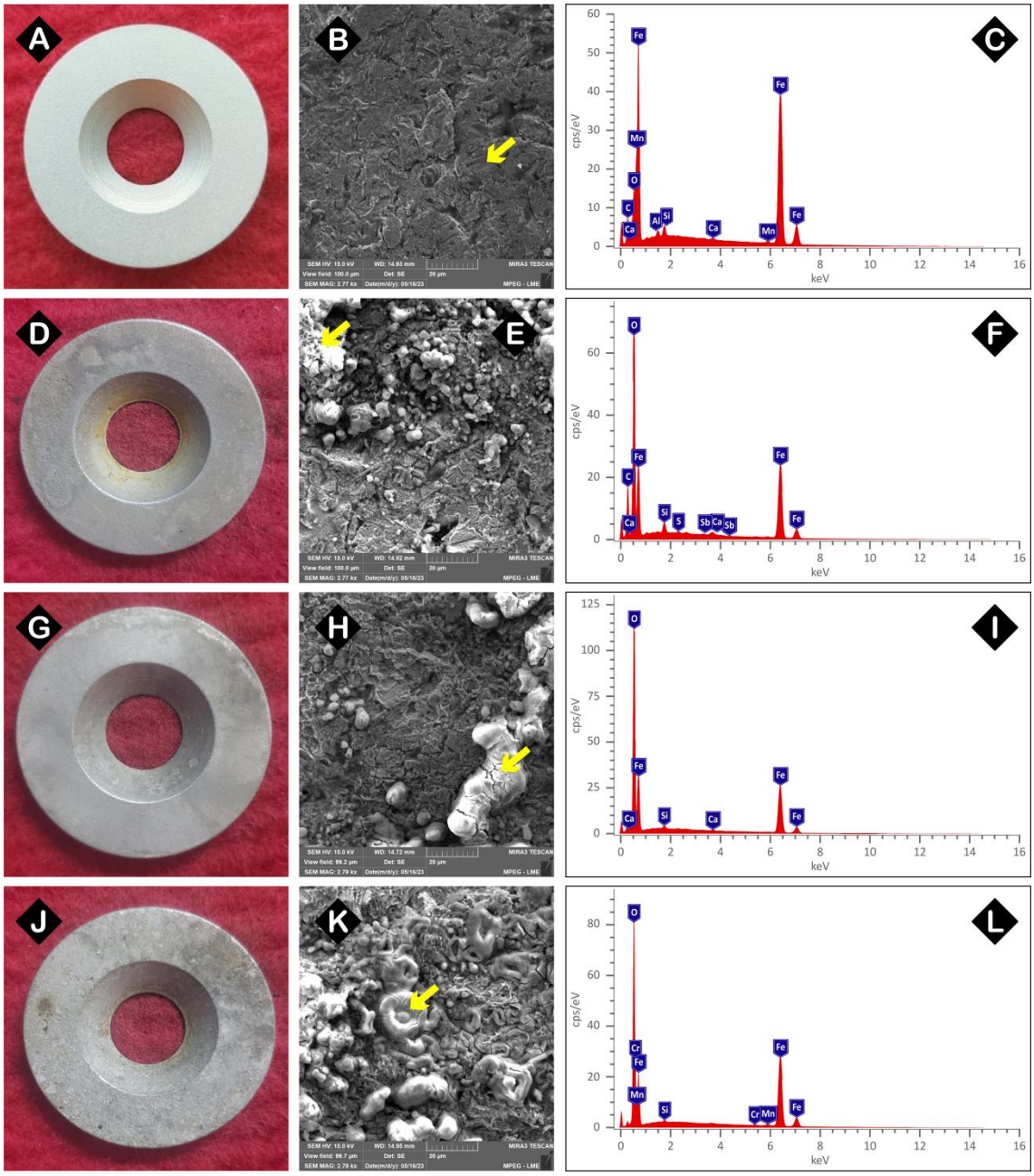

Figure 2. Macroscopic images, scanning electron microscopy (SEM) at 20 kV, and energy-dispersive spectroscopy (EDS). (a, b, c) Control coupon (not exposed), (d, e, f) Coupon exposed to the inner wall of the Crude duct, (g, h, i) Coupon exposed to the inner wall of the Stagnant duct, and (j, k, l) Coupon exposed to the inner wall of the Slop duct. The yellow arrows indicate the selected region for compositional analysis (EDS).

Comparative photographs and scanning electron microscopy (SEM) images of the carbon steel 1018 coupons, represented by Figures 2d-e, 2g-h, and 2j-k, displays the effect of exposure of the coupons to the inner wall of the Crude, Stagnant , and Slop duct, respectively, analyzed in comparison to the control coupons (Figures 2a-b), which were not

exposed to the interior of the ducts. Following the exposure period, changes in the coupons were observed, including variations in coloration in a heterogeneous manner and an increase in material porosity, characterized by the presence of reduced pores. Through SEM, it was possible to observe that the surface of the control coupon (Figure 2b) exhibited an irregular texture with depressions. Additionally, the images revealed transformations on the surface of the three coupons exposed to the ducts (Crude, Stagnant , and Slop), with the occurrence of different heterogeneous morphological modifications compared to the control coupon (Figure 2b, e, h, and k). The coupon from the Slop duct exhibited the highest degree of physical and morphological transformations, as illustrated in Figure 2j and 2k, with a greater formation of surface irregularities and disorganization of the carbon steel structures compared to the other coupons.

During the EDS analysis, the presence of oxygen was observed in all coupons, including the control coupon. To facilitate the presentation of results, the area with the highest weight percentage of oxygen (O) was selected, indicating the region with the highest oxygen content in the exposed and non-exposed coupons (Figure 2c, f, i, and l). The regions with the highest oxygen content, which may indicate a high oxidation rate and the formation of oxides, were identified by yellow arrows in the scanning electron microscopy (SEM) images (Figure 2b, e, h, and k).

The elemental composition of these coupons, expressed in weight percentage (% Weight), is shown in Figure 4. The exposed coupons exhibited significantly higher weight % of oxygen, as illustrated in Figure 3c, f, i, and l, and also in Figure 3, where the percentages were 33.46%, 42.19%, and 34.28% for the Crude, Stagnant, and Slop coupons, respectively. These values represent a significant increase compared to the control coupon, which had only 5.64% oxygen by weight.

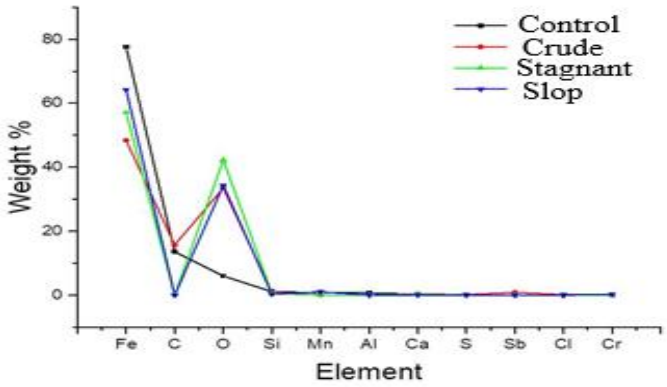

Figure 3. Percentage of chemical elements (Element) relative to the total weight of the metallic coupons (Weight %). The Control sample refers to the coupon not exposed to the duct conditions. The Crude sample indicates the coupon exposed to the inner wall of the Crude duct. The Stagnant sample represents the coupon exposed to the inner wall of the Stagnant duct, and the Slop sample indicates the coupon exposed to the inner wall of the Slop duct. The different colors indicate different samples.

Regarding the element iron (Fe), it was observed that the Crude coupon had the lowest weight percentage of this element, followed by the Stagnant and Slop coupons. A peculiar behavior was also observed regarding the element carbon (C), where the Crude coupon, which had a lower corrosion rate, exhibited a higher percentage compared to the control coupon, with a 23% increase in carbon content (Figure 4). In contrast, the coupons exposed to the interior of the Stagnant and Slop ducts did not show detection of carbon (C) in the analyzed area.

3.4. UNIQUENESS, SHARING, AND DIVERSITY OF MICROORGANISMS IN PLANKTONIC AND SESSILE ENVIRONMENTS

All metagenomic analysis proceeded with the samples from produced water and the samples from Slop duct as the samples from the other 2 duct did not present DNA of good quality to proceed the analysis. For hereafter coupon is referred only as the Slop duct samples. Figure 4A provides information about the exclusive and shared genera between PW and Coupon samples. Additionally, alpha diversity indices, such as Shannon_H diversity, dominance (Dominace_D), and evenness (Equatability_J), are displayed in Figure 4B.

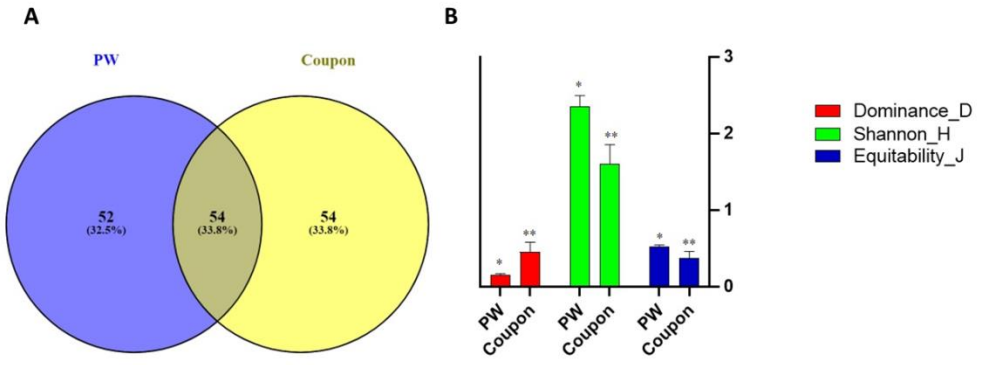

Figure 4. Integrative analyses comparing Produced Water (PW) and biofilm from the coupon exposed to the inner wall of the Slop pipeline (Coupon). (A) Venn diagram indicating the exclusive and shared genera between the evaluated samples. (B) Alpha-diversity analysis and comparison of means using ANOVA and Tukey's test, with statistical differences at $p \leq 0.05$; *No statistically significant differences within the groups (Shannon_H, Dominance_D, and Equitability_J); **Statistically significant differences within the groups (Shannon_H, Dominance_D, and Equitability_J).

The analysis of the Venn diagram revealed that the PW sample exhibited a significant proportion of 32.50% of exclusive genera compared to the Coupon sample (Figure 4A). Additionally, it was found that 33.80% of the genera were shared between the two analyzed samples. In terms of diversity indices, a significant difference was observed between the Coupon and PW samples in terms of Dominance_D, Shannon_H, and Equitability_J indices (Figure 4B).

3.5. ABUNDANCE OF ORGANISMS IN PLANKTONIC AND SESSILE SAMPLES

Distinct abundance patterns of phyla and genera of microorganism groups were identified in the PW and Coupon samples (Figure 5).

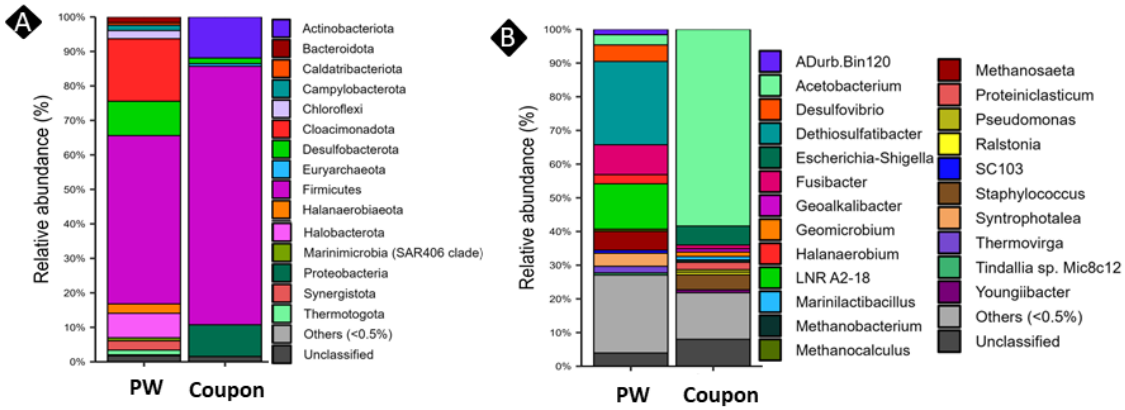

Figure 5. Relative abundance of phyla and genera revealed by metabarcoding in Produced Water (PW) and biofilm removed from the coupon exposed to the inner wall of the Slop pipeline (Coupon). Bar chart. (A) Each color represents a different phylum, and the proportions indicate abundances above 0.5%. (B) Each color represents a different genus, and

the proportions indicate abundances above 0.5%.

We observed that the phylum Firmicutes was identified in higher abundance in both PW, with 48.82%, and Coupon, with 74.97%. The phylum Desulfobacterota also stood out with significant relative abundance in both communities, with 9.94% in PW and 1.63% in Coupon.

In the produced water sample, microbial groups associated with sulfidogenesis (*Dethiosulfatibacter* - 24.72% and *Desulfovibrio* - 4.91%), methanogenesis (*Methanosaeta* - 5.61%), acid production (*Fusibacter* - 8.85%), and organisms predominant in anoxic environments (LNR-A2 18 - 13.46%) were found (Dutra, Garc, et al., 2023; M. Lu et al., 2021; Singh et al., 2021).

On the other hand, in the Coupon sample, the genus *Acetobacterium* (58.38%), an acid-producing organism (Shimizu et al., 2011) was predominant. A high abundance of the genera *Escherichia-Shigella* (5.63%) and *Staphylococcus* (4.47%) was also observed.

3.6. MULTIVARIATE STATISTICAL ASSOCIATION OF MICROORGANISMS IDENTIFIED THROUGH METABARCODING IN ENRICHMENT CULTURES

The multivariate statistical association of microorganisms identified through metagenomic barcoding in enrichment cultures was investigated. Principal Coordinate Analysis (PCoA) was conducted to assess the relationship among the samples. The results of the PCoA revealed that Coordinate 1 accounted for 55.46% of the total variability in the data, while Coordinate 2 explained 44.53% of the variability (Figure 6).

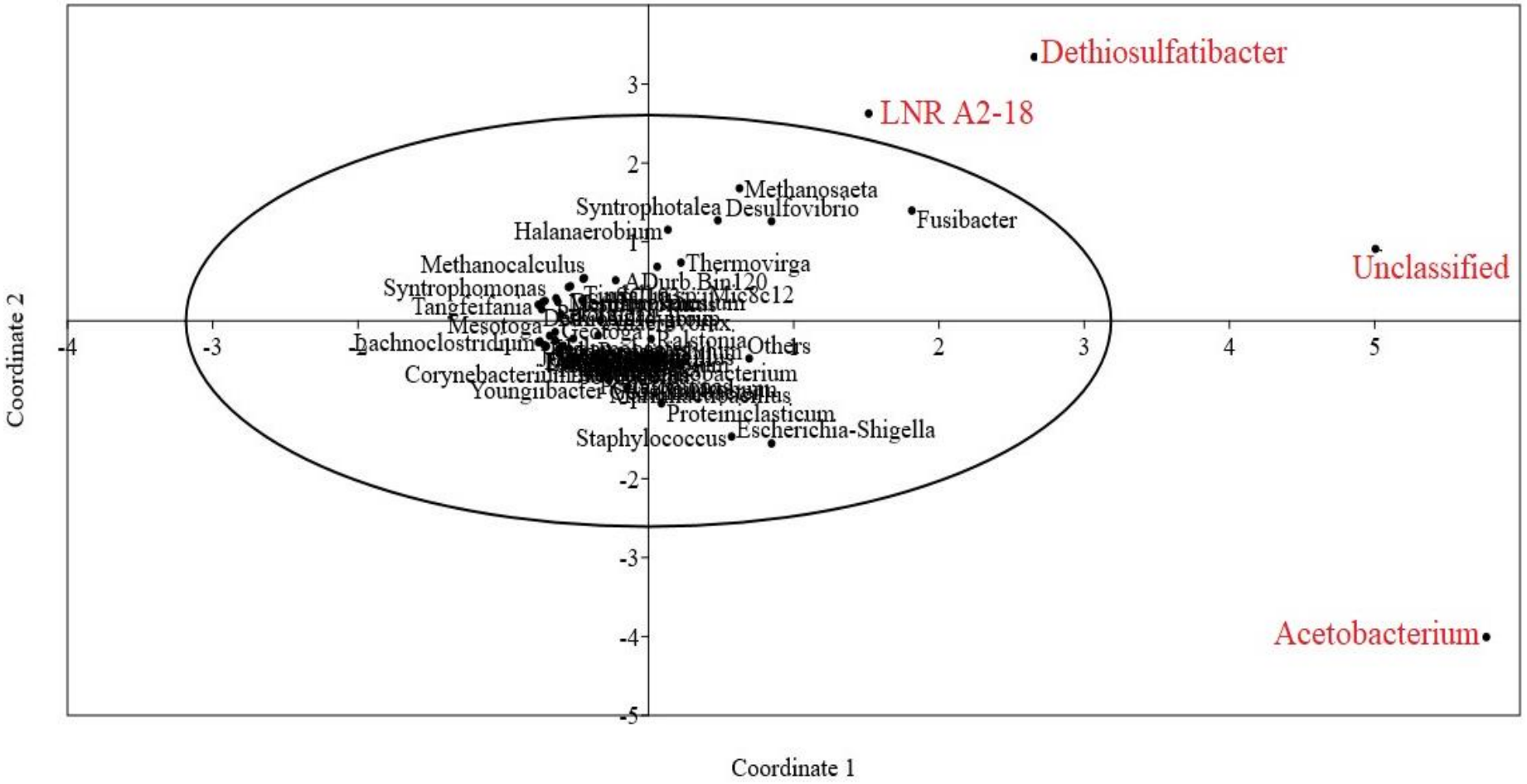

Figure 6. PCoA plot of the microbial community composition in Produced Water (PW) and biofilm from metallic coupon exposed to the inner wall of the Slop pipe. The plot was constructed using Euclidean distance. The black dots represent the microorganisms clustered within the circle. The red dots indicate groups with higher dissimilarity.

The majority of genera (92.72%) were found within the circle, indicating a high similarity between the analyzed samples. These genera are clustered close to each other, suggesting a common association in terms of biological or environmental characteristics. However, three genera (*Dethisulfatibacter*, LNR A2-18, and *Acetobacterium*) are located outside the circle, displaying higher dissimilarity compared to others, along with the unclassified organisms. *Acetobacterium* exhibited the highest dissimilarity as it was furthest from the groups. These genera represented outside the circle may possess unique or atypical characteristics.

3.6. FUNCTIONAL ANALYSIS

A total of 414 metabolic pathways were detected, with 1.5% exclusive to PW, 4.1% exclusive to Coupon, and 94.4% present in both samples, indicating a high correlation (r = 0.77%) (Figure 7).

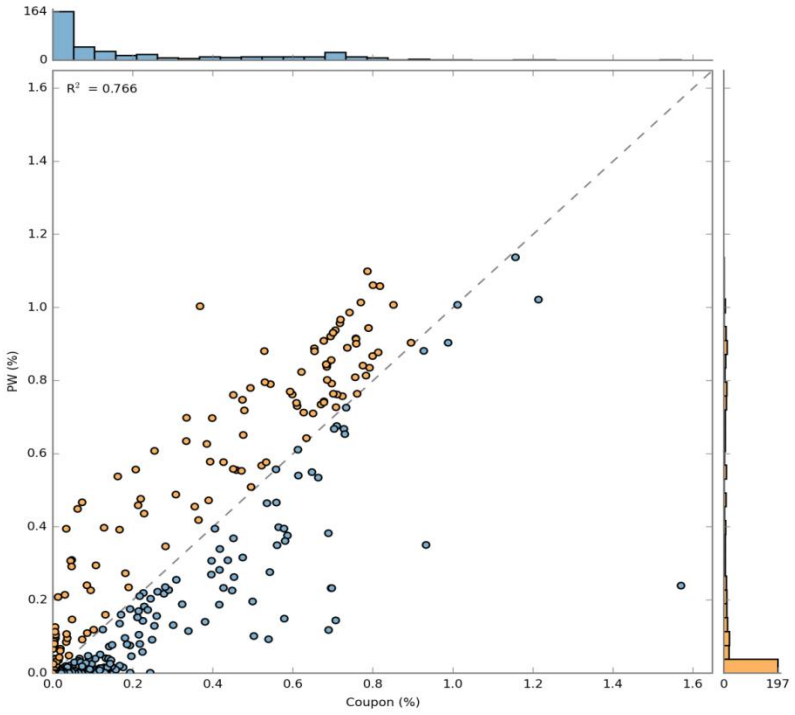

Figure 7. Scatter plot of maximum-level metabolic functions found in Metacyc and inferred by Picrust 2 in the samples of produced water (PW) and biofilm removed from the metal coupon exposed to the internal wall of the Slop pipe (Coupon). PW and Coupon. The histograms display stacked bars, where each column is divided into a portion corresponding to the abundance of metabolic functions detected by the PW sample (blue) and another portion relative to the genera detected by the metal coupon sample (Coupon). The Pearson correlation coefficient (r) is calculated to assess the functional association between the samples.

Upon grouping the metabolic pathways at the level 2 of Metacyc, a total of 28

metabolic pathways were identified. Removal of statistically non-significant metabolic pathways ($p \geq 0.05$) resulted in a total of 22 pathways, as shown in Figure 8.

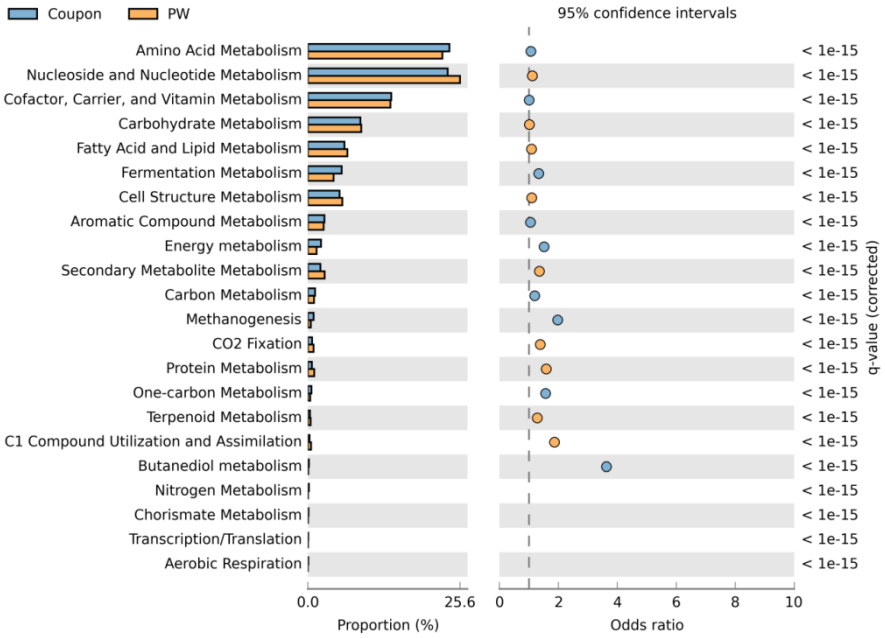

Figure 8. Comparison between the analysis module of two functional data groups at level 2 of Metacyc predicted in Picrust 2 for the samples of produced water (PW) and biofilm removed from the metal coupon exposed to the internal wall of the Slop pipe (Coupon).

The amino acid, nucleoside and nucleotide metabolism, cofactors, transporters and vitamins, carbohydrate, and fatty acid and lipid metabolism pathways were dominant in both PW and Coupon samples (Figure 8). The pathways associated with fermentation, such as fermentation metabolism, energy metabolism, methanogenesis, and butanediol metabolism, showed higher abundance in the Coupon sample (Figure 8).

When evaluating the metabolic pathways at the last level of Metacyc, considering an effect size above 0.5 in the Stamp software, only 5 significant metabolic pathways were observed, with the 1,2-propanediol degradation pathway being the most significant in the Coupon sample (Figure 9).

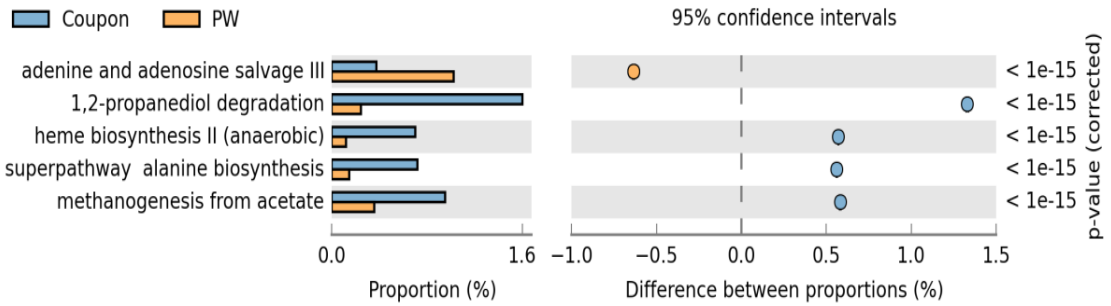

Figure 9. Comparison between the analysis module of two functional data groups from Metacyc predicted in Picrust 2. The comparison is based on samples of produced water (PW) and biofilm removed from the coupon exposed to the inner wall of the Slop pipeline (Coupon). The comparison focuses on data points with an effect size above 0.5.

4. DISCUSSION

In the present study, pit corrosion was predominantly observed in the coupon exposed to the interior of the Slop pipeline. The corrosion rate in the Slop coupon was categorized as low to moderate, with a value of 0.038 mm/year, indicating a low but significant level of metal degradation (NACE SP0775, 2018; NACE standard SP0775, 2005). Additionally, a high severity of corrosion was observed in terms of color variations, pore formation, and disorganization of carbon steel structures. These results support the findings of a study conducted by. (Nogueira et al., 2017), which identified heterogeneous discoloration in severely corroded coupons. Furthermore, (Silveira et al., 2014) found pore formation (pit corrosion) along the entire length of the coupon after corrosion testing. These significant modifications in the Slop coupon were possibly due to the higher water content in the coupon holder fluids, 60.44%, compared to the other samples, which may facilitate natural electrochemical oxidation and/or intensify the proliferation and activity of microorganisms (Santos et al., 2022; Yafei Wang et al., 2016).

The water content in the system can determine the corrosion mechanism. In the present study, the presence of thiols, compounds generated from the biological degradation of organic matter commonly found in petroleum (Liem-Nguyen et al., 2019) was detected. These compounds are undesirable in oil fields as they can cause operational problems such as corrosion. However, the presence of H_2S was not detected, which could be the result of thermochemical processes and/or microbial action of sulfidogenic organisms in petroleum reservoirs, posing a risk when present in the environment (Lahme et al., 2019). It is important to highlight that sulfidogenic metabolism is generally associated with MIC due to the presence of sulfate-reducing bacteria (SRB) in locations where corrosion occurs (Beech, 2003; Giorgi-Pérez et al., 2021; H. Li et al., 2015; H. Liu et al., 2015; Santos et al., 2022). However, there are other groups besides SRB that can cause biocorrosion but are often overlooked in microbiological analyses aimed at diagnosing MIC (Dutra, Gomes, et al., 2023; Gu, 2014).

Nevertheless, the presence of a higher amount of iron (Fe) in the Slop pipeline coupon compared to the Crude and Stagnant coupons contradicts the results obtained for the corrosion rate, as well as the observed physical and morphological characteristics. This

discrepancy may be attributed to the significant reduction of other chemical elements in the Slop coupon, resulting in a higher proportion of iron in the analyzed region.

It was observed that the Crude pipeline coupon exhibited a higher percentage of carbon (C) compared to the control coupon. This phenomenon can be attributed to the possible deposition of carbon on the metal surface. Metal surfaces have the ability to act as catalysts for dehydrogenation reactions present in most fossil fuels, resulting in the formation of carbon deposits (Altin & Eser, 2004). These deposits can be removed during the pickling process (Glück, 2016). However, when these deposits interact with the metal surface, it can be challenging to remove them completely, and they may end up incorporating into the metal through surface porosities (Martins, 2009).

The presence of 33.8% shared genera between the biofilm on the surface of the coupon exposed to the inner wall of the Slop pipeline (Coupon) and the planktonic community in the produced water (PW) aligns with the hypothesis that planktonic microorganisms are commonly found in biofilms. Biofilms form through the adhesion of microorganisms to solid surfaces in contact with water (Clark et al., 2012). On the other hand, the presence of exclusive genera in the Coupon sample can be attributed to variations in environmental conditions, including the presence of specific carbon and energy sources, which may have selected different types of microorganisms in each sample (Dutra, Gomes, et al., 2023). Furthermore, there may be specific characteristics in each environment, such as the presence of microorganisms originating from different sources of contamination, which would not naturally inhabit the environment in question. Examples of such microorganisms include the genera *Escherichia-Shigella* and *Staphylococcus*, commonly found in the gastrointestinal systems of humans and animals and exhibiting high abundance in the biofilm (Coupon).

The higher dominance in the Coupon sample can be explained by the potential complexity of the biofilm structure formed on the metallic coupons, which consists of water channels, extracellular polymeric substances (EPS), proteins, nucleic acids, lipids, corrosion products, and metal ions (Dall'Agnol et al., 2014; Santos et al., 2022). This complex matrix may limit the mobility of microorganisms and restrict the colonization of a greater diversity of species. Additionally, although biofilm formation offers advantages such as protection against biocides, interactions with other microorganisms (in microconsortia or syntrophy), uptake and transport of nutrients through diffusion, provision of physical support, and surface adhesion (Santos et al., 2022) biofilms can also be subject to competitive interactions among the present microorganisms. Thus, there may be limited nutrient availability within biofilms,

which can contribute to lower diversity and higher dominance compared to the planktonic community.

Despite the planktonic community (PW) showing higher diversity, evenness, and lower dominance compared to the sessile community (Coupon), 92.72% of the genera were associated and exhibited similar characteristics in terms of distribution and relative abundance, indicating ecological affinity between the groups. The abundant presence of sulfate-reducing bacteria such as thiosulfate-reducing bacteria (TRB), sulfate-reducing bacteria (SRB), acid-producing bacteria (APB), and methanogenic bacteria suggests a syntrophic environment where species depend on each other to obtain benefits and survive, working together to perform metabolic processes that would be unviable individually (Dutra, Garc, et al., 2023; Dutra, Gomes, et al., 2023). Only three genera showed significant dissimilarity in the communities: *Dethiosulfatibacter*, LNR A2-18, and primarily *Acetobacterium*, along with unclassified organisms. This may represent a dominant syntrophic complex or metabolic dominance in the environment where they are found.

The high abundance of the genera *Dethisulfatibacter* and LNR A2-18 in the planktonic community (PW) may indicate corrosion in a specific area of the storage tank. The genus *Dethiosulfatibacter* belongs to the group of sulfate-reducing bacteria, specifically TRB, which may be related to high levels of thiosulfate (Parks et al., 2014). During the initiation of pitting corrosion, through the electrochemical mechanism of steel's MnS dissolution, thiosulfate can be produced (Dutra, Garc, et al., 2023). With an increased availability of this specific nutrient, the development of the *Dethiosulfatibacter* genus is favored, which justifies its predominance in this community. Furthermore, although the LNR A2-18 genus has not been adequately characterized, these organisms have been mentioned in the literature as non-culturable and, despite limited available information, may play a significant role in anoxic environments (Dutra, Garc, et al., 2023; M. Lu et al., 2021; Singh et al., 2021) potentially engaging in syntrophic relationships with other microorganisms.

In contrast, the dominance of the genus *Acetobacterium* (58.38%) in the biofilm (Coupon), which explains the higher dominance index observed in this study, may be related to specific conditions on the metal surface that select organisms better adapted to these conditions [6]. This genus belongs to the group of APB, specifically acetogenic bacteria, which are known to cause corrosion under anoxic conditions (Dutra, Garc, et al., 2023; Kato et al., 2014). Additionally, this genus has been associated with biofilm formation that can enhance metal corrosion (Gu, 2014; Kato et al., 2014). The abundant presence of enteric

bacteria such as *Escherichia-Shigella* (7.30%) and *Enterococcus* (6.00%) in the environment can be explained by the presence of oil processing residues, equipment washing, and rainwater (uncontrolled environment) in the Slop pipe, which may result in contamination by genera that are typically uncommon in these environments.

The planktonic (PW) and sessile (Coupon) communities were highly correlated metabolically (r = 0.77). These correlations were related to primary metabolic pathways responsible for bacterial survival (Dutra, Gomes, et al., 2023; Pereira et al., 2021). Metabolic pathways associated with potentially corrosive metabolisms were particularly prominent in the biofilm (Coupon), including fermentative metabolism. Considering this result along with the dominance of the *Acetobacterium* genus, it can be inferred that the predominant metabolism in the biofilm (Coupon) is organic acid production. Fermentation products typically include organic acids such as lactic acid and acetic acid, as well as alcohols that lower the pH within biofilms. The acidity caused by APB is derived from the protons released by organic acids (Gu, 2014) which can cause localized corrosive damage.

The reduction of protons and free acids, along with iron oxidation, creates favorable thermodynamics and unhindered kinetics (Gu, 2014). Additionally, the concentration of free acids can exceed that of protons, rendering the environment corrosive. For example, a solution of acetic acid with a pH of 2 is more corrosive than a solution of sulfuric acid with the same pH (Gu, 2014).

Specifically, a higher abundance of the 1,2-propanediol degradation pathway was detected in the biofilm sample (Coupon). It has been shown that the acetogenic bacterium *Acetobacterium carbinolicum* degrades diols to form acetate, with the alcohol being oxidized to the corresponding acid and the electrons being channeled to CO_2 using the Wood-Ljungdahl pathway (Eichler & Schink, 1984). However, (Kai et al., 2014) demonstrated that the species *Acetobacterium woodii* is capable of degrading 1,2-propanediol independent of acetogenesis, yielding byproducts such as propanaldehyde, propanol, and propionate. They concluded that the metabolism of 1,2-propanediol independent of the Wood-Ljungdahl pathway may be an adaptation and ecological advantage in anoxic habitats where 1,2-propanediol is present as a substrate. This pathway may be related to the enzyme ADH4 (Kai et al., 2014; Moon & Müller, 2021) which catalyzes the reduction of propanaldehyde to propanol during 1,2-propanediol metabolism (Chowdhury et al., 2020).

The significant presence of the acetate-derived methanogenesis pathway in the coupon biofilm (Coupon) is intriguing, considering that only the strictly hydrogenotrophic

methanogenic archaeon (*Methanobacterium*) (He et al., 2021)showed significant abundance in the biofilm (Coupon). However, in complex ecosystems where there may be a scarcity of H_2/CO_2 sources, affecting ATP generation and cellular compounds, acetate can be assimilated by cells with constant and uniform growth over time, such as *Methanobacterium* (Durrens et al., 1980). Aghtaei et al. (2022) evaluated a discontinuous H_2/CO_2 feeding system in a biogas reactor and observed that the community of hydrogenotrophic methanogenic archaea formed a complex with homoacetogenic fermentative bacteria, suggesting acetate assimilation by these hydrogenotrophic archaea (Aghtaei et al.,2022).

5. CONCLUSION

The results obtained indicate that the coupon exposed on the inner wall of the Slop Slop pipe, used for the transportation of various waste fluids, exhibited a higher corrosion rate compared to the other analyzed coupons. The predominant form of corrosion observed was pitting corrosion, characterized by the formation of localized pits and notable heterogeneous distribution modifications on the metal surface. These surface alterations were accompanied by a visible change in color of the material, indicating significant damage at localized points. When comparing the planktonic community present in the produced water (PW) with the corrosive biofilm evaluated from the coupon exposed in the Slop pipe (Coupon), it was found that 33.8% of the genera were present in both samples.

Although the majority of organisms (92.72%) showed similarity according to Euclidean distance, three genera stood out, namely *Dethisulfatibacter*, an SRB, and LNR A2-18, uncultivated, representing the produced water, and *Acetobacterium* (56.08%), a APB, dominant in the corrosive biofilm. It was observed that *Acetobacterium* was mainly involved in the degradation metabolism of 1,2-propanediol, likely utilizing it as the primary energy source for acetate and/or propanol production. These results indicate that the main groups causing localized corrosion in areas with low levels of sulfide compounds may be due to APB rather than sulfidogenic bacteria. These findings reinforce the importance of monitoring damage and controlling corrosive biofilm. Understanding the metabolic pathways and corrosive activities of APB groups can provide valuable insights for the development of more effective protection and maintenance strategies against corrosion in equipment and metallic structures.

These discoveries contribute to improving practices for the prevention and mitigation of biocorrosion, aiming to prolong the lifespan of equipment and reduce costs associated with corrosion.

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CONCLUSÕES FINAIS

A tese de doutorado intitulada "AMEAÇA BIOCORROSIVA: Monitoramento da biocorrosão e diagnóstico metagenômico em campos de extração de petróleo" teve como objetivo o desenvolvimento de um pacote tecnológico metagenômico padronizado para o monitoramento da corrosão influenciada por microrganismos (CIM) em campos de petróleo. A pesquisa atingiu o objetivo proposto ao abordar questões específicas relacionadas aos ambientes encontrados nesses campos, utilizando uma abordagem baseada na compilação da literatura metagenômica disponíveis. Para alcançar resultados mais eficientes, foram estabelecidos critérios rigorosos para a seleção do protocolo de extração mais eficaz, considerando também a viabilidade econômica. Além disso, foram exploradas abordagens de sequenciamento adequadas e a identificação da amostragem mais eficiente, levando em consideração a possibilidade de coleta direta ou o uso de cultivos.

Através da aplicação direta da tecnologia desenvolvida, foi possível realizar a caracterização direta dos dutos de transporte de compostos de petróleo e resíduos. Essa caracterização permitiu a compreensão da dinâmica populacional do ambiente e a definição de estratégias mais eficientes para o monitoramento de ambientes com propriedade similares. A padronização dessa tecnologia viabiliza a implementação de protocolos específicos para cada ambiente presente nos campos petrolíferos, proporcionando um diagnóstico mais eficiente e alinhado com a realidade.

Essa abordagem é de extrema importância, uma vez que evita erros potenciais, como a resistência microbiana causada pelo uso inadequado de produtos químicos. Esses erros podem resultar em desequilíbrios ambientais que, por sua vez, podem promover condições favoráveis ao desenvolvimento de microrganismos altamente corrosivos ou que possuam metabolismos corrosivos, como evidenciado na revisão sistemática realizada neste estudo “Corrosion-influencing microorganisms in systematic review, analysis, and scientific petroliferous regions on a global scale: synthesis of 16s amplicon metagenomic studies”

Dessa forma, a tese de doutorado contribui para o avanço no entendimento e controle da biocorrosão em campos de extração de petróleo, ao estabelecer uma abordagem metodológica consistente e integrada que permite o diagnóstico preciso e a implementação de medidas de mitigação adequadas. A aplicação desses protocolos padronizados tem o potencial de reduzir os impactos econômicos e de segurança associados à biocorrosão, promovendo a sustentabilidade e a eficiência operacional na indústria do petróleo.

PERSPECTIVAS

As perspectivas da presente tese incluem a aplicação do pacote tecnológico no campo de petróleo usando as abordagens descritas nesta e monitorar os resultados apresentados para ser criado um histórico de resultados e verificar a evolução e a eficiência na prática, desse pacote tecnológico.

Realizar a adaptação do pacote tecnológico através de testes para monitoramento em amostras de óleo, que além de monitoramento da biocorrosão será possível aplicar para o monitoramento da biodegradação do óleo.

A implementação e atualização anual da metodologia, do estudo abordado no Capítulo 1, desta tese: “Corrosion-influencing microorganisms in systematic review, analysis, and scientific petroliferous regions on a global scale: synthesis of 16S amplicon metagenomic studies”, que utiliza *scripts* para buscas na literatura específica de metagenômica de campos de petróleo. Para atualização dessa metodologia é preciso seguir os passos descritos abaixo.

1. Utilizar o script desenvolvido na presente tese: com os descritores apresentados que são específicos para encontrar artigos de metagenômica de campos de petróleo, para automatizar a busca de artigos e informações relevantes. Esse *script* permite varrer de forma eficiente e sistemática a literatura científica, garantindo que nenhuma fonte relevante seja deixada de lado;

2- Atualizar os descritores: é importante considerar a necessidade de atualização desses descritores para abranger novos termos e avanços na área. Essa busca contínua permite a identificação de novos gêneros de microrganismos potencialmente envolvidos na corrosão influenciada por microrganismos (CIM) e também proporciona informações valiosas sobre os protocolos de extração de DNA. Como evidenciado na tese, o kit comercial para solos é o mais utilizado, mas é importante estar atento às demandas da indústria de petróleo e considerar a possibilidade de empresas estarem desenvolvendo um kit específico de extração de DNA para amostras de campos de petróleo.

2- Adaptação do *script*: o *script* pode ser adaptado para incluir filtros específicos, como a seleção de artigos publicados em determinado período de tempo ou em periódicos específicos. Essa abordagem sistemática e rigorosa permite não apenas a identificação de novas informações, mas também oferece uma visão panorâmica do cenário mundial em relação às pesquisas de petróleo. Isso pode servir como um guia valioso para o

desenvolvimento e aprimoramento da indústria como um todo, fornecendo insights sobre os países mais envolvidos e interessados em estudos relacionados ao petróleo.

Ao aplicar esses passos, será possível manter o pacote tecnológico atualizado, incorporando novos conhecimentos e avanços científicos relevantes. Essa abordagem contínua e atualizada é crucial para acompanhar o ritmo acelerado das pesquisas na área de metagenômica do petróleo e garantir que o monitoramento da corrosão influenciada por microrganismos seja eficiente e alinhado com os avanços mais recentes.

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PRODUÇÃO CIENTÍFICA DURANTE O DOUTORADO (

IDENTIFICAÇÃO

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Início Doutorado: Junho de 2019
Conclusão: 28 Junho de 2023
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Durante o periodo do Doutorado foram publicados 12 artigos científicos, incluindo os que fazem parte desta Tese: 7 como autora principal e 5 como co-autora. Também foram submetidos 4 artigos, sendo 2 como autora principal, 1 como pesquisador Sênior e 1 como co-autora.

***Artigos primeira autora Publicados (2019-2023)**

1. Anaerobic digestion using cocoa residues as substrate: Systematic review and meta-analysis. *Energy For Sustainable Development* – 2023. Fator de Impacto (2021 JCR): 5,65 v. 72, p. 265-277, 2023. https://www.sciencedirect.com/science/article/pii/S097308262200237X
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***Artigos co-autora publicados (2019-2023)**

1 1- *Lactococcus lactis* as an Interleukin Delivery System for Prophylaxis and

Treatment of Inflammatory and Autoimmune Diseases. *Probiotics and Antimicrobial Proteins*, Fator de Impacto (2021 JCR): 5,26, v. 1, p. 1-15, 2023. https://pubmed.ncbi.nlm.nih.gov/36746838/

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***Artigos primeira Autora Submetidos**

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***Artigos co-autora submetidos**

1 Systematic Review of Reverse Vaccinology and Immunoinformatics Data for Non-Viral Sexually Transmitted Infections. *Annals of the Brazilian Academy of Sciences*.

***Revisão de periódicos**

1- Revisora atual da revista “Water Process Engineering”. Editora Elsevier; FI: 7,34, 5 artigos.

2- Revisora atual da revista “Bioenergy Research”. Editora: Springer; FI: 2.81, 2 artigos.

***Relatórios Petrobras “Estudo Integrado de Metaômicas e Cultivo de Microrganismos Causadores de Biocorrosão em Dutos”.**

1- 1° relatório parcial - Relatório da Petrobras. Relatório referente à data de 07/fev/2019 a 07/jan/2020. Inclui parte do referencial teórico da presente tese e a etapa 1 do capítulo 2. file:///C:/Users/joyce/Dropbox/PETROBRAS-

UFMG%20META%C3%94MICAS%20E%20BIOCORROS%C3%83O/RELAT%C3%93RIOS%20T%C3%89CNICOS/UFMG%20Omicas%20-%201o%20Relat%C3%B3rio%20T%C3%A9cnico%20Parcial%20(mar-2020).pdf

2- 2°relatório parcial- Relatório da Petrobras. Relatório referente à data de 08/jan/2019 a 10/mar/2020. Inclui os estudos iniciais da revisão sistemática apresentada no capítulo 1 desta tese. file:///C:/Users/joyce/Dropbox/REVIS%C3%83O%20SISTEM%C3%81TICA%20GERAL_%20Petr%C3%B3leo_biocorrs%C3%A3o/Revisao%20Vinicius%20Petrobras/(2021-05-21)%20UFMG%20-%202%C2%BA%20Relat%C3%B3rio%20T%C3%A9cnico%20Parcial.pdf

3- 3° relatório parcial Relatório da Petrobras. Relatório referente à data de 11/mar/2021 a 04/fev/2022. Inclui parte do referencial teórico da presente tese e a revisão sistemática apresentada na qualificação, parte do material foi demonstrada no capítulo 1 desta tese. https://drive.google.com/file/d/1clgjEvcjo6uckU6E-hZTS5ISNhO9D96g/view?usp=drive_link

4- 4° relatório da Petrobras. Este relatório não pertence aos relatórios obrigatórios a serem entregues na Sigetec. Esse relatório pe referente à coleta dos cupons apresentados no capítulo 4 desta tese. https://drive.google.com/file/d/1xpTrQX-syZils4H-xjWJF_LhRUSF565U/view?usp=drive_link

***Cursos**

1- Curso “Meta-Análise: Revisão Sistemática - Roteiro Completo https://drive.google.com/file/d/13fcCDCtBhtMBmUeveWfbXU0hCcA7DAT/view?usp=sharing

2- Curso “Filogenia: pequena-escala (Filogenética) e larga-escala (Filogenômica)”, RECOM https://drive.google.com/file/d/1VBh2jEacoamULtaeZ0649sq8xfUM4vFt/view?usp=sharing

3- Curso Proteômica “Proteomic High-Throughput Data Analysis” https://drive.google.com/file/d/1QlckM0pbdVCZlcX2ZNY2TmmN2cwbgMbG/view?usp=sharing

4- Curso “Desvendando a redação científica Básico I, com Gilson Volpato. https://drive.google.com/file/d/1PZZsF8DVkXs7oKEN-V_-Lrke835UkjUH/view?usp=sharing

5- Curso “Desvendando a redação científica Basico II, com Gilson Volpato. https://drive.google.com/file/d/1NRWtvBQNxEKFWu3bwl6WAApz9MdT8M_Q/view?usp=sharing

6- Curso “Competências Profissionais, Emocionais e Tecnológicas para tempos de mudança” https://api.accredible.com/v1/auth/invite?code=ee8782be0a6f3310c457&credential_id=5b8fcee3-4827-4db4-93b7-eb123df841e8&url=https%3A%2F%2Fcertificado.pucrs.br%2F5b8fcee3-4827-4db4-93b7-eb123df841e8&ident=d0e7a6f53d0f78598b17fc946961253d2cfa8f57

***Congressos e simpósios**

1 1st Symposium on Mass Spectrometry Applied to Proteomics and Structural

Biology
https://drive.google.com/file/d/1q_CqnkOpG8R2SF2exk5q4q-T_qHQy4Bk/view?usp=sharing

2 2nd Associated International Laboratory Meeting (LIA 2019) - Bact-Inflam Conference
https://drive.google.com/file/d/1CIG66lyEyrntrc7VTfTdiYYRORKjM6j-

/view?usp=sharing

***Organização de cursos**

1 2nd Associated International Laboratory Meeting (LIA 2019) - Bact-Inflam Conference, 2019
https://drive.google.com/file/d/1CIG66lyEyrntrc7VTfTdiYYRORKjM6j-

/view?usp=sharing