

Desulfovibrio and Pseudomonas Dominated Enriched Produced Water Reinforcing their Importance in Oilfields and Production Processes

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Research Article

Keywords: Sulfate-reducing bacteria, Metagenomics, Produced-water, Proteobacteria, Microbial communities, Oilfields, MEOR

Posted Date: December 7th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-117969/v1>

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Abstract

Enrichment based isolation methods and molecular identification were used to investigate the microbial communities in produced water obtained from Periquito (PQO) and Galo de Campinas (GC) onshore oilfields in Brazil. Produced water was enriched with Postgate B, Postgate C and Baars media and incubated. DNA was extracted, PCR amplified and 16S rDNA fragments were sequenced using Illumina TruSeq. 4.2 million reads were analysed and deposited at the Sequence Read Archive at NCBI. No significant differences in microbial community composition could be attributed to the enrichment but significant differences were observed from two oil fields. The dominant Bacterial Orders detected from both oilfields were Desulfovibrionales, Pseudomonadales and Enterobacteriales. *Pseudomonas* were found predominantly in Periquito oilfield (19.8%) with only 2.4% at Galo de Campinas. *Pleomorphominas* (3.76%) and *Shewanella* (4.69%) were exclusive to and possible biomarkers for the Periquito Oilfield. 11.05% and 7.62% of the sequences were not classified at genus level and detected at GC and P. Abundances changed for *Desulfovibrio* from P, 29.8% at PQO and 16.08% at GC. The *Clostridium_sensu_stricto* varied from 2.8% at PQO and 2.4% at GC). *Pseudomonas* were found predominantly in Periquito oilfield (19.8%) with only 2.4% at Galo de Campinas. *Pleomorphominas* (3.76%) and *Shewanella* (4.69%) were almost exclusive to and possible biomarkers for the Periquito Oilfield. These data provide a bacterial biodiversity benchmark for future produced water treatment and microbially enhanced oil recovery (MEOR).

Introduction

Petroleum is found in reservoir rocks typically between a gas cap at the top and water at the bottom¹. Oil extracted from a reservoir rock reaches the surface as a mixture with some sediment, gas and water. Water naturally confined in a reservoir is denominated Formation Water; after it has been extracted with oil and gas, it is denominated Produced Water². Due to technical considerations (equipment and reservoir) and environmental legislation, produced water requires special attention and demands specific management actions³. Oil from a reservoir is pumped through pipelines to a primary processing unit, where density is used to separate particles, water, oil and gas and the emulsified zone between the oil and water. At the beginning of the operation, the liquid phase is separated from the gas phase and the water and the emulsified zone are separated from the oil phase⁴. Before disposing of, or injecting the produced water into back into a well, the operator must treat it to meet physical, chemical and biological quality control legislation that aims to protect the natural environment and to prevent fouling of the reservoir. If an operator decides to reinject the water into the reservoir, it is treated for: bacteria, the presence of salts, and the potential generation/deposition of fouling materials. Bacteria can create integrity problems for assets and safety problems for personnel⁵. Salts and solids can create flow assurance problems causing strangulation within the tubes and pipes. Solids will also create permeability problems within the reservoir rock⁶. Regulations covering the management and fate of produced water are increasingly stringent but not globally standardised. Treatment is required before disposing of produced water to remove concentrations of oil and grease. In Brazil, produced water is legally regarded as an “effluent” by the

National Environmental Council (CONAMA). CONAMA Resolution No. 357/2005 and its amendments (393/2007 and 397/2008) establish acceptable oil limits in produced water but do not mention microbial contamination.

Qualitative and quantitative characterization of the bacteria in produced water has not been a focus of studies in Brazil, nor globally and yet bacteria can cause significant problems and are sometimes the solution to oil production problems (MEOR). It is interesting to identify bacteria present in produced water to understand the microbial community as well to detect harmful bacteria such as sulphate reducing bacteria (SRB). Many papers discuss the origin of microbial populations in oil reservoirs. It is accepted that microorganisms are introduced into the wells during the drilling process, and/or by the injection of produced water or naturally through venting with ocean water offshore and ground water onshore⁷. In these instances, the microbial communities are a combination of native microorganisms in the well, and microorganisms present at the surface portion of the well added by drilling or injection^{8,9}. Oil production faces four microbially attenuated SRB problems and they include: (i) microbial induced corrosion (MIC) in and on pipelines, metal structures and equipment¹⁰; (ii) contamination of injection wells; (iii) impact of bioaccumulation (biofouling) on production and flow rates¹¹; and (iv) biogenic acidification (souring), which is caused by sulphide production in the form of Hydrogen Sulphide (H₂S)¹². When a high sulphate source is introduced into an oil reservoir already colonized by SRB, sulphate is reduced to sulphide thus oxidizing the organic electron donors present in crude oil, causing souring and a loss in commercial value of the oil¹³. To avoid this problem, biocides have been and are used, but without microbiological monitoring and resistance studies to assess the efficacy and impact. In this context, research that seeks new technologies and new biocidal substances must be combined with microbiological studies and not just toxicological effects for a more efficient management and maintenance of assets. Numerous studies on biofilms show that most of the biocidal substances have limited biofilm penetration, thus killing superficial cells and are more effective on planktonic cells¹⁴⁻¹⁶. Accurate knowledge of the microorganisms present in an oil field and its produced water is critical for selecting and monitoring the action of antimicrobial substances. It is also critical in order to establish the required/optimum amount of biocide, its duration of use, and stage of application. There is a clear need to stop biofilms establishing because treating them subsequently is much more challenging.

A wide variety of SRB and other microorganisms have been isolated or detected in samples from oil reservoirs and produced water, including aerobic bacteria, facultative anaerobes, microaerophilic bacteria, strict anaerobes, archaea, thermophilic organisms, mesophilic and hyper thermophilic organisms⁸. Some functional groups of microorganisms such as SRB are frequently described as present. Other bacteria routinely found in reservoirs, include members of genera *Clostridium*, *Pseudomonas* and *Bacillus* and functional groups including the Acid Producing Bacteria (APB), Oxidizing Bacteria (OB), Sulphur Reducing Bacteria (SRB), Oxidizing Iron Bacteria (OIB), and Methanogenic Archaea (MA)¹⁷⁻²¹.

Petrobras (Brazil's State controlled International Oil Company) is a major global oil producer and exporter and of great economic and technological importance for Brazil. Petrobras proactively researches all

aspects of exploration and production and very often with academic partners. Petrobras's studies on bacterial communities and especially SRB in produced water are ongoing and to date bacterial strains from the following genera have been isolated from Brazilian oilfields *Curtobacterium*, *Brevundimonas*, *Brachymonas*, *Streptomyces*, *Bacillus*, *Pseudomonas* amongst others^{22,23}. Bacteria belonging to the Firmicutes are commonly isolated and within them the Bacilli and Clostridia Classes are most often reported. Specific studies that have isolated bacteria associated with pipeline corrosion and biofilms reported strains from the genera *Marinobacter*, *Colwellia* and *Pseudomonas*. Strains from these genera are known to stimulate corrosion, producing large amounts of extracellular polysaccharides that protect sulphate-reducing bacteria (SRB) from biocides. Isolates from these genera have been shown to contribute to corrosion and to produce hydrogen sulphide impacting negatively on the market value of the crude oil and a threat to staff safety²²⁻²⁶.

In this study, we isolated cultivable bacteria from two Brazilian oilfields, Galo de Campinas and Periquito, both located in Rio Grande do Norte. Very little was known about the bacterial component of these wells. Metagenomic molecular biology methods were used to investigate samples and to establish a biodiversity benchmark as well as identifying potential biomarkers for the oilfields.

Materials And Methods

Sampling collection

Produced water was sampled from the Galo de Campina (GC) and Periquito (PQO) oilfields. The Galo de Campina oilfield is located in the Potiguar Basin (Lat - 05:26:44,532 Long - 37:36:16,235), Rio Grande do Norte State, about 50 km from the urban Centre of Mossoró city. The Periquito oilfield is also located in Rio Grande do Norte State, in the southwest part of the Potiguar Basin (Lat - 05:30:01,409 Long - 37:26:06,855). Three 50 ml samples of produced water were collected from the oil treatment separator unit of each oilfield using a sterile plastic container. Some characteristics of the two oilfields are in shown in Fig. 1.

Enrichment media

Produced water samples were stored refrigerated between at 0- 4 °C until transported under aseptic conditions to the lab. Given the low bacterial density and small volume of the samples, 3 different growth media (g/L) were added to the produced water samples to encourage the growth of bacteria of interest, the media were: i) Postgate B (K_2HPO_4 , 1.0 / NH_4Cl , 2.0/ $CaSO_4 \cdot 2H_2O$, 1.3 / $MgSO_4 \cdot 7H_2O$, 4.0 / Lactic acid (88%) 2.7); Postgate C²⁷, Sodium Lactate, 6.0/ Na_2SO_4 , 4.5 / NH_4Cl , 1.0 / Yeast Extract, 1.0 / K_2HPO_4 , 0.5 / $C_6H_5Na_3O_7 \cdot 2H_2O$, 0.3;/ $CaCl_2 \cdot 6H_2O$, 0.06 / $MgSO_4 \cdot 7H_2O$, 0.06 / $FeSO_4 \cdot 7H_2O$); 0.004 and Baars media²⁸, ($MgSO_4 \cdot 7H_2O$, 4.096 / $C_6H_5Na_3O_7 \cdot 2H_2O$, 5.7 ;/ $CaSO_4$, 1.0 NH_4Cl , 1.0 / K_2HPO_4 , 0.5 / Sodium lactate 4.5 ml / Yeast extract 1.0 / $Fe(NH_4)_2(SO_4)_2$, 6.72 g 50 ml 5 ml, for 1000 ml medium). Using a sterile syringe, 10 ml of media plus 1 ml of produced water samples was injected into a vial and

purged with nitrogen free oxygen gas for 2-minutes, before being clamped with rubber and aluminium cap. The sealed tubes were incubated for 20 days at 30 ° C under anaerobiosis in Gaspak jar. For each oil field there were nine samples, 3 of each type of medium. After 20 days bacteria from each of the 18 samples was collected by centrifugation for DNA extraction.

DNA extraction and Molecular identification of bacteria

Genomic DNA was extracted with the Wizard® Genomic DNA Purification Kit (PROMEGA) and quantified using QUBIT fluorometer (Thermo Fisher Scientific). DNA purity and quality were evaluated by 1% agarose gel electrophoresis containing SYBR® Safe DNA gel stain (Life Technologies™) at 90 V in 0.5X TBE buffer for 1 h and visualized by transillumination under ultraviolet light. DNA was PCR amplification of the 16S rDNA. The V3 and V4 variable region of the 16S rDNA was amplified by PCR, using the primers 338F²⁹ and 806R³⁰ with a barcode on the forward primer, in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 minutes, followed by 28 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds and 72 °C for 1 minute, after which a final elongation step at 72 °C for 5 minutes was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Samples were purified using calibrated Ampure XP beads. Purified PCR product was used to prepare the DNA library by following the Illumina TruSeq DNA library preparation protocol. Paired-end Sequencing was performed at MR DNA (www.mrdnlab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. After sequencing, the two reads from the paired end sequencing were joined together after q25 trimming of the ends, with the MrDNA pipeline prior to further analyses.

Sequence identification and bioinformatic analyses

The raw joined sequences were processed using mothur v.1.36.1 software³¹. The sequences were trimmed using trim.seqs command, with the following parameter: qwindowaverage = 30, qwindowsize = 50, maxambig = 0, minlength = 410, maxlength = 500. The sequences were then aligned using the Silva database as a reference³² and the resultant alignments were submitted to screen.seqs and filter.seqs to remove sequences with bad alignments and to remove uninformative columns of the alignment. The sequences were then pre-clustered using the command pre.cluster with parameter diffs = 3. Chimeras were detected with the chimera.uchime command, which uses the Uchime software³³, chimeric sequences were removed from further analyses. The remaining sequences were then classified using classify.seqs command, with RDP database as reference and a bootstrap cut-off of 80. Sequences classified as chloroplasts, mitochondria, eukaria, archaea and those not assigned to any Kingdom were removed. The resultant sequences, were used as input for the construction of a distance matrix and for clustering the sequences into operational taxonomic units (OTUs), with a cut-off of 3% of dissimilarity. The samples were then randomly normalized to the same number of sequences. Then the taxonomy summary was used to identify the bacterial compositions of each sample, and the OTU distributions were used to calculate the diversity indices and to establish the relationship between samples, using a NMDS analysis.

Heat map analysis

To illustrate the heat map results, some modifications were made to the methodology proposed by Pagé et al.³⁴. A heatmap and a dendrogram with the Bray-Curtis dissimilarity index were generated from the most abundant bacterial families and the community relationships calculated using the `vegdist` function of the `Vegan` package³⁵ for R. Heat maps were created using the `heatmap.2` of the `gplots` package³⁶ and the dendrograms generated by the `hclust` function from R's statistical package. Bacterial families whose relative reading abundance was less than 1% of at least one sample were removed. These analyses were performed in R software³⁷.

Results And Discussion

Bacterial biodiversity of produced water

The sequence data were deposited in the NCBI Sequence Read Archive (SRA) and are available under accession number SRP149784. In this study 4.206.240 sequence reads were generated, all the sequences studied were bacterial. The presence of some Archaea sequences would not have been a surprise as they are commonly found with this methodological approach in DNA derived from oil reservoirs³⁸⁻⁴⁰.

Crenarchaeota, Euryarchaeota were the most common archaea phyla found in produced-water¹⁹. The bacterial diversity indices of the produced water for the Galo de Campina (GC) and Periquito (PQO) oil fields are shown in Table 1.

Table 1
Biodiversity of the Produced Water samples.

Sample	Oil Field	OTUs	Chao	ACE	Shannon	NP Shannon
GCa-BA	Galo	101	176	263	0.98	0.99
GCa-PB	Galo	110	270	637	0.74	0.75
GCa-PC	Galo	119	275	508	0.96	0.97
GCb-BA	Galo	108	244	407	0.86	0.87
GCb-PB	Galo	95	169	221	0.72	0.73
GCb-PC	Galo	126	280	593	0.86	0.88
GCC-BA	Galo	143	405	760	1.29	1.30
GCC-PB	Galo	130	380	517	1.08	1.09
GCC-PC	Galo	150	370	730	1.42	1.43
PQOa-BA	Periquito	119	253	537	1.06	1.08
PQOa-PB	Periquito	119	272	437	1.17	1.18
PQOa-PC	Periquito	136	484	987	1.12	1.13
PQOb-BA	Periquito	144	443	980	0.99	1.01
PQOb-PB	Periquito	133	276	478	1.17	1.18
PQOb-PC	Periquito	128	349	1016	0.91	0.92
PQOc-BA	Periquito	120	243	411	0.91	0.92
PQOc-PB	Periquito	101	231	362	0.97	0.98
PQOc-PC	Periquito	93	179	289	0.86	0.87

BA = Baars medium, PB = Postgate B and PC = Postagatate C. Galo de Campina (GC) and Periquito (PQO) = Periquito oilfield. a,b, and c = number of samples

Based on a 3% dissimilarity cut off, a total of 992 OTU's were defined and compared. The number of OTUs (species richness/alpha diversity) varied between 95 and 150 at Campo de Galo and between 93 to 144 at Periquito. Comparisons of the Chao, ACE e Shannon indices were made and there was no significant differences in biodiversity between the oil fields and within the oilfields. At the 3% sequence dissimilarity cut off, the effects of enriching the produced water with three different SRB growth media were not significant. The diversity indices results and the results of a non-metric multidimensional scaling analysis (Fig. 2) indicate that differences seen between samples is explained by differences between the two oilfields and not the enrichment media. This support an inference that differences at the genus taxonomic rank between the two oilfields is real and not methodological.

Taxonomic distributions

The phylum level distribution of SRB at both the Galo de Campina and Periquito oilfields was dominated by the Proteobacteria, 93.8%, and Firmicutes with 6.2%. This was not a surprise and has been reported previously Belgini et al.⁴¹ also identified Proteobacteria, Firmicutes and Bacteroidetes as dominant when analysing samples from the Gabriel Passos Refinery (REGAP) in the city of Betim (MG, Brazil). Proteobacteria (29.2%), Firmicutes (8.3%) and Bacteroidetes (8.3%) were found in produced water of Diyarbakır oil Fields in Turkey⁴². In Turkey, the Proteobacteria were the predominant phylum in all samples, similar to the GC and PQO samples. In a study from the Mae Soon Luang Field, Fang Basin, Thailand⁴³, Proteobacteria, Firmicutes and Actinobacteria dominated the bacterial communities in most samples. This has also been reported from Texas, USA by Santillan et al.⁹, Li et al.⁴⁴ and Lan et al.⁴⁵ in China. Silva et al.²³ studied the microbial communities in petroleum samples from Brazilian oil fields and found that the Proteobacteria was the most abundant phylum, followed by Bacilli and Clostridia which belong to the Firmicutes phylum. Lipus et al.⁴⁶, analyzed microbial abundance in produced water samples in the Bakken region by qPCR. the analysis was done directly from produced water, without a pre-enrichment medium. The anaerobic, fermentative Firmicutes orders (Bacillales and Halanaerobiales) and the Proteobacteria Order Pseudomonadales were the most abundant taxa across all evaluated samples, representing between 57% and 99% of the total microbial population. Despite the differences in geographical locations of oilfields, their physicochemical properties and methods used for analyses, it is interesting to note that these two phyla dominate and perhaps not surprising if we consider conditions within a reservoir will strongly select for certain groups of bacteria. No differences in bacterial diversity were observed by using three enrichment media at phylum level. The dominant phyla are dominant regardless. Use of enrichment is a deliberate strategy for recovering cultivable bacteria with desired metabolic rates⁴⁷. The main Orders detected in the produced water from Galo de Campinas e Periquito were Desulfovibrionales, Pseudomonadales, Enterobacteriales, Clostridiales, Alteromonadales, Rhizobiales and Bacillales and their distributions are shown in (Fig. 3). Li et al.⁴⁴, in his work on biofilms in water injection systems in the Daqing oil field (China), also identified Pseudomonadales, Enterobacteriales, Clostridiales and Desulfovibrionales orders, some organisms of these orders are already known to cause problems associated with hydrogen sulphide production, biofilm formation and biocorrosion^{8,9,19}. Sequences from the order Desulfovibrionales were present in all samples and it was the dominant order found in both oil fields accounting for 46% of all sequences. The majority of bacteria in this order are SRB and are responsible for biocorrosion and possibly related to problems with hydrogen sulphide at the Periquito oilfield (personal communication). Pseudomonadales was predominant in Galo de Campinas oilfield and the, Enterobacteriales in both oil fields. In the Galo de Campinas oilfield, no significant difference was observed among the media. The Firmicutes, and Proteobacteria Orders seem to have a worldwide distribution in oilfields and are abundant in produced water. Sun et al.⁴⁸ isolated 61 phylogenetic groups that belong to 32 genera in the phyla Actinobacteria, Firmicutes, and Proteobacteria in oil-production water from the Karamay Oilfield, Xinjiang, China. The Enterobacteriales and Alteromonadales orders that were detected in this study are in agreement with other reports in the

literature that relate these orders to oil environments^{49,50}. Liu and Liu⁵¹ analyzed the bacterial community of oil collected from the sea surface of the northern Gulf of Mexico, as a result they found high proportions of *Alteromonas*, *Marinobacter*, *Thalassospira*, *Bartonella*, *Rhodovulum* and *Stappia*. And they point out that *Marinobacter* and *Alteromonas*, Gammaproteobacteria, are common oil-degrading microorganisms. This result is in agreement with Bacosa et al.⁵² who analyzed the bacterial diversity of the same region and concluded that *Alteromonas* is an important class of bacteria in the fate of oil, being effective in degrading the alkanes in oil. Wang et al.⁵³ studied the microbial community of oil-polluted soil on agricultural land in Fushun, Liaoning province in China, where they attested that the abundance of Enterobacteriales was greater in areas with oil-contaminated soil.

Figure 4 provides analysis genus level diversity of the produced water and the main genera are summarized in Table 2. *Desulfovibrio* is the main and dominant genus in produced water from Galo de Campinas (16,08%) and Periquito (29,7%) followed by *Pseudomonas* in Galo de Campinas (19,88%) and *Shewanella* in Periquito (4,69%). Some bacterial sequences were too divergent for classification and that was the case for both oilfields. Clostridia were detected in low numbers from both oil fields. Postgate C, in the majority of the samples, favoured *Desulfovibrio* isolation over the other media used. *Pseudomonas* was detected only in groups GCc and GCb, being a possible biomarker for these sites. *Pleomorphomona* were identified only at Periquito. *Desulfovibrio*, *Pseudomonas* and *Clostridium* are routinely found in produced water samples where strains from the Genus *Desulfovibrio* are noted as of major concern for the oil and gas industry. Comparing Fig. 4 with Fig. 3 we note that the figures look similar. This happens because a limited number of dominant genera are responsible for the Order level diversity. What stands out immediately is the importance of the *Desulfovibrio* sequences^{40,54}. *Desulfovibrio* impact negatively causing corrosion, hydrogen production, souring and biofouling caused by biofilm formation within the operational plants. More than 220 species from 60 genera of SRB have been reported; of which, the most commonly isolated mesophilic SRB from produced water are from the *Desulfovibrio* genus⁴⁰. Also of note were the *Pseudomonads* from two of the three Galo de Campinas sites, and, the as yet unidentified bacteria at genus level were from the Enterobacteriales. *Pelobacter*, *Marinobacterium* and *Geotoga* were detected in produced water from Petrobras Ilha Grande Bay Oil Terminal in Brazil, as well as the SRB *Desulfoplanes formicivoran*⁵⁵. Bacteria of the genera *Desulfovibrio* and *Clostridium* are producers of hydrogen sulphide, it is toxic and accelerates the corrosion of metallic structures^{20,56}. The most frequently described SRB genera from produced water are the Delta Proteobacteria *Desulfovibrio* and *Desulfomicrobium*²¹. Although *Desulfovibrio* was the dominant SRB in this study other related genera have been reported from petroleum derived samples, including *Desulfomicrobium*, *Desulfobacterium*, *Desulfosarcina*, *Desulfococcus*, *Desulfotignum*, *Desulfobotulus*, *Desulfobulbus*, *Desulfacinum*, *Thermodesulforhabdus*, *Desulforhabdus*, *Desulfatibacillum*, *Desulfoglaeba*, *Desulfonauticus*, *Desulfocurvus* (Delta-proteobacteria) *Desulfotomaculum* (Firmicutes), *Thermodesulfobacterium* (Thermodesulfobacteria), *Thermodesulfovibrio* (Nitrospira), *Archaeoglobus* (Euryarchaeota) and *Caldivirga* (Crenarchaeota)^{44,57-59}. The genera *Pleomorphomonas* and *Shewanella* were more abundant in samples from the Periquito oil field. *Pleomorphomonas* was described in 2005 by Xie and Yokota⁶⁰

and is composed of Gram-negative, pleomorphic, nitrogen-fixing, non-spore-forming, non-motile rods^{61,62}. At the time of writing, there is no data correlating this genus to problems in oil industry. *Shewanella* are facultative anaerobic, gram-negative, motile and rod-shaped bacteria, most of which have been isolated from marine environments, such as seawater, marine sediments or sand, tidal flats or marine invertebrates. Some species have, however, been isolated from clinical samples, oilfield fluids, activated sludge and coal-mine sludge^{63,64}. *Shewanella* strains were described as a potential hazard to the oil industry causing souring of crude oil⁶⁵.

Table 2
Top 5 dominant genus found in Galo de Campinas e Periquito oilfield

Genera	Oil well	
	Galo de Campinas	Periquito
<i>Desulfovibrio</i>	16,08%	29,87%
<i>Pseudomonas</i>	19,88%	0,18%
<i>Clostridium_sensu_stricto</i>	2,40%	2,81%
<i>Shewanella</i>	0,07%	4,69%
<i>Pleomorphomonas</i>	0,07%	3,77%
* unclassified genera from Galo de Campinas is 11,05% and Periquito 7,62		

In Fig. 5, the heat map provides a clearer representation of the genus level differences between the sites and oilfields. The heatmap illustrates the mutual occurrence of *Pseudomonas* and *Desulfovibrio* only at the GCb site. The genera *Anaerobaculum*, *Pleomorphomonas* and *Shewanella* are important and exclusive biomarkers of the Periquito oilfield. Non-metric multidimensional scaling analysis of Galo de Campina and Periquito oil fields (Fig. 2) was used to study the correlation between the three sampling locations with the two oil fields (PQO and GC) and culture media (Postgate B, Postgate C and Baars) and the influence of each on the diversity detected. That analysis indicated that sampling points within each oil field and culture media did not significantly influence the microbial populations detected and the main difference in microbial distributions at the genus level was related to the two oil fields.

The metagenomic data obtained here by enriching produced water and then large scale PCR-DNA sequencing is consistent with the results reported in literature based on isolation methods and on methods where organisms were neither enriched nor isolated^{21,23,66}.

It is only at the genus level that differences between individual wells and oil fields become evident. Bacterial strains of the Genus *Pseudomonas* are producers of extracellular polymeric substances that form biofilms within oil facilities¹⁷. The presence of mesophilic *Pseudomonas* strains that are sensitive to high temperatures is not believed to originate from pristine oil reservoirs. Their presence in systems with high temperature oil wells is likely to follow flooding with cooler produced water, and contamination

with *Pseudomonas* strains that are very versatile heterotrophs with the competitive capability to survive including formation of biofilms in oil/water mixtures²¹.

Zdanowski et al.⁶⁷ analyzed the anaerobic microbiome of subglacial samples. In their work, the authors compared the phylogenetic diversity of native samples with enriched ones. This enrichment was done by incubating native sediments in Postgate C medium for 8 weeks using airtight bottles to emulate subglacial conditions. As a result, they found that the following genera were found more abundantly in the enriched medium: *Psychrosinus*, *Clostridium*, *Paludibacter*, *Acetobacterium*, *Pseudomonas*, *Carnobacterium*, and *Desulfosporosinus*. *Pseudomonas* and *Carnobacterium* were found only in the enriched medium. Thus, the authors suggest that it may be that bacteria of the genus *Pseudomonas* have proliferated under enrichment conditions, depleting available oxygen in the early stages of the process, and then helping anaerobes to develop. Kliushnikova et al.⁶⁸ described a microorganism of the genus *Pseudomonas* with sulphate-reducing activity. According to the authors, this strain when grown under strictly anaerobic conditions was able to reduce sulphate more intensely than under aerobic conditions. In a study by Guo et al.⁶⁹, the authors demonstrated that *Pseudomonas* strain sp. C27 has an enzymatic system to perform sulfide removal. The authors cultivated the C27 strain in an anaerobic environment and demonstrated that the sulfide metabolism occurred through the expression of succinate dehydrogenase, iron–sulfur protein, oxidoreductase, serine hydroxymethyltransferase, and iron superoxide dismutase. Brahmacharimayum and Ghosh⁷⁰ analyzed the removal of sulfate in an anaerobic environment by metagenomics. As a result, they show that the *P. aeruginosa* strain was predominant in the consortium and that it was involved in reducing Sulfate. Tüccar et al.⁴², found *Pseudomonas* as the dominant genus in produced water from Diyarbakir oil fields in Turkey and the authors suggested that these strains may have been inoculated into the oil reservoirs through the injection of fluids, and point out that these strains may adapt to the conditions of the reservoir to survive. Several studies have found the occurrence of the genus *Pseudomonas* in oil reservoirs^{71–77}, according to Cui et al.⁷⁸, *Pseudomonas* and *Acinetobacter* are genera that can effectively use crude oil as a carbon source, being able to survive and reproduce at the oil-water interface. Species of the genus *Pseudomonas* are facultative anaerobes capable of performing nitrification and nitrate reduction using various carbon substrates^{79,80}. In a study by Braun and Gibson^[11], the authors reported two bacteria of the genus *Pseudomonas* that were able to degrade, under anaerobic conditions, 2-aminobenzoate (anthranilic acid) to CO₂ and NH₄⁺. According to Cai et al.⁸¹, in the petroleum area, despite the fact that the oil is considered a hostile and toxic environment for microorganisms, there is lot of evidence that demonstrates the presence of microbes in the crude oil. The authors analyzed the microorganisms found in oil and water samples from four oil wells. According to them, as an unexpected result, they found that the genus *Pseudomonas* dominated the oil samples, where several groups of functional genes were identified.

According to Arai (2001)⁸², *Pseudomonas aeruginosa* has a remarkable ability to grow in the most diverse environmental conditions, such as in soil and water and on and in animals, humans and plants. This versatility is related to its metabolic flexibility through a branched respiratory chain with multiple

terminal oxidases and denitrification enzymes. Its set of denitrification enzymes are capable of reducing nitrate to nitrogen via nitrite, nitric oxide (NO), and nitrous oxide. Nitrogen oxides function as electron acceptors, which allows *P. aeruginosa* to grow in anaerobic conditions. It is interesting to note that in the absence of nitrate, *P. aeruginosa* is able to metabolize arginine via arginine deiminase and in the absence of both, it can ferment mixed acid pyruvate and survive for long periods in anoxic conditions⁸³.

Conclusion

DNA analysis of over 4 million sequences from the Galo de Campina and Periquito oil fields detected a dominant phylum, the Proteobacteria (93.8%) followed by the Firmicutes (6.2%). These phyla are typically described in all global studies of reservoir and produced water bacterial biodiversity. The rDNA data in this study supports previous findings based on isolation studies. Seven major orders were detected, Clostridiales and Bacilliales belong to the phylum Firmicutes, and Desulfovibrionales, Pseudomonadales, Enterobacteriales, Alteromonadales and Rhizobiales that belong to the phylum Proteobacteria. Among the genera detected, we noted that *Pseudomonas*, *Desulfovibrio* and *Clostridium* were the dominant genera, bacteria that are routinely isolated from samples of produced water and are known to be involved in biofilm formation processes, production of hydrogen sulphide and corrosion of metallic structures were also detected. For these two oil fields we now know which bacteria in the produced water responded to the experimental conditions and that could possibly respond to attempts to manage those bacteria. Microbial dynamics and ecological studies will facilitate monitoring and guide the best use biocides in order to avoid biocorrosion and related processes. Microbial communities are both the problem and the solution to corrosion and souring; to decrease maintenance/operating costs as well as to minimise environmental preservation. These results can be used to help identify and target specific antimicrobial agents, biotechnologies that are more efficient and less toxic to the environment resulting microbially enhanced oil recovery.

Declarations

Funding

This work was supported in part by the Coordenação de Aperfeiçoamento Pessoal de Nível Superior-Brasil (CAPES), Finance code 001, by grants from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, grant code “CIENTISTA DO NOSSO ESTADO” 26/ 202. 630/2019 [247088]), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (MCTI-CNPq, grant code 309461/2019-7).

Declaration of Competing interests

The authors report no declarations of interest.

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Figures

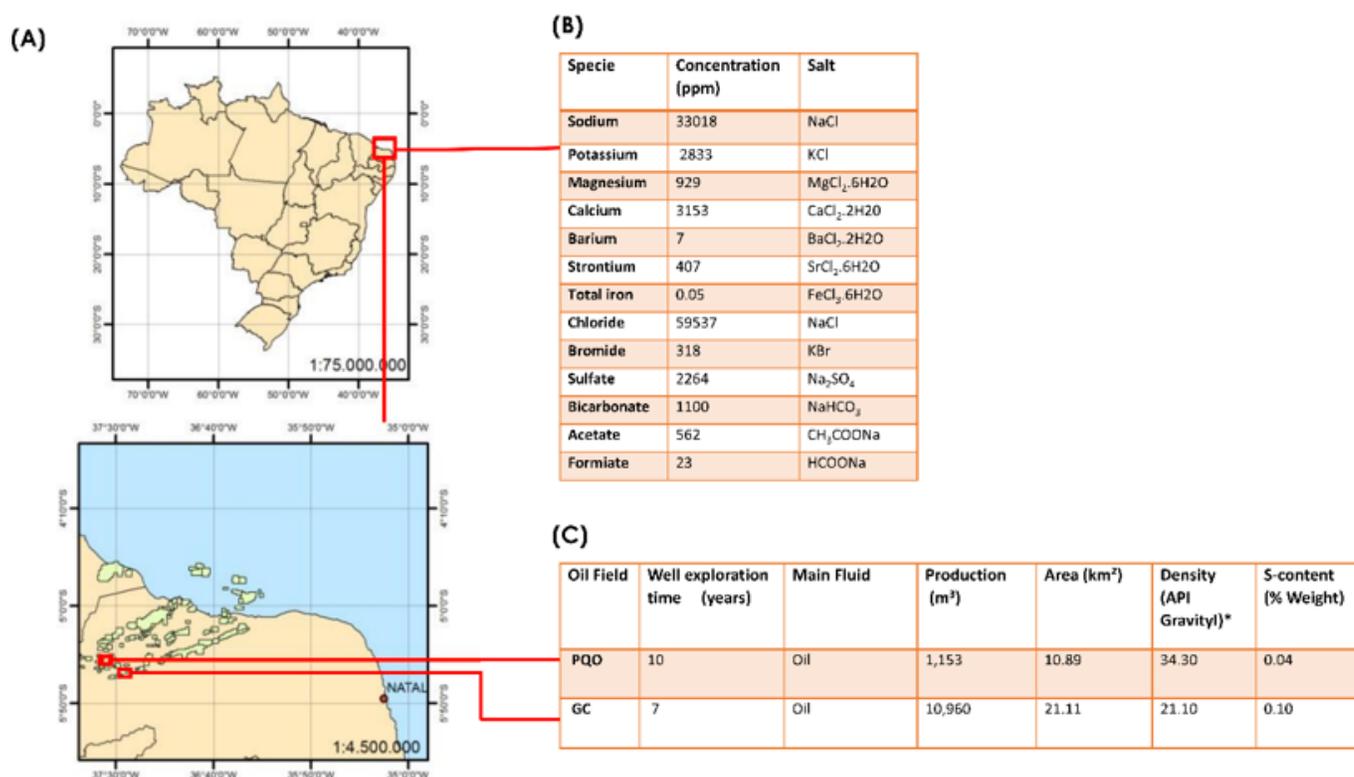


Figure 1

(A) Geographical location, (B) Table of chemical composition of produced water at Periquito, (C) Characteristics of Periquito (PQO) and Galo de Campinas (GC) onshore oilfields from Brazil.

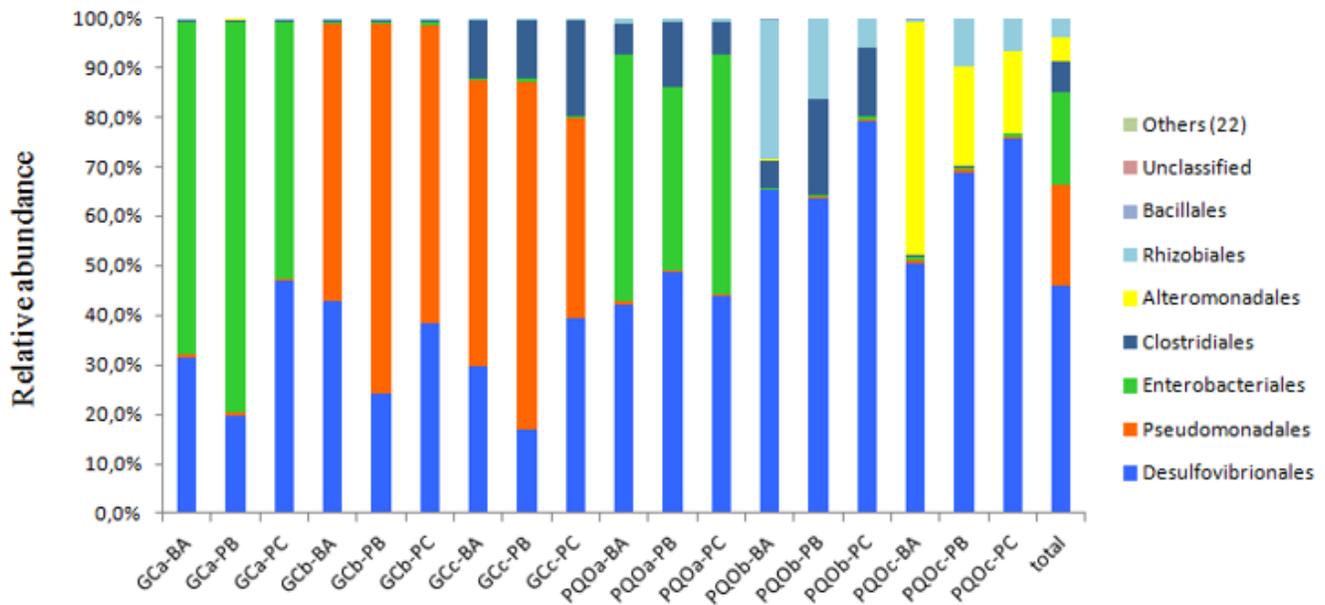


Figure 3

Bacterial Orders found in produced water from the GC and PQO oilfields. GCa, GCb and GCc represents the replicas from GC oilfield. PQOa, PQOb and PQOc represents the replicas from PQO oilfields. BA, PB and PC represents Baars, Postgate B and Postgate C media, respectively.

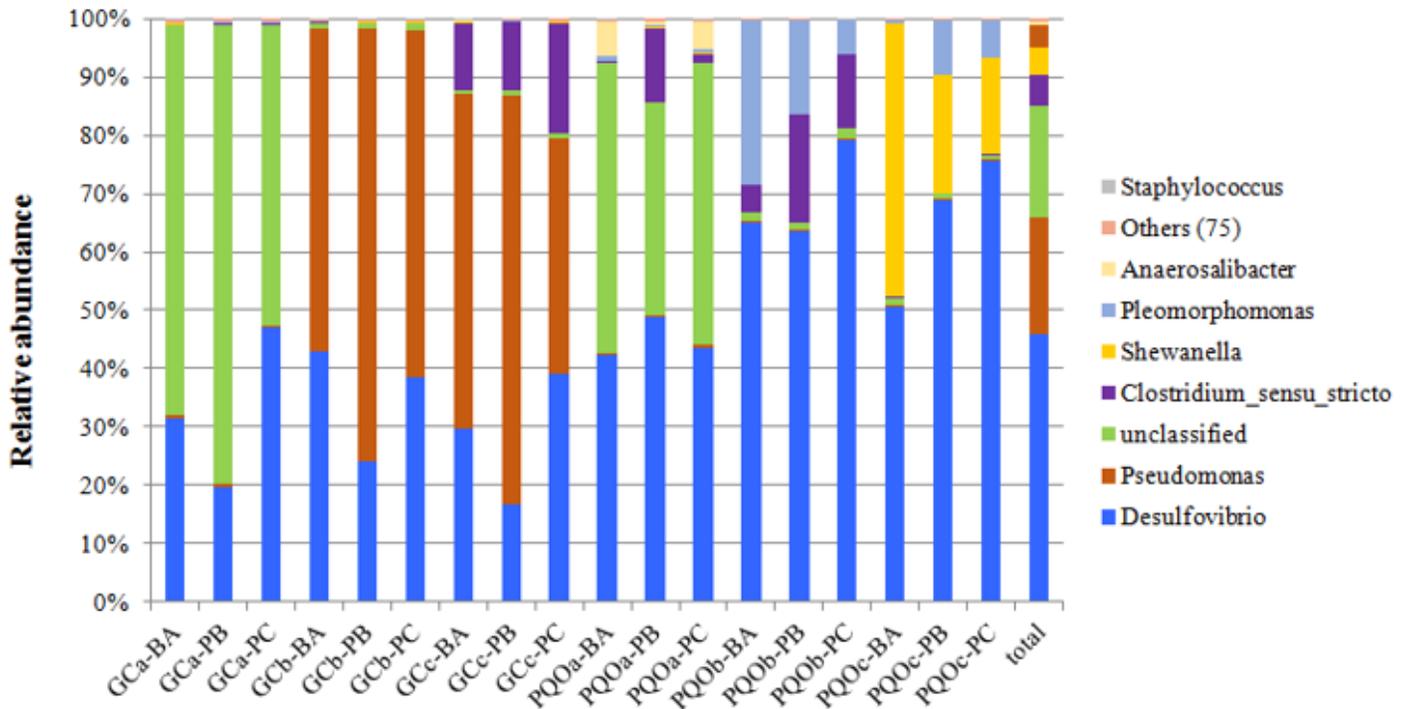


Figure 4

Bacterial genera found in the samples from GC and PQO oilfields. GCa, GCb and GCc represents the replicas from GC oilfield. PQOa, PQOb and PQOc represents the replicas from PQO oilfields. BA, PB and

PC represents Baars, Postgate B and Postgate C media, respectively.

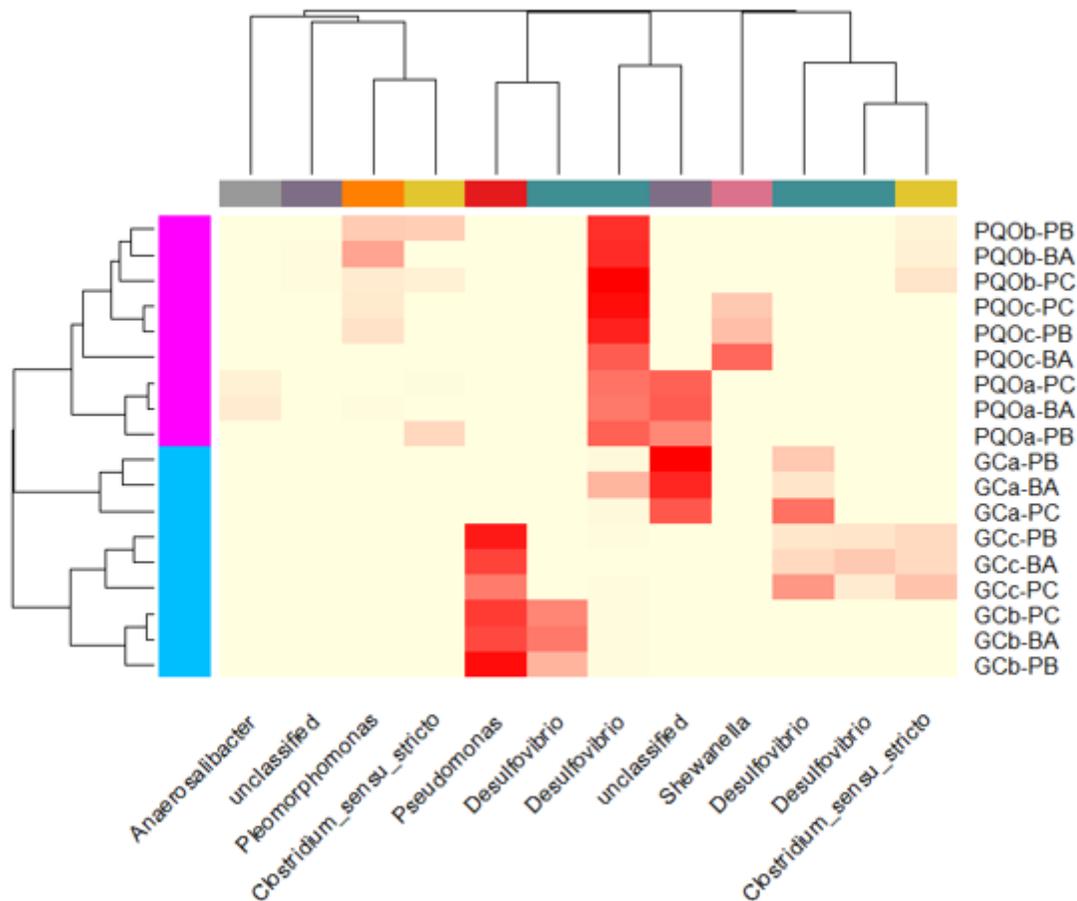


Figure 5

Heatmap showing the relative abundances of the most relevant operational taxonomic units (OTUs) derived from bacteria in the oil wells. The samples and OTUs were clustered according to Complete distances and were similar using Bray–Curtis dissimilarity. GCa, GCb and GCc represent the three sites from GC oilfield. PQOa, PQOb and PQOc represent the sites from PQO oilfields. BA, PB and PC represent Baars, Postgate B and Postgate C enrichment media.