

# Biological treatment of produced water coupled with recovery of neutral lipids

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## ARTICLE INFO

### Article history:

Received 1 June 2018

Received in revised form

30 August 2018

Accepted 20 September 2018

Available online 27 September 2018

### Keywords:

Produced water

Biological treatment

Alkane biodegradation

Neutral lipids production

## ABSTRACT

Produced water (PW) is the largest waste stream generated by oil and gas industry. It is commonly treated by physical-chemical processes due to high salt content and poor biodegradability of water insoluble compounds, such as n-alkanes. N-alkanes can represent a major fraction of organic contaminants within PW. In this study the possibility of simultaneous n-alkane biodegradation and production of neutral lipids in a concentrated PW stream with *A. borkumensis* SK2 as the sole reactor inoculum was investigated. N-alkane removal efficiency up to 99.6%, with influent alkane COD of 7.4 g/L, was achieved in a continuously operated reactor system. Gas chromatography results also showed that the majority of other non-polar compounds present in the PW were biodegraded. Biodegradation of n-alkanes was accompanied by simultaneous production of neutral lipids, mostly wax ester (WE)-like compounds. We demonstrate, that under nutrient limited conditions and 108.9 ± 3.3 mg/L residual n-alkane concentration the accumulation of extracellular WE-like compounds can be up to 12 times higher compared to intracellular, reaching 3.08 grams per litre of reactor volume (g/L<sub>reactor</sub>) extracellularly and 0.28 g/L<sub>reactor</sub> intracellularly. With residual n-alkane concentration of 311.5 ± 34.2 mg/L accumulation of extracellular and intracellular WE-like compounds can reach up to 6.15 and 0.91 g/L<sub>reactor</sub>, respectively. To the best of our knowledge simultaneous PW treatment coupled with production of neutral lipids has never been demonstrated before.

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## 1. Introduction

Water co-produced with oil and gas is referred to as produced water (PW) (Cakmakce et al., 2008). It is a complex mixture of organic and inorganic compounds (Tellez et al., 2002; Fakhru'l-Razi et al., 2009) with varying composition depending on the reservoir characteristics, operation strategy, production process, geological structure of the formation, lifetime of reservoirs, chemicals used in production facilities (Ozgun et al., 2013a, 2013b; Rehman et al., 2018) and has been reported as the largest waste stream in oil and gas industry (Fakhru'l-Razi et al., 2009).

Contaminants, such as organics and salts, within PW have negative effects on the environment upon direct discharge. Hence, several organisations (e.g., OSPAR, USEPA) and countries (e.g., Venezuela, People's Republic of China, Australia) have set stringent

discharge rules with respect to both salinity and organic content of treated PW (Díaz et al., 2000; Rincón et al., 2003; Tellez et al., 2005; Lefebvre and Moletta, 2006; Fakhru'l-Razi et al., 2009; Igunnu and Chen, 2012). Due to the stringent discharge limits for PW many of the oil and gas wells in the U.S. were reported to reach their economic limit, since production, maintenance, treatment and disposal costs were higher than the selling price in 2005 (Tellez et al., 2005), while the selling price in the early 2000s was high (Millard et al., 2017). Since historically substitutes for oil exist, its price may decrease rapidly (Millard et al., 2017). For example, the price of crude oil dropped by more than a half from July 2014 to January 2015 (Millard et al., 2017), meaning that the costs of extraction and processing should be minimised.

Conventionally PW is treated by a combination of several physical and chemical processes, such as coagulation, hydrocyclones, flotation units and membrane processes, but they are expensive to apply due to high start-up and running costs (Lefebvre and Moletta, 2006; Cakmakce et al., 2008; Knutsen, 2011; Ozgun et al., 2013a). Moreover, chemical processes can produce

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hazardous sludge (Knutsen, 2011). Another drawback of current physical/chemical treatment technologies of PW is their inability to remove small suspended oil particles and dissolved organics (Lefebvre and Moletta, 2006; Fakhru'l-Razi et al., 2009; Dong et al., 2011).

Space limitations offshore do not allow for implementation of large bioreactors. However, onshore it has been proposed to incorporate biological removal of organic contaminants from PW because of cost-effectiveness of biological compared to physical/chemical treatment (Tellez et al., 2005; Lefebvre and Moletta, 2006; Fakhru'l-Razi et al., 2009; Lu et al., 2009; Fakhru'l-Razi et al., 2010). PW is often a highly saline stream (Díaz et al., 2000; Freire et al., 2001; Lefebvre and Moletta, 2006; Fakhru'l-Razi et al., 2009) and around 90% of total hydrocarbons within the stream are dispersed oil-alkanes (Tellez et al., 2002). The salinity of PW introduces microorganisms to a challenge of dealing with osmotic stress, if biological treatment is applied. The low solubility of hydrocarbon substrates makes them difficult to biodegrade, because all known hydrocarbon-oxidizing enzymes are cell-bound (Tellez et al., 2002). Hydrocarbons with chain lengths above C12 are almost insoluble in water and bacteria capable of degrading them need to produce biosurfactants either to attach to the substrate or to solubilize it for uptake (Singh et al., 2012). These molecules are also chemically very inert and need to be activated by specific enzymes for biological conversion (Singh et al., 2012).

Anaerobic biological treatment has several advantages over aerobic biological treatment, such as net energy recovery, reduced surplus sludge production and possibility to apply higher loading rates (Xiao and Roberts, 2010). Some researchers have demonstrated the efficiency of, for example, addition of anaerobic zone to waste stabilization ponds for removal of heavy metals, oil and grease (Shpiner et al., 2009a, 2009b) or application of anaerobic baffled reactors for removal of oil and suspended solids (Su et al., 2007) from PW. Despite the potential advantages of anaerobic biological treatment over aerobic, thus far most researchers have been focusing on aerobic biological treatment of PW (Su et al., 2007; Lu et al., 2009; Fakhru'l-Razi et al., 2010; Dong et al., 2011; Mancini et al., 2012). The research focus on aerobic treatment of PW is mainly due to high sensitivity of anaerobic biomass towards osmotic stress and other potentially toxic compounds within PW (Khong et al., 2012). In addition, anaerobic degradation of alkanes and other petroleum hydrocarbons proceeds at much slower rate and is less efficient than degradation under aerobic conditions (Heider et al., 1998; Berdugo-Clavijo and Gieg, 2014). Application of pure cultures of specialised marine hydrocarbonoclastic microorganisms as inoculum in bioreactors has not been a widely recognised approach for PW treatment, neither in aerobic nor in anaerobic biological treatment.

*Alcanivorax borkumensis* SK2 is a marine hydrocarbonoclastic bacterium with a highly specialised metabolism for n-alkane degradation (Kasai et al., 2002; Head et al., 2006; Schneiker et al., 2006; Cappello and Yakimov, 2010). One of its peculiar features is the ability to produce intracellular and extracellular neutral lipids in the form of wax esters (WE) and triacylglycerols (TAG) upon exposure to abundance of hydrocarbons (Kalscheuer et al., 2007; Manilla-Pérez et al., 2011). Alvarez (2010) reported an increasing interest in bacterial production of WE and TAG due to increasing global demand for fats, oils and WE. Industrially TAG can be used for production of, for example, personal care products, plastics, detergents, soaps, resins and lubricants. Wax esters can be used in production of lubricants, surface coatings and cosmetics (Alvarez, 2010). The unique features of *A. borkumensis* SK2 can potentially allow for aerobic biological treatment of PW coupled with recovery of valuable compounds (WE and TAG).

In this study we explore the applicability of *A. borkumensis* SK2

as inoculum for biological treatment of oily part of PW obtained from a full-scale physical separation process and investigate the conditions allowing for simultaneous production of intracellular and extracellular neutral lipids. This contribution is not aiming to give detailed mass balances with respect to production of neutral lipids, but is rather aiming to demonstrate the concept with real PW for the first time. We demonstrate efficient (more than 90%) n-alkane biodegradation both in batch and continuous experiments, biodegradation of other non-polar compounds as well as nutrient non-limited production of intracellular and extracellular neutral lipids.

## 2. Materials and methods

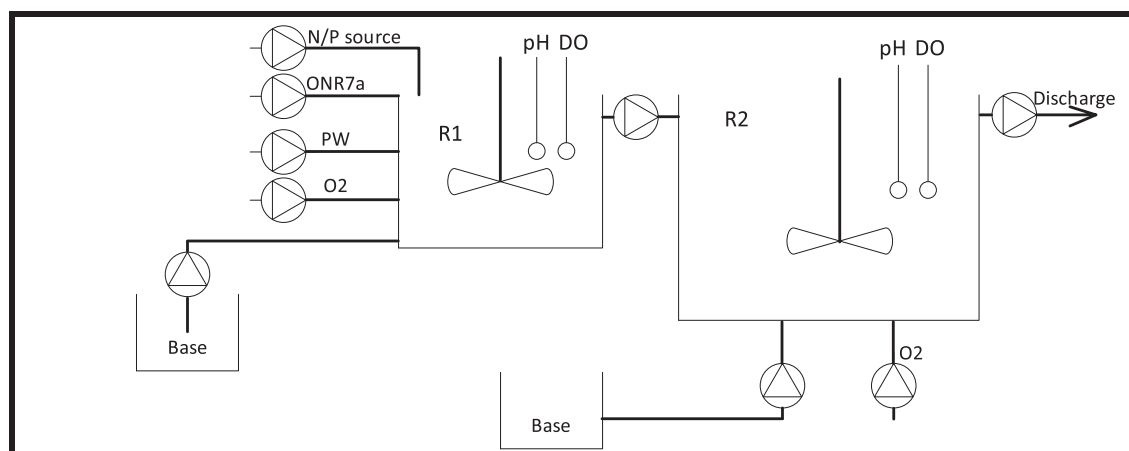
### 2.1. Reactor system, inoculum and produced water

Two continuously stirred tank reactors (CSTRs) without sludge retention connected in series were inoculated with *A. borkumensis* SK2 (DSM number 11573). The culture was previously adapted to the produced water concentrate as the sole carbon and energy source in batch experiments using saline medium (ONR7a) prepared according to DMSZ, Braunschweig, Germany. A simplified reactor scheme is shown in Fig. 1. Reactor 1 (R1) with a working volume of 2 L was operated at 28 °C, 40% oxygen saturation, pH of 7.5 and with nitrogen non-limited conditions. The detection of undissolved matter, such as microbial cells, emulsified alkanes and hydrophobic matter produced by bacteria, was measured in real-time as optical density (OD<sub>600</sub>) at the near-infrared region by a photometric probe. Full details on the control and monitoring system can be found in Da Silva et al. (2016). Reactor 2 (R2) with a working volume of 4 L was operated at room temperature (19–21 °C) under oxygen depleted and saturated conditions, pH of 7.5 and nitrogen limiting conditions. Oxygen depleted and saturated conditions were studied, because dissolved oxygen concentration of 2%–100% has been reported to be suitable for WE production in other hydrocarbonoclastic bacteria, such as *Fundibacter jadensis* T9 (Bredemeier et al., 2003). If this is true for *A. borkumensis* SK2, it allows to optimize the aeration costs for production of neutral lipids.

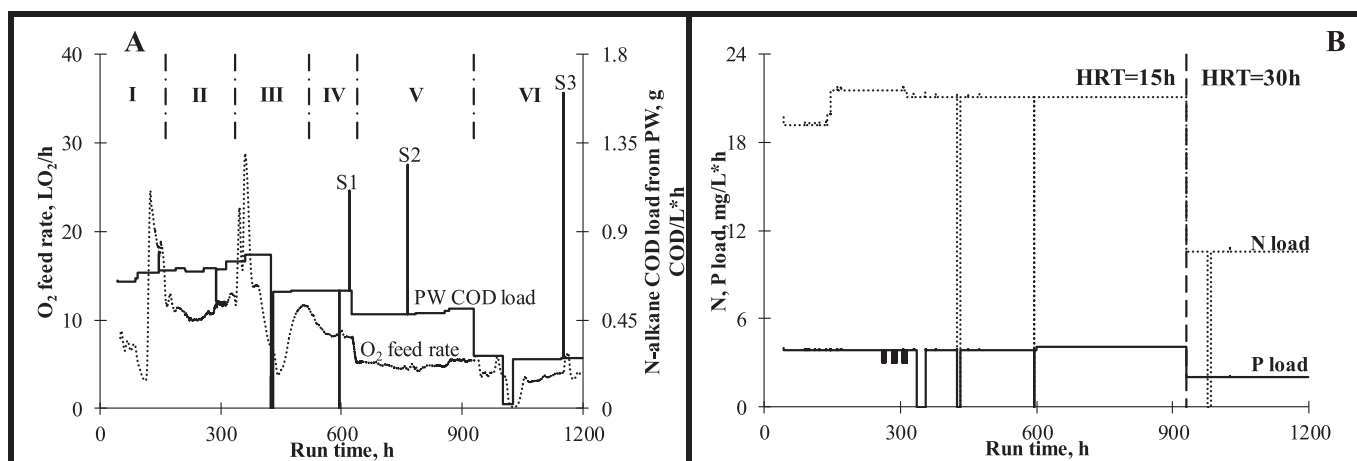
Mixing of R1 was achieved by a metal propeller with a diameter of 4 cm at 900 rpm, whereas mixing of R2 was achieved by two metal propellers attached to one shaft at 500 rpm.

Concentrated oily part of PW from a full-scale physical separation process was obtained from Shell (The Netherlands) and fed to the reactor system as a separate stream. The total C<sub>10</sub>–C<sub>33</sub> n-alkane concentration of the PW concentrate was determined as described below and was 98 g/L, which accounts for a COD of 339.2 g COD/L. Detailed analyses of n-alkanes in PW are provided in Table S1. The PW fed to R1 was mixed with ONR7a medium and phosphate source. The ONR7a medium was modified for nitrogen concentration of 334 mg/L and phosphorous concentration in the phosphorous solution source was adjusted to reach influent concentration of 62 mg/L.

In order to have sufficient amount of iron for microbial growth, FeCl<sub>2</sub>·4H<sub>2</sub>O concentration was adjusted from 0.00001 M in the original recipe to 0.00021 M after the first 112 h of continuous R1 operation in this study. The increase of iron concentration was found to be necessary after observing decreasing oxygen feed rate in R1 with the 40% oxygen saturation as the set-point, indicating decreasing microbial activity in the reactor (Fig. 2). Oxygen uptake rate is defined here as oxygen flow measured with ez-Control device from Applikon Biotechnology, Inc. (Delft, The Netherlands) to maintain the set-point of 40% oxygen saturation in the reactor.



**Fig. 1.** Scheme of reactor set-up. R1 – CSTR with a working volume of 2 L operated at nitrogen and phosphorous non-limiting conditions, R2 – CSTR with a working volume of 4 L operated at nitrogen and/or phosphorous limited conditions. Base – 3M NaOH solution, O2 – oxygen, PW – concentrated produced water (Shell, Netherlands), ONR7a – nutrient medium, N/P source –  $\text{NH}_4\text{Cl}$  and  $\text{Na}_2\text{HPO}_4$  solution, pH – pH sensor, DO – dissolved oxygen sensor.



**Fig. 2.** Continuous operation of R1. A – moving average of oxygen uptake rate and produced water COD load. Vertical dotted lines separate different periods within R1. Periods II, IV, V and VI were studied with respect to n – alkanes removal. S1, S2, and S3 are time points at which additional PW was spiked directly in the reactor to see, if carbon source is limiting and period VI was found carbon limiting. B - applied nitrogen and phosphorous load. Vertical line shows increase of HRT in R1 to 30 h. Effluent of R1 was directly pumped to R2 without additional dosing of nutrients or carbon source.

## 2.2. Reactor operation

Two experiments were performed during this study. In the first experiment, the performance of R1 in a controlled batch operation was studied in terms of n-alkane removal to evaluate if efficient n-alkane biodegradation can be achieved within the complex PW matrix with the chosen inoculum. The batch experiment lasted for 54 h. *A. borkumensis* SK2 was inoculated in ONR7a medium containing 238 mg/L  $\text{NH}_4\text{-N}$ , 34 mg/L  $\text{PO}_4\text{-P}$  and 23.5 mL (19.8 g) of PW. The resulting n-alkane COD:N:P ratio at the beginning of the batch was 234.4:7:1. The inoculum was prepared as described above.

During the second experiment R1 and R2 were operated continuously in series and performance of both reactors was studied in terms of n-alkane biodegradation and production of neutral lipids. Concentrated stream of oily PW, ONR7a medium and phosphorous source were fed as separate streams to R1, resulting in influent concentrations of 334.9 and 62 mg/L for  $\text{NH}_4\text{-N}$  and  $\text{PO}_4\text{-P}$ , respectively. The flow rate of PW was reduced in steps resulting in influent n-alkane COD concentrations of  $10305 \pm 66$ ,  $8766 \pm 40$  and  $7205 \pm 160$  mg/L and a HRT in R1 of  $14.9 \pm 0.09$  h. Finally, the HRT in R1 was decreased down to  $28.9 \pm 0.18$  h, while keeping the influent  $\text{NH}_4\text{-N}$  and  $\text{PO}_4\text{-P}$  concentration constant and n-alkane COD influent

concentration of  $7411 \pm 197$  mg/L. The resulting N, P and COD loads are depicted in Fig. 2-A,B. The nitrogen and phosphorous load was kept such in order to achieve carbon limiting conditions within R1. Before decreasing the loading rate, PW was spiked into the reactor to assess carbon limiting conditions. A responsive increase in oxygen uptake rate indicates the previous carbon limiting conditions. The entire experiment was divided into six periods, shown in Fig. 2-A. Periods II, IV, V and VI were periods with stable oxygen uptake rate for at least 3 hydraulic retention times. The removal of n-alkanes and production of neutral lipids was studied during these stable periods. Only period VI was carbon source limited, since spiking of additional carbon source induced an immediate increase of the oxygen uptake rate from 4 to 6.24 L/h (Fig. 2-A). The phosphorous and nitrogen loading rates applied in R1 are depicted in Fig. 2-B. No additional dosing of nutrients was performed in R2.

## 2.3. Analytical methods

### 2.3.1. Ammonium nitrogen, total particulate nitrogen and phosphorous

Ammonium nitrogen in the reactors was measured on daily bases in triplicate for R1 and in duplicate for R2. Two mL samples

were taken directly from the reactors, and immediately filtered through 0.45 µm filters. Further analysis was performed with LCK 302, LCK 303 or LCK 304 (HACH GMBH, Germany) ammonium kits. The relative standard deviation for ammonium nitrogen analysis in triplicate was up to 28% in the concentration range below 1 mg NH<sub>4</sub>-N/L and up to 8% in the higher concentration range. The deviation range for duplicate samples of R2 was up to 28% in the lower range and up to 4% above 1 mg/L NH<sub>4</sub>-N. Biomass concentration in the bioreactors was measured as total particulate nitrogen (TPN) (Da Silva et al., 2016). Samples taken from bioreactors were centrifuged at 20,350 xg for 5 min and washed with PBS 1x for 3 times. Finally the washed pellet was re-suspended in milli-Q and total nitrogen was measured with LCK 338 kit (HACH GMBH, Germany). The relative standard deviation for TPN measurements in triplicate was 2–19% in the TN range of 20–40 mg N/L and 4–16% in the higher range.

Phosphorous concentration in the bioreactors was detected with ICP elementary analysis. Sampling was performed once a day and measurements were made in triplicate for R1 and in duplicate for R2. Samples were filtered through 0.45 µm filters, diluted with deionised water to result in TDS levels smaller than 1 g/L and acidified to result in 2% nitric acid content within the samples. These samples were analysed with Perkin Elmer Optima 5300 DV ICP apparatus equipped with Perkin Elmer software package WinLab 32 3.3.1.0210. The relative standard deviation of triplicate measurements in R1 ranged from 1 to 14% throughout the operational period with only one exception, where 27% deviation was measured. The absolute deviation for duplicate measurements in R2 ranged from 1 to 13%.

### 2.3.2. Extraction and analysis of hydrocarbons

Two times 15 mL samples for hydrocarbon analysis were collected on daily bases. Analysis was performed on samples taken during the stable operation periods II, IV, V and VI. Extraction of hydrocarbons and analysis of C<sub>10</sub>–C<sub>33</sub> n-alkanes was done as described in Da Silva et al. (2016). Extractions were performed on mixed liquor samples, including the volatile suspended solids, therefore the effluent n-alkane concentration adsorbed to microbial cells was taken into account to measure the total n-alkanes in the effluent. The chromatograms and data for hydrocarbon quantification were obtained with Galaxie software version 1.9.302.530. In the sequence of sample injection also standard solutions of C<sub>8</sub>–C<sub>20</sub> and C<sub>21</sub>–C<sub>40</sub> with 20 and 40 ppm of each compound as well as standard solutions of C<sub>10</sub>, C<sub>20</sub>, C<sub>30</sub> and C<sub>40</sub> with concentrations from 25 to 200 ppm were injected. The quantification of hydrocarbons was done based on area for C<sub>8</sub>–C<sub>20</sub> and based on response factor (area divided by theoretical concentration in the standards) for C<sub>21</sub>–C<sub>40</sub>. Relative standard deviations for n-alkane analysis in raw PW concentrate are shown in Table S1. The deviation range was 1–14% with higher variation in the range C<sub>10</sub> to C<sub>14</sub>. In the effluent samples the relative standard deviations between duplicate measurements of C<sub>10</sub> to C<sub>12</sub> ranged between 0.03 up to 141%. For C<sub>13</sub> to C<sub>29</sub> the deviation range was between 0.04 and 26.4% and for C<sub>30</sub> to C<sub>33</sub> between 1.2 and 141.4%.

### 2.3.3. Extraction and thin layer chromatography (TLC) of neutral lipids

Twenty five mL duplicate samples for extraction and analysis of neutral lipids were taken on daily bases. Analysis was performed on samples taken during the stable operation periods II, IV, V and VI. Samples were taken directly from R2, whereas composite effluent samples were collected from R1 to avoid interference with the process performance.

Solid and liquid phase of the reactor samples were separated by centrifugation for 10 min at 15,000 xg and 10 °C by a centrifuge

Avanti J25, Beckman Coulter. Both phases were freeze-dried and the extraction of non-polar compounds was done by use of Chloroform:Methanol (3:1, v/v) solution. The extraction of neutral lipids was adapted from Folch et al. (1957), whereas the removal of trace polar compounds was achieved with polarity-based fractionation on SPE columns (LC - Si SPE tubes 6 ml/1 g). The fractionation process was adapted from Revellame et al. (2012) as described in Da Silva et al. (2016).

The fractions obtained were analysed with thin layer chromatography (TLC). Silica gel plates (60 F<sub>254</sub> 10 × 20 cm by Merck KGaA) were used to perform TLC with n-hexane/diethyl-ether/acetic acid (80:20:1, v/v/v) serving as mobile phase. Lipids were highlighted on 8 × 2 cm elution area by staining with iodine vapour (Silva et al., 2010). For recognition of different non-polar compounds oleic acid (fatty acids), oleyl oleate, methyl hexanoate, methyl linoleate, methyl oleate (WEs), glyceryl tri-oleate, glyceryl trihexanoate, glyceryl trilinoleate (TAGs), glyceryl di-oleate (DAGs), glyceryl mono-oleate (MAGs) were used as references. Each fraction was re-suspended in 250 µl of chloroform and 5 µl were used for TLC analysis. Images of TLC plates were processed with ImajeJ software by converting them to 8 bit greyscale and increasing the contrast by 1% of saturated pixels.

### 2.3.4. Quantification of TAGs, WEs and fatty acids (FAs)

Each class of neutral lipids was scraped off from the TLC plate, transferred into a Pasteur pipet containing glass wool and eluted from the silica for three times with 0.5 mL of chloroform (Santala et al., 2011). The extracts were dried with gentle flow of nitrogen and their weight was measured daily till constant value. The quantification was done in one replicate for each sampling time.

### 2.3.5. DNA extraction and 16S rRNA gene sequencing

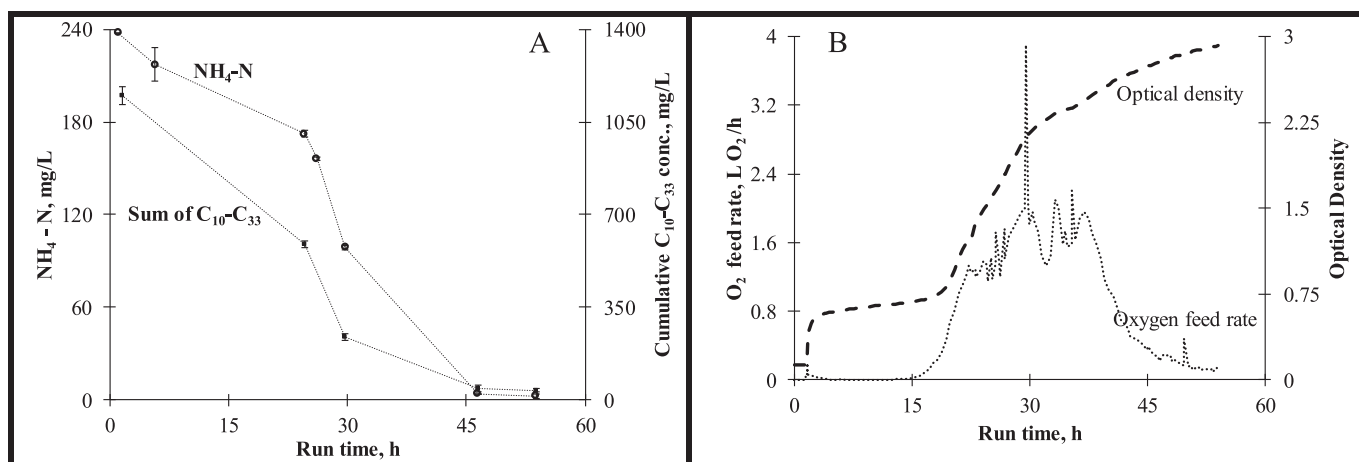
Samples of well-homogenized biomass were centrifuged (4000 rpm, 10 min at 4 °C) and preserved at –20 °C until DNA isolation. Total genomic DNA was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to instructions of the manufacturer. Amplification of DNA, Illumina libraries preparation, amplicon sequencing (Illumina MiSeq) and bioinformatics analysis of the data were performed at BaseClear BV (Leiden, The Netherlands) as described earlier (Roman et al., 2016).

## 3. Results and discussion

### 3.1. Efficient n-alkane biodegradation within the complex PW matrix in a controlled batch experiment

Dispersed oil-alkanes may represent as much as 90% of total hydrocarbons in PW (Tellez et al., 2002). Fig. 3 shows efficient C<sub>10</sub>–C<sub>33</sub> n-alkane biodegradation during the controlled batch experiment. The concentration of aliphatics dropped from 1150.3 ± 32.3 mg/L at the start of the experiment to 588.2 ± 11.1 mg/L after 24.6 h and finally to 20.6 mg/L ± 0.7 mg/L after 53.8 h. The final concentration of sum of C<sub>10</sub>–C<sub>33</sub> n-alkanes corresponds to a removal efficiency of 98.2%. Profiles of individual n-alkanes concentrations during the batch experiment are shown in Figure S1. The overall trend for individual n-alkanes was similar to the sum of n-alkanes shown in Fig. 3 resulting in low concentrations at the end of the batch experiment and overall removal efficiencies above 90%.

The downwards trend of NH<sub>4</sub>-N in Fig. 3-A suggests that the removal mechanism of n-alkanes is biodegradation, since no other carbon source was provided for microbial growth during the experiment. No nitrification was found in the reactor (data not shown). The headspace of the reactor was not monitored for ammonia stripping, or n-alkane stripping. Efficient ammonia



**Fig. 3.** A - Ammonium nitrogen ( $\text{NH}_4\text{-N}$ ) and n-alkane ( $\text{C}_{10}\text{-C}_{33}$ ) profiles during controlled batch experiment. Alkanes presented as a sum of all quantified chain lengths ( $\text{C}_{10}\text{-C}_{33}$ ). B - Optical density ( $\text{OD}_{600}$ ) and oxygen feed rate profile during the batch experiment. Error bars for ammonium nitrogen represent standard deviation for triplicate measurements. Error bars for n-alkanes represent the maximum and minimum measured values in duplicate samples.

stripping is only possible at high pH (9.5 and above), high gas flow rates and large contact area between liquid and gas (Guštin and Marinšek-Logar, 2011). Since the pH in our study was controlled at 7.5 and the maximum oxygen flow peaked at 3.9 L/h (Fig. 3-B), significant ammonia stripping is unlikely to occur. Shorter chain n-alkanes on the other hand have been demonstrated to partition into gas phase during air stripping (Cazoir et al., 2012). In a set-up of similar size as used in the present study Cazoir et al. (2012) demonstrated that with an air flow rate of 2.4  $L_{\text{air}}/L_{\text{reactor}} \cdot \text{h}$  (more than the maximum oxygen flow rate during the batch experiment) only hydrocarbons with chain lengths of  $\text{C}_{10}$  to  $\text{C}_{12}$  can be stripped from the aqueous phase.  $\text{C}_{10}$  to  $\text{C}_{12}$  contributed  $4.9 \pm 0.3\%$  of total hydrocarbons analysed in this study, therefore the impact of stripping on the overall  $\text{C}_{10}\text{-C}_{33}$  removal presented in Fig. 3-A is expected to be rather small, especially considering that the actual oxygen flow rate was much smaller than reported by Cazoir et al. (2012) (Fig. 3-B). Moreover partitioning of these alkanes into the gas phase can be even further reduced, because *A. borkumensis* is shown to solubilize them by producing biosurfactants (Perfumo et al., 2010).

Also the optical density profile in Fig. 3-B follows the trend of n-alkane disappearance, indicating dispersion of n-alkanes with biosurfactants and of microbial growth. The biosurfactants produced by *A. borkumensis* SK2 are glycolipidic molecules containing nitrogen (Perfumo et al., 2010). Interestingly, we demonstrate, that *A. borkumensis* SK2 is capable of biodegrading even tritriacontane ( $\text{C}_{33}$ ). Thus far *A. borkumensis* SK2 has been reported to biodegrade n-alkanes up to  $\text{C}_{32}$  (Schneiker et al., 2006).

The above results confirmed the ability of *A. borkumensis* SK2 to biodegrade n-alkanes even within the complex PW matrix at seawater salinity levels thus showing its affinity to oil hydrocarbons as reported before (Kasai et al., 2002; Cappello and Yakimov, 2010). Moreover, due to the fact that it is a marine microorganism (Yakimov et al., 1998) no adaptation period was necessary to the salinity levels of seawater applied in this study.

### 3.2. Continuous reactor operation: efficient alkane removal coupled with production of neutral lipids

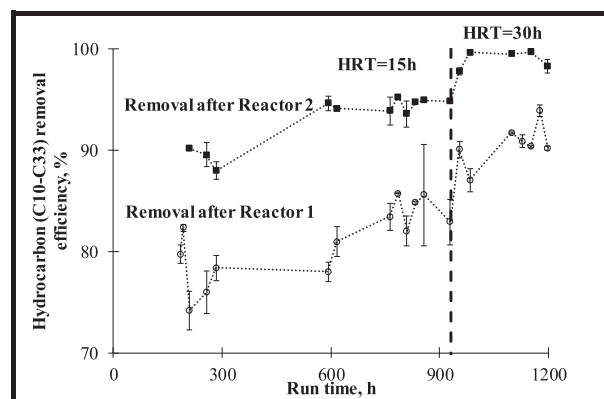
#### 3.2.1. Continuous system with efficient n-alkane biodegradation

Continuous system yielded good removal efficiency of n-alkanes both with the R1 and R2 combined as well as with R1 alone. N-alkane removal efficiency profiles in Fig. 4 clearly show the

possibility of efficient biodegradation in a continuously operated system. A HRT of 15 h in R1 (30 h in R2) could yield up to  $82.6 \pm 1.9\%$  removal efficiency within R1 alone and up to  $94.5 \pm 1.0\%$  removal efficiency within the total system. Upon increase of R1 HRT to 30 h (60 h in R2), the removal efficiency within R1 alone up to  $90.3 \pm 2.1\%$  could be achieved whereas in the entire system up to  $98.8 \pm 1.0\%$  of n-alkanes could be removed. Table 1 summarizes the average reactors performance during the periods of stable oxygen uptake rates in R1.

The good removal efficiency of n-alkanes was achieved while operating R1 at organic loading rates relatively high for an aerobic activated sludge system. The organic loading rates applied here are approximately four times higher than those reported before. In previous studies the organic loading rates applied in biological treatment systems ranged from 0.3 kg COD/( $\text{m}^3$  d) in a pilot scale hydrolysis acidification/bio contact oxidation system treating PW from oilfield in China (HA/BCO) (Lu et al., 2009) up to 4.2 kg COD/( $\text{m}^3$  d) in an activated sludge and MBBR system treating PW from an oilfield injection pipe (Dong et al., 2011). The organic loading rate applied to R1, considering only the n-alkanes contribution, was 6.2–17 kg COD/( $\text{m}^3$  d) (Table 1).

It is difficult to compare the performance of bioreactors in



**Fig. 4.** Removal efficiency of n-alkanes during continuous operation of R1 and R2. The removal efficiency is presented after R1 alone as well as after R2. Vertical line represents increase of HRT in R1 to 30 h (corresponding HRT of R2 is 60 h). The error bars represent the maximum and minimum measured removal efficiency between duplicate samples.

**Table 1**  
Average measured biomass concentration in bioreactors, average influent COD concentrations and loading rates originating from n-alkanes (C<sub>10</sub>–C<sub>33</sub>), average influent n-alkane removal in R1 and over the entire reactor system during steady operation periods of R1. Values given with standard deviations.

Operating Parameter	Period II	Period IV	Period V	Period VI
Average alkane COD <sub>in</sub> , mg/L	10305 ± 66	8766 ± 40	7205 ± 160	7411 ± 197
Loading kg COD/(m <sup>3</sup> d) in R1	17.0 ± 0.1	14.4 ± 0.05	11.7 ± 0.3	6.2 ± 0.2
VSS conc. In R1, mg VSS/L	793 ± 178	367.0 ± 28	258.8 ± 61	344.6 ± 70
Average COD concentration from R1 to R2, mg/L	2319 ± 41	1822.0 ± 171	1258 ± 142	680 ± 244
Loading kg COD/(m <sup>3</sup> d) in R2	1.8 ± 0.2	1.5 ± 0.1	1.0 ± 0.1	0.3 ± 0.07
VSS conc. In R2, mg VSS/L	875 ± 364	883 ± 94	882 ± 121	856 ± 168
<b>Performance</b>				
Alkanes rem. in R1, %	79.0 ± 2.1	79.3 ± 2.0	82.6 ± 1.9	90.3 ± 2.1
Alkanes rem. in R1 + R2, %	89.1 ± 1.3	94.3 ± 0.3	94.5 ± 1.0	98.8 ± 1.0

different studies, since often pre-treatment/post – treatment is applied and the performance is calculated over the entire system including the pre-treatment steps rather than reporting the biodegradation efficiency (Lu et al., 2009). Moreover, often COD measurements are made to assess the reactors performance instead of analysis of specific target compounds (Dong et al., 2011). Thus, it is not always possible to conclude, if the bioreactors are capable of removing the insoluble n-alkanes within PW and what is the actual efficiency of the biological treatment.

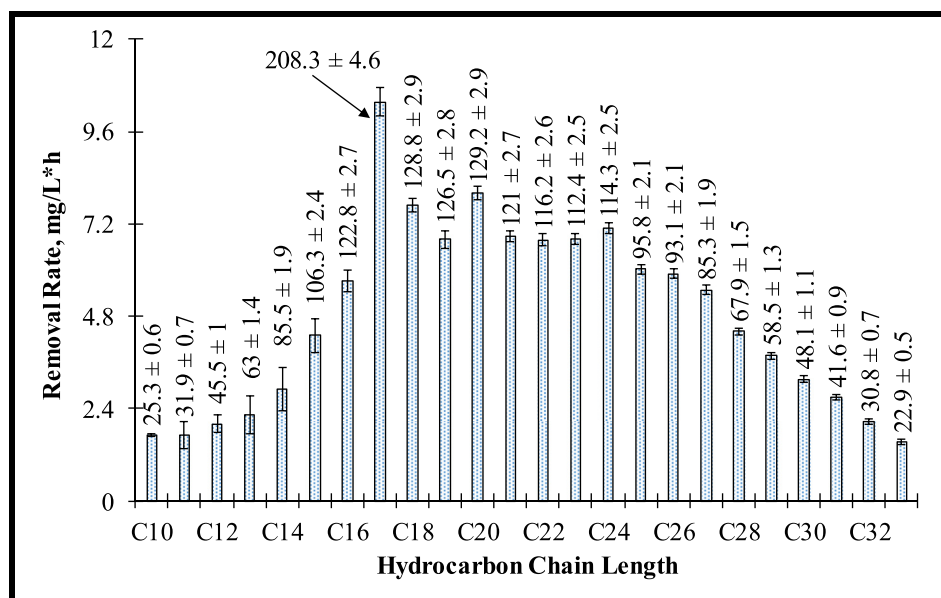
With reactors as presented in this study and PW of a similar composition, the volume of aeration basins to treat e.g., PW with a flow rate of 20,000 m<sup>3</sup>/day would be approximately 37,500 m<sup>3</sup>, if both reactors would be connected in series with an HRT of 15 h in R1. However, potentially the size of the reactors can be reduced by 25,000 m<sup>3</sup>, because most of n-alkanes are biodegraded already in R1 (Table 1) and the capacity of the reactors can be even higher, if biomass concentration would be increased by implementing a sludge retention system. Another consideration, when choosing an activated sludge system for treatment of PW is the excess sludge production. With a sludge yield reported for refinery wastewater ranging from 0.3 to 0.5 kg SS/kg COD (Fakhru'l-Razi et al., 2010), very large amounts of excess sludge would be produced if PW with high influent COD concentrations would be treated. If intracellular accumulation of neutral lipids can be achieved as shown in section 3.2.2 and a cheap extraction method of neutral lipids can be

developed, this sludge can become of value. Otherwise, a large amount of excess sludge needs to be treated and disposed of.

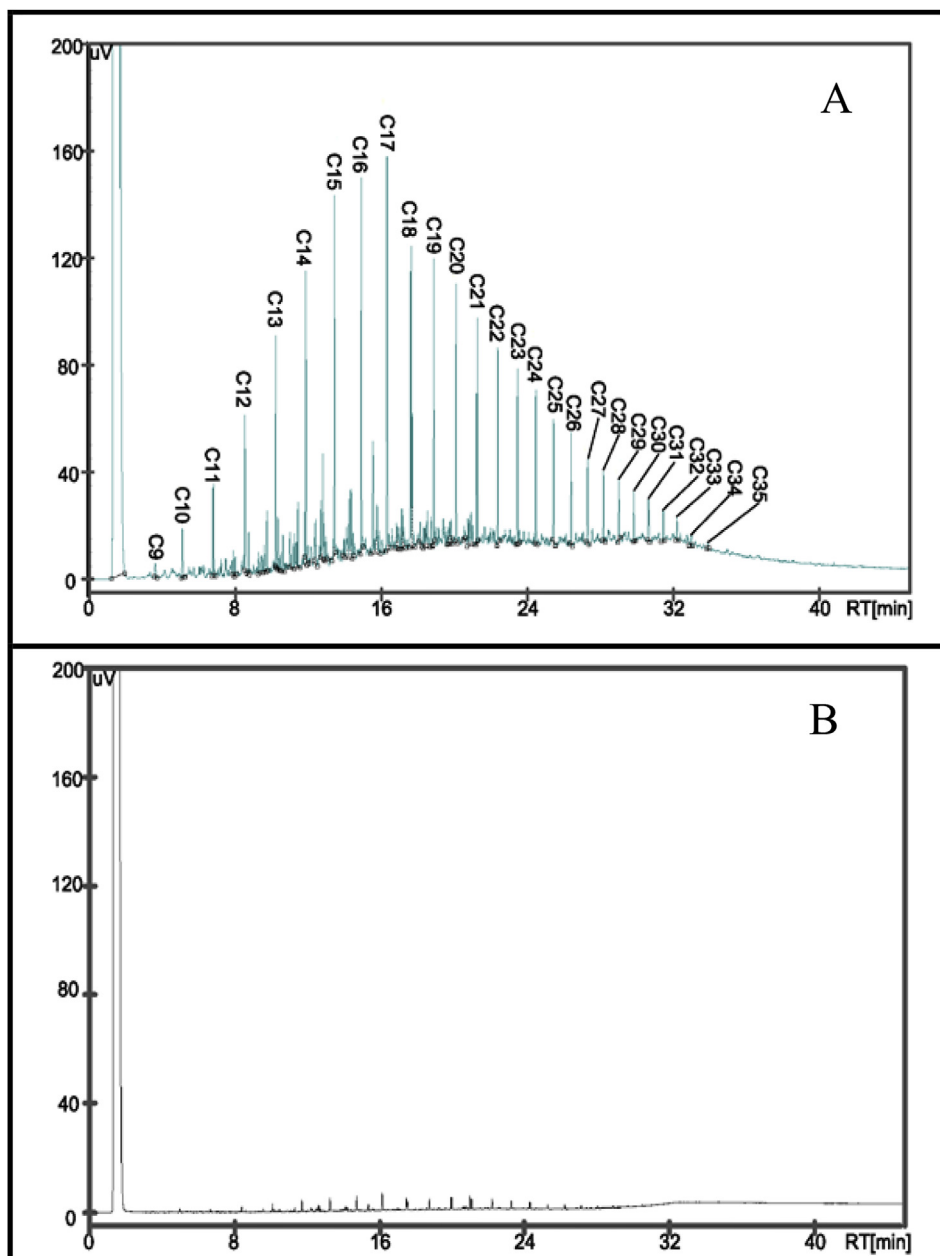
Fig. 5 illustrates the conversion rates of different n-alkane chain lengths during period V. The conversion rates of individual n-alkanes in R1 followed the trend of influent concentrations with increasing influent concentrations corresponding to an increased removal rate. The maximum conversion rate was found for C<sub>17</sub> (10.37 mg/L·h), whereas the minimum for C<sub>33</sub> (1.53 mg/L·h). Both, the maximum and minimum conversion rates correspond to minimum and maximum influent concentrations of 22.9 and 208.3 mg/L, respectively.

Interestingly, our results provide evidence that besides n-alkane biodegradation, also other non-polar compounds were biodegraded. Fig. 6 contains chromatograms of untreated PW (Fig. 6-A) and a sample taken from R1 (Fig. 6-B). The dilution factor of a raw PW sample was 130, whereas the dilution factor of treated sample was 45 by taking into account also the dilution within the reactor. The unresolved area as well as unidentified peaks shown in Fig. 6-A were practically absent in the chromatograms obtained for samples taken from R1 (Fig. 6-B). The disappearance of these is an indicator of biodegradation of those compounds in the reactor.

*A. borkumensis* SK2, has been reported to grow on several hydrocarbons other than alkanes, including branched aliphatic as well as isoprenoid hydrocarbons, alkylarenes, organic alkyl-sulphuric compounds, alkylcycloalkanes, volatile fatty acids, pyruvate and



**Fig. 5.** Average removal rates of C<sub>10</sub>–C<sub>33</sub> n-alkanes and their respective average influent concentrations during period V in R1. Numbers above the removal rate bars indicate the influent concentration of the corresponding n-alkane chain length with standard deviation. Error bars indicate the standard deviation for removal rates of individual alkanes.



**Fig. 6.** A – chromatogram of 130 times diluted PW sample. B – chromatogram of a sample taken from R1 during period IV. Both samples have been treated in the same way. The response signals on Y-axis are given in thousands.

its methyl ether (Yakimov et al., 1998; Sabirova et al., 2006; Schneiker et al., 2006). Here we demonstrate removal of a mixture of n-alkanes as well as other non-polar compounds in a complex PW matrix and in a continuously operated bioreactor. This is of great importance if real wastewater needs to be treated, since one of the biggest challenges for physical-chemical post-treatment is not only removal of small suspended oil particles, but also removal of dissolved organic compounds (Dong et al., 2011).

It is important to realise that the operated system was non-sterile and the removal of other non-polar compounds may be attributed to different microorganisms than *A. borkumensis* SK2 eventually evolving in R1. However, given the high abundance of n-alkanes within the concentrated PW (Table S1) and the reported competitive advantage of *A. borkumensis* SK2 to scavenge on the rather insoluble substrate (Kasai et al., 2002; Cappello and Yakimov,

2010), it should have remained as the dominant microorganism within the system throughout the operational period. Indeed, 16S rRNA gene sequencing analysis revealed that *A. borkumensis* was the dominant microorganisms in both R1 and R2, representing about 40–50% of the total microbial community (Table S2).

### 3.2.2. Production of intracellular and extracellular neutral lipids coupled to n-alkane biodegradation

Table 2 summarizes the conditions under which production of neutral lipids was observed in R1 and R2 as well as concentrations of intracellular and extracellular WEs, TAGs and FAs. Besides WE-like and TAG-like neutral lipids, other lipophilic compounds such as diacylglycerol-like and monoglyceryl-like compounds were also detected, in both intracellular (Figure S2) and extracellular fractions (Figure S3).

**Table 2**  
Concentrations of extracellular and intracellular neutral lipids and conditions within the bioreactors. Samples analysed only during steady state conditions in R1. DO – dissolved oxygen, n.d. – data not available. The error values are given as deviation from the mean for R2 n-alkanes and measurements of R2. For R1 standard deviations for NH<sub>4</sub>-N and P are presented.

Reactor 1													
Period	Run time, hours	DO, %	NH <sub>4</sub> -N, mg/L	P, mg/L	C <sub>10</sub> -C <sub>33</sub> /P	C <sub>10</sub> -C <sub>33</sub> /NH <sub>4</sub> -N	C <sub>10</sub> -C <sub>33</sub> , mg/L	Extracellular lipids, g/ L <sub>reactor volume</sub>			Intracellular lipids, g/ L <sub>reactor volume</sub>		
								WEs	TAGs	FAs	WEs	TAGs	FAs
II	259.0	39.7	15.1 ± 0.7	10.9 ± 0.2	66.1	47.5	716.7 ± 61.8	6.70	0.20	0.11	1.75	0.14	0.05
II	287.5	40.5	20.6 ± 0.7	13.1 ± 0.8	49.3	31.3	645.5 ± 37	1.02	0.11	0.09	3.49	0.11	0.08
IV	593.8	40.3	117.3 ± 0.6	6.3 ± 0.2	88.1	0.5	555.2 ± 24.3	1.53	0.07	0.05	0.56	0.04	0.00
V	929.9	40.4	119.2 ± 2.6	21.3 ± 0.6	17.5	3.1	371.9 ± 49.1	1.24	0.04	0.02	0.04	0.05	0.02
VI	1151.7	40.0	93.2 ± 2.5	19.0 ± 0.0	10.8	2.2	204.5 ± 2.1	0.49	0.07	0.04	0.27	0.03	0.01
Reactor 2													
II	263.9	67.3	N/A	5.4 ± 0.2	57.7	n.d	311.5 ± 34.2	6.15	0.33	0.17	0.91	0.07	0.04
II	283.2	67.0	1.2 ± 0.2	2.6 ± 2.6	138.2	307.2	359.4 ± 25.5	5.42	0.17	0.13	2.37	0.10	0.09
IV	592.6	1.3	0.8 ± 0.1	<5.15	n.d	178.8	135.9 ± 18.6	3.31	0.14	0.11	0.56	0.04	0.03
V	833.2	61.0	0.4 ± 0.0	<5.15	n.d	265.6	108.9 ± 3.3	3.08	0.09	0.07	0.26	0.01	0.01
V	929.3	24.8	0.4 ± 0.0	<5.15	n.d	295.3	112.2 ± 4.1	2.64	0.09	0.07	0.84	0.06	0.04
VI	1151.8	92.0	41.5 ± 0.4	10.4 ± 0.0	0.6	0.2	6.3 ± 6.3	0.03	0.03	0.12	0.37	0.03	0.02

Accumulation of neutral lipids was observed both in R1 and R2. Table 2 shows, that in general more WE-like neutral lipids compared to TAG-like and FAs were produced in both reactors. For example, maximum concentration of extracellular TAG-like and WE-like neutral lipids reached 0.2 and 6.7 g/L, respectively in R1. Similarly, in R2, the maximum concentration of TAG-like and WE-like neutral lipids were 0.33 and 6.15 g/L. Interestingly, accumulation of these compounds within R1 occurred even though residual soluble concentrations of both NH<sub>4</sub>-N and P were present at sufficiently high levels. The accumulation of neutral lipids in R1 as well as R2 decreased once the carbon source became limiting during period VI (Table 2). In R2 extracellular WE-like lipids concentration dropped from approximately 2.6 g/L during period V to 0.03 during period VI.

It becomes clear that accumulation of neutral lipids within R1 occurred in a broad range of residual total n-alkane/phosphorous or total n-alkane/NH<sub>4</sub>-N ratios. The ratio ranges at which accumulation occurred were 0.5–47.5 and 10.8–88.1 for NH<sub>4</sub>-N and P, respectively. However, from Table 2 it is also obvious, that more neutral lipids, in particular WEs, was produced in R2 compared to R1. With the amount of data gathered in this study good correlation between individual parameters within the reactors and the amounts of neutral lipids quantified could not be established. The only exception with a reasonable linear correlation ( $R^2 = 0.89$ ) could be found between residual n-alkane concentration in R2 and the extracellular WE content. With higher residual n-alkane concentration in the reactor, more extracellular WE could be quantified.

Intracellular accumulation of neutral lipids in R2 was lower than extracellular production throughout the operational period. The highest intracellular concentrations of WE-like and TAG-like lipids could be observed during period II, when n-alkane concentration from R1 effluent was above 650 mg/L and residual n-alkane concentration inside the reactor was above 300 mg/L. Also in the case of R2 it seems that accumulation of neutral lipids only takes place during periods with sufficient residual n-alkane concentration within the reactor (Table 2).

The results obtained in our study suggest that intracellular accumulation of neutral lipids by *A. borkumensis* SK2 in a complex PW matrix is not only nitrogen or phosphorous limitation related, since accumulation in both R1 and R2 occurred during carbon non-limiting conditions with sufficient nutrients available. A similar phenomenon was previously observed by Da Silva et al. (2016)

when treating lubricant waste rich in n-alkanes using *A. borkumensis* SK2 as inoculum. It was hypothesized that the accumulation of neutral lipids could be associated with removal of n-alkane oxidation intermediates toxic to *A. borkumensis* SK2, namely fatty alcohols, which are known to be the first products from n-alkane biodegradation (Da Silva et al., 2016).

It has been reported by several authors that production and excretion of neutral lipids has an advantage over intracellular accumulation, because extracellular production is not cell volume limited and in fact is a key to make the process economically viable (Alvarez, 2010; Manilla-Pérez et al., 2010).

The excretion of produced neutral lipids took place at the same conditions as intracellular accumulation, throughout the operational period in both reactors. Interestingly, we demonstrate that conditions within R2 were created under which extracellular WE-like lipids were accumulated at concentrations up to 12 times higher than intracellular. Characteristic for these periods are low nitrogen concentrations, phosphorous concentrations below 5.15 mg/L and around 100–140 mg/L of residual n-alkanes. This is an important outcome, since extracellular production of neutral lipids has been suggested as one of the main aspects to make the process economically viable due to expensive downstream processing when these compounds occur intracellularly (Alvarez, 2010; Manilla-Pérez et al., 2010). Manilla-Pérez et al. (2010) demonstrated the ability of *A. borkumensis* SK2 to produce and excrete lipids when grown on hexadecane or pyruvate as the sole carbon source. Here, we show, that this can be achieved with a complex PW matrix as the carbon source.

To better evaluate the economic viability of the production of neutral lipids coupled to PW treatment, detailed mass balances are needed to understand the fraction of influent organics being converted to both intracellular and extracellular neutral lipids. The current paper aims to demonstrate the feasibility of efficient PW treatment coupled to simultaneous production of neutral lipids for the first time rather than performing detailed mass balances.

#### 4. Conclusions

The results presented here suggest that aerobic biological treatment of PW while using a specialised inoculum may be an interesting alternative. Efficient n-alkane biodegradation in continuously operated bioreactor while treating real PW is possible with *A. borkumensis* SK2 as the dominant microorganism within the



reactors. Besides n-alkanes, other non-polar organic compounds are biodegraded in bioreactors inoculated with *A. borkumensis* SK2. When treating PW, simultaneous accumulation and excretion of neutral lipids by microbial consortia can be achieved and with adequate conditions the process can be steered to predominant accumulation of extracellular neutral lipids.

The simultaneous process of hydrocarbon biodegradation and production of neutral lipids, while treating PW could offer an interesting alternative for the oil and gas industry, especially if extracellular neutral lipids can be produced. Further investigations with detailed mass balances may reveal an alternative for treatment of PW coupled to bio-recovery of valuable compounds.

## Acknowledgements

This work was performed in the cooperation framework of Wetsus, European Centre of Excellence for Sustainable Water Technology ([www.wetsus.nl](http://www.wetsus.nl)). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and the Environment, the European Union Regional Development Fund, the Province of Fryslân, and the Northern Netherlands Provinces.

The authors would like to thank Wetsus analytical staff for the help with analysis and experimental set-up. A special thanks to Jelmer Dijkstra for n-alkane quantification during this study. We would also like to thank Chloé Fleith for the work performed during her internship period at Wetsus.

The authors also thank the Portuguese Science Foundation (FCT) Strategic Project of UID/BIO/04469/2013 unit, COMPETE 2020, projects POCI-01-0145-FEDER-006684, POCI-01-0145-FEDER-030180 and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.watres.2018.09.050>.

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