Research Paper

Sequencing batch airlift reactors (SBAR): a suitable technology for treatment and valorization of mineral oil wastewaters towards lipids production

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\textbf{A B S T R A C T}

Produced water (PW) and spent oil-based wastewaters are some of the largest mineral oil wastewaters produced. Due to the high toxicity of hydrocarbons, several countries set stringent discharge limits and its treatment is compulsory before discharge. In this work, biological treatment of mineral oil wastewaters coupled with the production of bacterial lipids is demonstrated in sequential batch airlift reactors (SBAR). Two SBAR (2 L working volume) were used for treatment of PW and lubricant-based wastewater (LW), inoculated with \textit{Alcanivorax borkumensis} SK2 (SBAR\textsubscript{PW}) and \textit{Rhodococcus opacus} B4 (SBAR\textsubscript{LW}), respectively. A total petroleum hydrocarbon removal (TPH) efficiency up to 96% and 80% were achieved for SBAR\textsubscript{PW} and SBAR\textsubscript{LW}, respectively. Intracellular lipids production in SBAR\textsubscript{PW} increased when lower TPH/N ratios and higher feast stage duration were applied (up to 0.74 g g\textsuperscript{-1} cell dry weight (CDW)), whereas in SBAR\textsubscript{LW} higher lipids production was observed for higher TPH/N ratios (0.94 g g\textsuperscript{-1} in CDW). Triacylglycerols (TAG) were the main intracellular lipid accumulated in both SBAR\textsubscript{PW} and SBAR\textsubscript{LW} operations, while wax ester (WE) production was only observed extracellularly in the SBAR\textsubscript{PW}.

\textbf{1. Introduction}

The increasing demand for petroleum and petroleum derived products had led to the production of large amounts of mineral oil wastewaters, being spent oil-based wastewaters (SOW) and oilfield produced water (PW) the most significantly produced. Lubricant-based wastewater (LW) is a common type of SOW that is produced during the manufacture and the application of lubricants in industrial activities (Castro et al., 2018). On the other hand, oilfield PW is the largest by-product generated during the process of oil and gas extraction with a worldwide production up to 39.5 billion liters per day (Jim\textsuperscript{enez} et al., 2018). Industrial petrochemical wastewater treatment is an important study area in environmental engineering. Mineral oil wastewaters are very recalcitrant streams and due to the presence of large concentrations of petroleum hydrocarbons are considered an environmental problem with serious consequences to the environment and human health (Camarillo and Stringfellow, 2018). Therefore, the treatment of these wastewaters is compulsory and limits and rules regarding the organic content had been set for its discharge into the environment (Fakhru Razii et al., 2009).

Nowadays, mineral oil wastewaters are commonly treated by different physical and/or chemical processes such as coagulation, adsorption, hydrocyclones, centrifugation and membrane processes (Aljuboury et al., 2017). However, these methods require high capital and operation costs and it is more appropriate to use biotechnological methods to diminish costs, improve treatment efficiency and recover valuable components from mineral oil wastewaters (Dong et al., 2011). Biological treatment of mineral oil-wastewaters offers some
advantages to the current physical and chemical processes. It is a more cost-effective method, allowing to remove the small suspended oil particles that cannot be eliminated otherwise and environmental friendly, since it relies in bacteria capability to transform hydrocarbons into non-toxic by-products such as carbon dioxide (CO₂), water or bacterial reserve compounds (Dong et al., 2011; Fakhru’l-Razi et al., 2009).

Several works have been focused on the aerobic treatment of hydrocarbon contaminated wastewaters since it occurs at a faster rate and is more efficient than under anaerobic conditions (Da Silva et al., 2016; Heider et al., 1998; Sudmalis et al., 2018). Mineral oil wastewaters, like for instance PW and LW, differ from each other relatively to the type and content of hydrocarbons, salinity levels and presence of some other specific compounds. For example, PW consists mainly on aliphatic hydrocarbons from C₁₁ to C₄₀, which are more easily biodegradable than aromatic hydrocarbons (Sudmalis et al., 2018), while LW is composed by a complex matrix that includes a mixture of straight-chain alkanes ranging from C₁₀ to C₃₄ and a complex and unresolved hydrocarbon mixture (Da Silva et al., 2016). Another important feature of mineral oil wastewaters is the salinity levels which in the case of offshore PW can be challenging when a biological treatment is applied. Therefore, the use of hydrocarbonoclastic bacteria (HCB) and in the particular case of offshore PW, halotolerant HCB, can be a suitable strategy for aerobic treatment of mineral oil wastewaters such as PW and LW (Da Silva et al., 2016; Sudmalis et al., 2018).

One particular feature of HCB is the accumulation of storage compounds in response to nutrient limitation in the environment. Alcanivorax borkumensis SK2 and Rhodococcus opacus B₄ are two HCB species capable of degrading a wide range of hydrocarbons and use them as carbon source for the production and accumulation of neutral lipids (Alvarez and Steinbüchel, 2002; Manilla-Pérez et al., 2011). The accumulation of lipids occurs as a survival strategy during starvation periods, when an essential nutrient (i.e. nitrogen and/or phosphorous) becomes unavailable or at lower concentrations comparing with the carbon source (Kalscheuer et al., 2007; Manilla-Pérez et al., 2011). Several factors can influence the production of storage compounds by bacteria. Some studies reported that the carbon to nitrogen ratio (COD/N ratio) is the most important parameter influencing the production and accumulation of lipophilic compounds by HCB (Manilla-Pérez et al., 2011; Zhang et al., 2019). Currently, bacterial neutral lipids are of great interest as raw materials for production of biofuels or other oleochemical products (Castro et al., 2017; Kumar et al., 2015).

Previous studies performed by our research group reported A. borkumensis SK2 ability to produce intracellular and extracellular lipids, namely WE and TAG, as well as R. opacus PD630 and B₄ ability to produce intracellular TAG, from mineral oil wastewaters (Castro et al., 2016; Da Silva et al., 2016; Sudmalis et al., 2018). Due to these remarkable characteristics, A. borkumensis SK2 (halophilic) and R. opacus B₄, were chosen for the treatment of PW and LW respectively. Moreover, A. borkumensis SK2 was chosen due to its wide presence in marine environments.

Combining aerobic biological treatment with the valorization of mineral oil-based wastewaters can be an environmentally sustainable alternative for the management of these wastewaters. After showing that A. borkumensis SK2 and R. opacus B₄ are suitable candidates for the treatment process, it is important to study the most appropriate process conditions and bioreactor configurations for the optimization and application of a full-scale treatment.

In this work the suitability of the sequencing batch airlift reactor (SBAR) configuration to biologically treat different types of mineral oil wastewaters namely PW (saline wastewater) and LW (non-saline wastewater) was explored. A specialized inoculum focused in PW and LW characteristics, namely salinity level, was used and the most suitable conditions for the production and accumulation of bacterial lipids were assessed, in order to achieve a balance between neutral lipids production and wastewater treatment costs and efficiency.

2. Materials and methods
2.1. Experimental set-up, inoculum and carbon source

A SBAR with a working volume of 2 L, designated as SBARₐₜₜ₋ₚW, was inoculated with A. borkumensis SK2 (DSM 11573), fed with oilfield produced water (PW) and operated at room temperature (20 ± 5 °C) and pH around 7.8. Another SBAR with the same working volume, designated as SBARᵣₒ₋₁₋ₚW, was inoculated with R. opacus B₄ (NBRC 108011), fed with lubricant-based wastewater (LW) at different concentrations and operated at room temperature (20 ± 5 °C) and pH around 7.3. PW concentrate was obtained from Shell (The Netherlands), from a full-scale physical separation process. LW concentrate (floating phase separated in a gravimetric oil/water separation unit) was obtained from a wastewater treatment plant located in a heavy machinery maintenance service unit (ALSTOM Portugal, S.A., Maia, Portugal). The chemical characterization of the concentrates of lubricant-based wastewater (LW) and oilfield produced water (PW) is presented in Table 1.

<table>
<thead>
<tr>
<th>Characterization</th>
<th>LW</th>
<th>PW</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD (g L⁻¹)</td>
<td>317 ± 14</td>
<td>n.d.</td>
</tr>
<tr>
<td>Nitrate (mg L⁻¹)</td>
<td>94 ± 9</td>
<td>n.d.</td>
</tr>
<tr>
<td>Nitrite (mg L⁻¹)</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>Total ammonia nitrogen (mg L⁻¹)</td>
<td>0.13 ± 0.4</td>
<td>1.2 ± 0.08</td>
</tr>
<tr>
<td>Total nitrogen (mg L⁻¹)</td>
<td>172 ± 0.4</td>
<td>43 ± 2.7</td>
</tr>
<tr>
<td>Salinity (g kg⁻¹ of seawater)</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>Total petroleum hydrocarbons (TPH) (g L⁻¹)</td>
<td>177 ± 17</td>
<td>202 ± 16</td>
</tr>
</tbody>
</table>

a Value not determined due to interferences of the high chloride levels in the quantification method.
characterization of LW and PW is described in Table 1.

A. borkumensis SK2 was pre-grown in saline medium (ONR7a) according to DMSZ, Braunschweig, Germany and R. opacus B4 in Mineral Salts (MS) medium according to Schlegel et al. (1961) (please see Supplementary information for details and Fig. S1).

A short adaptation period of the microorganisms to the respective carbon source was performed before SBAR operations. In this period both carbon (PW or LW) and nitrogen source (ammonium chloride (NH4Cl)) were added to the bioreactors at the conditions of 0.26 g L−1 of TPH and 30 mg L−1 of nitrogen for SBARAb+PW and 1 g L−1 of TPH and 30 mg L−1 of nitrogen for SBARAb+LW. The wastewaters treatment process consisted in alternate cycles of feast (carbon addition) and famine (nitrogen addition) stages. In the famine stage, only nitrogen (at concentrations of 15 or 30 mg L−1) was added to the bioreactor. This stage was necessary to ensure that all the carbon from the previous cycle was totally consumed before the carbon source addition. In the feast stage, only the carbon source was added (Fig. S2, Supplementary material).

Different ratios of total petroleum hydrocarbons per nitrogen concentration (TPH/N ratios) and feast stage duration were tested for both wastewaters. The operational conditions are described in Table 2 for SBARAb+PW and Table 3 for SBARAb+LW. In both SBAR operations and for each condition tested, three cycles were performed with exception of the condition of 4.4 g L−1 of TPH and 15 mg L−1 of nitrogen in SBARAb+LW, where only two cycles were made. At the end of each cycle, analysis on neutral lipids, total nitrogen and total petroleum hydrocarbons concentration were performed.

2.2. Analytical procedures

Bacterial growth in the seed cultures used for bioreactors inoculation was monitored by optical density at 600 nm wavelength (OD600) in a DR 2800 Hach Lange spectrophotometer.

During reactors operation, 2 mL samples were collected daily and centrifuged at 10,000 rpm for 10 min for nitrogen determination (N-NH4+), using the commercial kit LCK 304 (Hach Lange®, Germany). At the end of each cycle total nitrogen (TN) determination was performed, using the commercial kits LCK 338 (Hach Lange®, Germany). Biomass concentration was measured as total suspended nitrogen (TSN), using the commercial kit LCK 304 (Hach Lange®, Germany).

Chemical oxygen demand (COD), nitrite and nitrate concentrations were determined using the commercial kit LCK 914, LCK 342 and LCK 339, respectively (Hach Lange®, Germany).

The pH was manually controlled and measured by using a benchtop metre inoLabVR pH 7110 (WTW, Weilheim, Germany). The adjustment was performed by adding hydrochloric acid (HCl) with a syringe to the bioreactor until reaching the optimal pH.

2.3. Extraction and quantification of total petroleum hydrocarbons (TPH)

Samples of 50 mL (in duplicate) were collected for hydrocarbons analysis. The samples were acidified with HCl to a pH below 2 and preserved at temperatures below 5 °C until further analysis.

Hydrocarbons extraction was performed according to the international standard method ISO 9377-2 at room temperature as described in Castro et al. (2016), with the following modifications: the extraction was performed three times with 25 mL of hexane; and after the cleaning step, the samples were dried under a gentle flow of nitrogen in a TurboVap®LV (Biolage) at 51 °C for 40 min and resuspended in 1 mL of n-hexane for further analysis by gas chromatography with a flame ionization detector (GC-FID). The extracts were injected in a GC Varian® star 3400CX, USA equipped with a VF-1 ms column (30 m long, 0.25 mm diameter and 0.25 µm film thickness). A sample volume of 2 µL was injected at 250 °C with a split ratio of 1:20 and helium was used as carrier gas at a flow rate of 1 mL min−1. The column temperature was set at 60 °C for 1 min and then increased until 270 °C at a rate of 8 °C min−1 in a total time run of 62 min. The detector was operated at a temperature of 315 °C.

2.4. Extraction and analysis of neutral lipids

30 mL samples were collected from the bioreactor for extraction and analysis of neutral lipids. The samples were centrifuged at 10,000 rpm for 10 min in a centrifuge Beckman allegra 64R. The pellet was freeze dried and used for intracellular lipids analysis. Intracellular lipids extraction was performed with chloroform: methanol (2:1, v/v) as described in Castro et al. (2016).

For extracellular lipid extraction, the supernatant was mixed with chloroform: methanol (2:1, v/v) in a separation funnel (1:1 vol/vol) for 2 min and left for 30 min to allow the separation between the aqueous and organic phases.

The organic phases were dried under a gentle flow of nitrogen in a TurboVap®LV (Biolage) at 51 °C for 40 min and resuspended in 500 µL of chloroform.

Extracellular lipid extracts were fractionated by polarity on SPE columns (SIOH 1 g/6 cc) from Supelco, as described in Revellame et al. (2012).

The total lipid content was quantified gravimetrically. The different classes of lipids present in the extracts was assessed by TLC (Supplementary material).

2.5. DNA extraction and sequencing by Illumina

At the end of each bioreactor cycle, samples of well-homogenized biomass were collected, centrifuged (10,000 rpm, 10 min at 4 °C), washed with PBS and stored at −20 °C. Total genomic DNA was extracted using a FastDNA SPIN kit for Soil (MP Biomedicals, LLC, Santa Ana, CA) according to manufacturer’s instructions.

Amplification, library preparation, sequencing and taxonomic assignment of 16S rRNA reads were done by STAB VIDA (Carapica, Portugal). Library construction was performed using the Illumina 16S Metagenomic Sequencing library preparation protocol with the specific bacterial primers set 027F-1492R (Lane, 1991). 16S rDNA genes from the variable V1-V2 regions were sequenced with MiSeq Reagent Kit v3 in
the Illumina MiSeq platform, using 300 bp paired-end sequencing reads.

Bioinformatic analysis were also performed by STAB VIDA (Caparica, Portugal). The analysis of the generated raw sequence was carried out using QIIME2 v2018.11.0 according to Caporaso et al. (2010). The reads were denoised using the DADA2 plugin and the following processes were applied: trimming and truncating low quality regions; dereplicating the reads and filtering chimeras (Callahan et al., 2016). The reads were organized in operational taxonomic units (OTUs) and the taxonomical classification was performed using SILVA database (release 132 QIIME) with a clustering threshold of 97% similarity. Only OTUs containing at least 10 sequence reads were considered as significant.

Sequencing reads were submitted to the European Nucleotide Archive (ENA) under the study accession number PRJEB37500 (sample SAMEA6791327 - cycle XI A. borkumensis SK2; sample SAMEA6791326 - cycle VIII A. borkumensis SK2; sample SAMEA6791328 - cycle VI R. opacus B4; sample SAMEA6791329 - cycle II R. opacus B4).

2.6. Attenuated total reflection-fourier transform infrared analysis (ATR-FTIR)

Extracts of the intracellular and extracellular lipids resuspended in 0.5 mL of chloroform were analyzed by ATR-FTIR (attenuated total reflectance-Fourier transform infrared) using a Perkin Elmer Spectrum Two™ IR spectrometer with a deuterated triglycine sulfate (DTGS) detector and KBr beam splitter, coupled with an UATR (single reflection diamond, Perkin Elmer) accessory. Samples were applied in the top of the diamond crystal and evaporated to form a film. Complete solvent evaporation was controlled by spectra acquisition until disappearance of the characteristic chloroform band. FTIR spectra were collected at room temperature, in the range of 4000 – 400 cm⁻¹, with a resolution of 4 cm⁻¹, accumulating 64 scans.

3. Results and discussion

3.1. Evaluation of SBAR performance in the treatment of LW and PW and on the production of lipids

Microbial growth in both bioreactors (SBAR_{Ab-PW} and SBAR_{R.o-l-LW}) was followed by the determination of N-NH₄ concentration in the culture medium (Fig. 1 (A) and (B)). N-NH₄ consumption in each cycle suggested that bacterial cells in SBAR_{Ab-PW} and SBAR_{R.o-l-LW} were able to degrade the hydrocarbons present in the wastewaters and use them as carbon source for growth (Fig. 1 (A) and (B)). NO₃ and NO₂ concentration in the SBAR_{Ab-PW} effluent was below 2 and 1 mg L⁻¹, respectively. In the case of SBAR_{R.o-l-LW} NO₂ concentration was below 2 mg L⁻¹ and NO₃ concentration was approximately 9 mg L⁻¹. The low NO₂ and NO₃ concentrations observed in both SBAR operations indicates that occurrence of nitrification was not significant. The N-NH₄ profile was a relevant monitoring parameter in the performance of the feast and famine stages, allowing to determine the moment of addition of the wastewater (carbon source).

In the SBAR_{Ab-PW} operation, the microbial growth was similar in all cycles, regardless the nitrogen concentrations used (approximately 0.6 g L⁻¹, Table 4), indicating that growth was not influenced by the nitrogen concentration and consequently by the TPH/N ratio. In the case of the SBAR_{R.o-l-LW} operation, accurate biomass quantification was not possible due to the formation of granules by the bacterial biomass.

In the SBAR_{Ab-PW}, a TPH removal efficiency up to 96% ± 1.8 was achieved (Table 4). The preference of A. borkumensis SK2 for aliphatic hydrocarbons as sole or main carbon source is well described (Yakimov et al., 1998). Moreover, the ability of A. borkumensis SK2 to remove n-alkanes and other non-polar compounds from PW was already demonstrated by Sudmalis et al. (2018) in batch and continuous stirred tank bioreactor (CSTR). In cycles IV to VIII, a similar TPH removal efficiency was obtained, independently of the feast stage duration. However, when a TPH/N ratio of 9 (cycles I to III and IX to XI) was applied to the bioreactor (0.26 g L⁻¹ TPH_{PW} and 30 mg L⁻¹ of nitrogen), the increase of the feast stage duration from 2 to 5 days led to an improvement on the TPH removal efficiency of approximately 10% (Table 4). This result suggests that the feast stage duration applied to the cycles I, II and III (approximately 2 days) was not enough for a complete hydrocarbon removal.

The high salinity levels present in the bioreactor (approximately 30 g L⁻¹) did not hinder PW treatment, which shows that A. borkumensis SK2 was fully adapted to these conditions. Therefore, this bacterium can be considered a robust candidate for the treatment of PW, making the process less time consuming and more cost-effective, since no adaptation period to the high salinity levels was required.

Intracellular and extracellular lipids production was detected for all the TPH/N ratios tested (Table 4). Intracellular lipids content was higher than extracellular, suggesting that accumulation inside the cell was promoted in all of the operational conditions.

Higher intracellular lipid contents were obtained when lower TPH/N ratios and higher feast stages durations were applied to the bioreactor. The highest intracellular lipid production (0.85 g g⁻¹ CDW) was achieved in cycle XI when a TPH/N ratio of 10 and a feast stage duration of 5 days were applied. During the SBAR_{Ab-PW} operation the maximum total lipids yield was 1.78 g of lipids produced per g of TPH_{PW} consumed in cycle XI.

For a TPH/N ratio of approximately 10 (0.26 g L⁻¹ TPH_{PW} and 30 mg L⁻¹ of nitrogen), the increase of the feast stage duration from 2 to 5 days (cycles I to III and IX to XI, respectively) led to an increase of approximately 7 times on the intracellular lipids production, increasing the bacterial cells were able to accumulate approximately 45% of its cellular dry weight in lipids (0.45 g g⁻¹ CDW) (cycles from VI to VIII).

The results indicate that intracellular lipids accumulation in A. borkumensis SK2 was dependent on the TPH/N ratio and on the duration of the feast stage, increasing when higher feast stage duration
and lower TPH/N ratio were applied. Although the production of storage compounds is usually associated with unbalanced growth conditions (excess of carbon and limited amount of an essential nutrient, such as nitrogen), the results suggest that accumulation of neutral lipids in *A. borkumensis* SK2 from simple substrates such as pyruvate and hexadecane in CDW. The results obtained in this work showed that from a real oilfield produced water and with a SBAR system *A. borkumensis* SK2 could reach up to 80% of its CDW.

Concerning the treatment of LW in the SBAR inoculated with *R. opacus* B4, the TPH removal efficiency varied between 55% and 79% (Table 5). The highest TPH removal efficiency (79%) was achieved in cycle III, which corresponded to the operational conditions of 1 g L\(^{-1}\) of TPH and 30 mg L\(^{-1}\) of nitrogen (Table 5). The increase of the TPH concentration from 1 to 4.4 g L\(^{-1}\) did not influence LW treatment, since similar TPH removal efficiencies were obtained throughout the process. Therefore, the results suggested that the microbial community was only capable to partially degrade the hydrocarbons present in LW. Lubricant oil is composed by a complex mixture of hydrocarbons and an unresolved complex matrix (Da Silva et al., 2016; Frysinger et al., 2003). This fraction, was reported as the most resistant to biodegradation (Frysinger et al., 2003), and may be the fraction not degraded in both TPH concentrations tested which explains the similar TPH removal efficiencies observed.

During SBAR\(_{R,o,LW}\) operation, higher neutral lipid concentrations were achieved when higher TPH/N ratios were applied (cycles III to VIII) (Table 5). The highest intracellular lipids production attained was 0.94 g g\(^{-1}\) CDW in cycle VI, for a TPH/N ratio of 476. The results showed that the carbon concentration was the most important factor for lipids accumulation in *R. opacus* B4. Indeed, the increase of the TPH concentration from 1 g L\(^{-1}\) to 4.4 g L\(^{-1}\) led to a correspondent increase in the lipid accumulation (approximately 4.4 times higher).

Intracellular lipid content in SBAR\(_{R,o,LW}\) operation was not significantly affected by the feast stage duration. The highest lipid production rate (of 0.34 g L\(^{-1}\) d\(^{-1}\)) and lipid yield (of 0.13 g g\(^{-1}\) PW consumed)

### Table 5

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Nitrogen (mg L(^{-1}))</th>
<th>TPH(_{LW}) (g L(^{-1}))</th>
<th>TPH/N Ratio</th>
<th>Feast stage duration (d)</th>
<th>TPH removal efficiency (%)</th>
<th>Intracellular lipids (g g(^{-1}) of CDW)</th>
<th>Lipids production rate (g L(^{-1}) d(^{-1}))</th>
<th>Lipids Yield (g g(^{-1}) of TPH(_{LW}) consumed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>30</td>
<td>1</td>
<td>61</td>
<td>4</td>
<td>56 ± 1.17</td>
<td>0.13</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>II</td>
<td>30</td>
<td>1</td>
<td>175</td>
<td>5</td>
<td>46 ± 0</td>
<td>0.18</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>III</td>
<td>30</td>
<td>1</td>
<td>3247</td>
<td>7</td>
<td>79 ± 1.49</td>
<td>0.28</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>IV</td>
<td>30</td>
<td>4.4</td>
<td>861</td>
<td>2</td>
<td>n.d</td>
<td>0.84</td>
<td>0.06</td>
<td>n.d</td>
</tr>
<tr>
<td>V</td>
<td>30</td>
<td>4.4</td>
<td>809</td>
<td>2</td>
<td>n.d</td>
<td>0.84</td>
<td>0.06</td>
<td>n.d</td>
</tr>
<tr>
<td>VI</td>
<td>30</td>
<td>4.4</td>
<td>476</td>
<td>4</td>
<td>71 ± 0.6</td>
<td>0.94</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>VII</td>
<td>30</td>
<td>4.4</td>
<td>815</td>
<td>2</td>
<td>55 ± 2.1</td>
<td>0.92</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>VIII</td>
<td>15</td>
<td>4.4</td>
<td>2821</td>
<td>1</td>
<td>57 ± 3.8</td>
<td>0.87</td>
<td>0.34</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Correspond to the real TPH/N ratio applied to the bioreactor at the start of the feast stage. This ratio was calculated by dividing the TPH concentration added to the bioreactor at the start of the feast stage by the nitrogen concentration inside of the bioreactor at that moment.
were obtained in the operational conditions of 4.4 g L\(^{-1}\) TPH\(_{\text{PW}}\) and 15 mg L\(^{-1}\) of nitrogen and shorter feast stage duration (1 day in cycle VIII). A similar outcome was observed for the operational condition of 4.4 g L\(^{-1}\) TPH\(_{\text{PW}}\) and 30 mg L\(^{-1}\) of nitrogen. A high lipid production rate of 0.23 g L\(^{-1}\) d\(^{-1}\) was achieved once again in the cycle with the lower feast stage duration (2 days in cycle V).

The SBAR\(_{\text{R,o}+\text{LW}}\) results showed that TPH/N ratio was the most relevant parameter in neutral lipid accumulation, which is in accordance with the results reported by Castro et al. (2018).

The obtained results showed the suitability of the SBAR design for the aerobic biological treatment of different types of mineral oil-based wastewaters and its valorization for the production of neutral lipids. SBAR systems are commonly used for the removal of nutrients and/or toxic compounds from several wastewaters, including saline wastewaters (Li et al., 2017; Nguyen et al., 2016). From the authors’ knowledge, this is the first time that the feasibility of a SBAR system for an efficient aerobic biological treatment of different types of mineral oil-wastewaters coupled with neutral lipids production is reported.

### 3.2. Neutral lipids identification by ATR-FTIR

The lipid extracts obtained from both reactors were analyzed by ATR-FTIR, in order to identify the different types of lipids produced during the biological treatment of PW and LW. Several studies have reported the feasibility of this technique for the determination of the lipid content of the obtained results showed the suitability of the SBAR design for the aerobic biological treatment of different types of mineral oil-based wastewaters and its valorization for the production of neutral lipids. SBAR systems are commonly used for the removal of nutrients and/or toxic compounds from several wastewaters, including saline wastewaters (Li et al., 2017; Nguyen et al., 2016). From the authors’ knowledge, this is the first time that the feasibility of a SBAR system for an efficient aerobic biological treatment of different types of mineral oil-wastewaters coupled with neutral lipids production is reported.

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#### Table 6

Assignment of the bands found in ATR-FTIR spectrum of extracellular and intracellular lipids extracts of SBAR\(_{\text{Ab}+\text{PW}}\) and SBAR\(_{\text{R,o}+\text{LW}}\) operations according to literature.

<table>
<thead>
<tr>
<th>Wavenumber range (cm(^{-1}))</th>
<th>Band assignment from the literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>2955</td>
<td>stretching vibration of acyl chains (v_{\text{as}}(\text{CH}_2))</td>
</tr>
<tr>
<td>2920</td>
<td>stretching vibration of asymmetric acyl chains (v_{\text{as}}(\text{CH}_2))</td>
</tr>
<tr>
<td>2850</td>
<td>stretching vibration of symmetric acyl chains (s(\text{CH}_2))</td>
</tr>
<tr>
<td>1708</td>
<td>stretching vibration of the ester group (\nu(\text{C} \equiv \text{O})) in fatty acids</td>
</tr>
<tr>
<td>1740</td>
<td>stretching vibration of the ester group (\nu(\text{C} \equiv \text{O})) in TAGs</td>
</tr>
<tr>
<td>1460</td>
<td>asymmetric bending vibration of acyl chains (\delta_{\text{as}}(\text{CH}_2))</td>
</tr>
<tr>
<td>1370</td>
<td>symmetric bending vibration of acyl chains (\delta_{\text{ss}}(\text{CH}_2))</td>
</tr>
<tr>
<td>1300–1100</td>
<td>stretching vibration of esters (\nu(\text{C} \equiv \text{O}))</td>
</tr>
<tr>
<td>720</td>
<td>methylene rocking vibration (\rho(\text{CH}_2))</td>
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</table>

**Fig. 2.** ATR-FTIR spectrum of the lipids produced by: (A) A. borkumensis SK2 and (B) R. opacus B4 during SBAR\(_{\text{Ab}+\text{PW}}\) and SBAR\(_{\text{R,o}+\text{LW}}\) operations, respectively.

and SBAR\(_{\text{R,o}+\text{LW}}\) revealed characteristic absorption bands around 2955 cm\(^{-1}\), 2925 cm\(^{-1}\) and 2850 cm\(^{-1}\), assigned to asymmetric \(\nu_{\text{as}}(\text{CH}_2)\), asymmetric \(\nu_{\text{as}}(\text{CH}_2)\) and symmetric \(s(\text{CH}_2)\) stretching vibrations of acyl chains from fatty acids present in TAGs, respectively (Shapaval et al., 2019); and a band at 1745 cm\(^{-1}\), attributed to the stretching vibration of the ester group \(\nu(\text{C} \equiv \text{O})\) in the carbonyl group of TAG (Fig. 2) (Esther Elizabeth Grace et al., 2020). This peak is a characteristic vibration representative of the presence of TAG (Esther Elizabeth Grace et al., 2020), suggesting that this type of neutral lipids was the most predominant type accumulated intracellularly by both microorganisms.

In the intracellular lipid spectrum of SBAR\(_{\text{R,o}+\text{LW}}\) and in the extracellular lipid spectrum of SBAR\(_{\text{Ab}+\text{PW}}\) a band at 1710 cm\(^{-1}\) corresponding to the stretching in esters \(\nu(\text{C} \equiv \text{O})\) was detected which can indicate the presence of free fatty acids (Shapaval et al., 2019).

The extracellular lipid spectrum profile of SBAR\(_{\text{Ab}+\text{PW}}\) showed characteristic absorption bands at approximately 2955 cm\(^{-1}\) \(\nu_{\text{as}}(\text{CH}_2)\), 2925 cm\(^{-1}\) \(\nu_{\text{as}}(\text{CH}_2)\) and 2850 cm\(^{-1}\) \(s(\text{CH}_2)\); additional peaks in the region between 1300 and 1100 cm\(^{-1}\) which are related to the stretching...
vibration of esters \( \nu(C=O) \), as well as the presence of a band at 1700 cm\(^{-1}\), typical of the absorption frequency for the carbonyl group in aliphatic esters \( \nu(C=O) \). This result suggests the extracellular production of WE by \textit{A. borkumensis} SK2 (Dubis et al., 1999; Forfang et al., 2017).

Other absorption bands characteristic of lipids were detected in the extracellular and intracellular spectrums of \textit{SBAR}_{Ab-PW} and \textit{SBAR}_{Ab-LW}. An absorption band at approximately 720 cm\(^{-1}\) that corresponds to the methylene rocking vibration \( \rho(CH_2) \), and two bands near 1460 cm\(^{-1}\) and 1380 cm\(^{-1}\), corresponding to the asymmetric \( \delta(CH_2) \) and symmetric \( \delta(CH_3) \) bending vibration of acyl chains, respectively (Forfang et al., 2017).

The ATR-FTIR spectra were in accordance with the neutral lipid analysis using TLC (Fig. 3S and 4S). Bands correspondent to TAG and FA were detected in the intracellular lipids fraction of \textit{SBAR}_{Ab-PW}, indicating that TAG was the dominant neutral lipid accumulated. Bands correspondent to WE were not observed (Fig. 3A). On the other hand, in the extracellular lipid profile a band correspondent to WE was detected in cycles VI, VII, IX and XI, confirming the ATR-FTIR results (Fig. 3S (B) and Table 4). Bands with very low intensity correspondent to TAG production were detected but only in cycles VI and IX (Fig. 3S (B)). In cycle VIII, the presence of the band correspondent to WE was not detected, which explains the absence of the absorption bands in the range of 1300–1100 cm\(^{-1}\) related to \( \nu(C-O) \) (data not shown).

TLC neutral lipid profile from \textit{SBAR}_{Ab-LW} also confirmed the presence of TAG and FA in the intracellular lipid extract from cycle VI (Fig. 4). Moreover, the TLC analysis showed that TAG and FA were produced in all cycles, with more intense bands in the cycles IV, V and VI, which corresponded to the operational conditions with higher lipid accumulation (4.4 g L\(^{-1}\) of LW and 30 mg L\(^{-1}\) of nitrogen) (Fig. 4S and Table 5).

During PW treatment, \textit{A. borkumensis} SK2 was able to produce WE and TAG. However, WE was detected in the extracellular fraction while TAG where predominantly detected in the intracellular content, which is in accordance with Sudmalis et al. (2018). The ability of \textit{Acanthovax} genus to produce neutral lipids such as TAG and WE, both intra- and extracellularly, from hexadecane, under unbalanced growth conditions, is reported in literature (Manilla-Pérez et al., 2011, 2010a). The genes \textit{atfA1} and \textit{atfA2} that encode for a homologous wax ester synthase/acyl coenzyme A: diacylglycerol acyltransferase (WS/DGAT) were detected in the genome of \textit{A. borkumensis} SK2, which is responsible for the synthesis of WE and TAG (Kalscheuer et al., 2007).

TAG production by \textit{A. borkumensis} SK2 was reported to be related to nitrogen limiting conditions while WE-like compounds were growth-linked (nitrogen limiting-independent) when cultivated in a bioreactor under controlled batch conditions (Da Silva et al., 2016). This explains the higher neutral lipids productions obtained in the present study when higher nitrogen concentrations were applied to the bioreactor.

During \textit{SBAR}_{Ab-LW} operation, \textit{R. opacus} B4 was able to intracellularly produce TAG, however WE production was not observed (Fig. S3). TLC of the intracellular lipids of both \textit{SBAR}_{Ab-PW} and \textit{SBAR}_{Ab-LW} showed additional bands correspondent to TAG precursors, such as acylglycerols (MAG), diacylglycerols (DAG) and fatty acids (FA) (Fig. S3 and S4).

The results obtained in this study indicate that bacterial neutral lipid profiles and its production depends on the characteristics of the mineral oil-wastewater used as carbon source (i.e hydrocarbons profile and the salinity levels), and the cellular machinery of the bacterial species present in the bacterial community.

### 3.3. Bacterial community development during PW and LW treatment

Since both \textit{SBAR}_{Ab-PW} and \textit{SBAR}_{Ab-LW} were operated under non-sterile conditions and LW and PW may have its own microbial community, it is likely that \textit{A. borkumensis} SK2 and \textit{R. opacus} B4 were not the only microorganisms inside the bioreactor. In \textit{SBAR}_{Ab-PW}, 16S rRNA gene sequencing analysis of samples collected at cycle VIII (15 mg L\(^{-1}\) of nitrogen) and XI (30 mg L\(^{-1}\) of nitrogen) showed that \textit{Acanthovax} sp. was the most dominant microorganism within the bioreactor throughout the operation process. \textit{Acanthovax} sp. relative abundance represented approximately 80% and 76% of the total bacterial community in cycle VIII and XI, respectively (Fig. 3). As described by Sudmalis et al. (2018) and Kasai et al. (2002), \textit{Acanthovax} sp. metabolism is fully adapted to \textit{n}-alkanes degradation and is known that \textit{Acanthovax} genus becomes dominant in the microbial community of petroleum-contaminated seawaters when nitrogen and phosphorus are supplied in adequate amounts (Kasai et al., 2002). Under these conditions, \textit{Acanthovax} sp. relative abundance can reach approximately 80–90% of the total oil-degrading microbial community (Martins dos Santos et al., 2010). Therefore, it is expected that \textit{Acanthovax} sp. remains the most dominant microorganism within the bioreactor.

\textit{Achromobacter} genus was also detected in the bacterial community with a relative abundance of 5% and 9% in cycles VIII and XI, respectively (Fig. 3). Members of \textit{Achromobacter} genus were described as capable of aliphatic and aromatic hydrocarbons degradation and also as biosurfactant producers (Gielnik et al., 2019; Joy et al., 2017). Moreover, in the presence of crude oil some \textit{Achromobacter} sp. produced a glycolipid with biosurfactant activity (Joy et al., 2017). Nevertheless, the high abundance of \textit{A. borkumensis} SK2 in the microbial community during bioreactor operation suggests that this bacterium is a key player during PW treatment in SBAR.

Regarding the \textit{SBAR}_{Ab-LW}, the 16S rRNA gene sequencing analysis showed that \textit{Pseudomonas} sp. and \textit{Rhodococcus} sp. were the most dominant microorganisms with a relative abundance of 42.8% and 20.6% in cycle II and 8.3% and 9.6% in cycle VI, respectively (Fig. 4). \textit{Pseudomonas} sp. have been reported as able to degrade aromatic and/or aliphatic hydrocarbons and also to be the most dominant microorganism in environments with high concentrations of \textit{n}-alkanes and cycloalkanes (Muriel-Millan et al., 2019). \textit{Pseudomonas} sp. is known to mainly accumulate polyhydroxalkanoates as reserve materials of carbon and energy (Mozejko-Gieleska et al., 2019).

Several aerobic bacterial genera reported in literature capable of hydrocarbons degradation were detected in the microbial community develop throughout the reactor operation (Fig. 4). The most represented genera, with relative abundances higher than 5% were \textit{Ochrobactrum}, \textit{Sphingobacterium} and \textit{Novosphingobium}. These bacterial genera have preferentially degraded aromatic hydrocarbons, particularly polycyclic
relative abundance %
100%
10%
30%
60%
90%
0%  
Relative abundance %

Fig. 4. Bacterial community composition during bioreactor inoculated with R. opacus B4 treating LW (SBARS1a-LW). Only bacterial families and genera with relative abundance above 1% were represented.

aromatic hydrocarbons (PAHs) that are commonly used for the manufacture of lubricating oils (Abou-shanab, 2015; Kertesz and Kawasaki, 2010; Satti et al., 2019). The lubricant wastewater used as carbon source in this study can explain the development of a bacterial community specialized in the biodegradation of these hydrocarbons. Due to n-alkanes low solubility and reactivity, and the complexity of PAHs, the biodegradation of these compounds represents a challenge to bacteria. Therefore, the development of bacterial communities in this work, able to degrade these compounds is an advantage for the treatment of both mineral oil-wastewaters used (PW and LW).

Although the microbial community analysis showed the presence of several bacterial genera able to degrade hydrocarbons, the ability for neutral lipids accumulation was only assigned to the Alcanivorax and Rhodococcus genera.

4. Conclusion

This work demonstrated the feasibility of a SBAR for the treatment of PW and LW and its valorization for neutral lipids production by A. borkumensis SK2 and R. opacus B4, respectively.

The results obtained suggest that a specialized inoculum needs to be used according to the mineral oil-wastewater characteristics. Moreover, the results demonstrated that the neutral lipid profile and its production is influenced by the inoculum and the operational conditions applied to the SBAR. Particularly, the nitrogen concentration is a determinant parameter for neutral lipids production by A. borkumensis SK2 from saline hydrocarbon wastewaters. For non-saline wastewaters, R. opacus B4 is a suitable candidate and the carbon source concentration is the most important factor for neutral lipids accumulation. TAGs were the main neutral lipids accumulated by R. opacus B4. In A. borkumensis SK2, WE production was mostly extracellular whilst TAG production was intracellular. This is an interesting result, since the different types of neutral lipids can be used for different industrial applications, in order to make the biological treatment of mineral oil-wastewaters more sustainable. In sum, this work shows that different operation strategies need to be applied depending on the type of mineral oil-wastewater and highlights the relevance of the bacterial lipid location (intracellular or extracellular) in defining the most suitable operational strategy to maximize neutral lipids production.

CRediT authorship contribution statement

All authors contributed intellectually via scientific discussions during the work and have been involved in drafting the manuscript and revising it critically. Silva RM helped to design the experiments, performed the experimental work, analysed and interpreted the data and wrote the manuscript. Fernandes AM and Fiume F performed the experimental work related to LW treatment. Castro AR helped with TPH and lipids analyses. Machado R helped with ATR-FTIR analysis and data interpretation. Pereira MA designed the experiments, supervised all the experimental work, contributed to data interpretation and reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1101/j.hazmat.2020.124492.

References


