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Valorization of lubricant-based wastewater for bacterial neutral lipids production: Growth-linked biosynthesis

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ABSTRACT

Lipids produced by microorganisms are currently of great interest as raw material for either biofuels or oleochemicals production. Significant biosynthesis of neutral lipids, such as triacylglycerol (TAG) and wax esters (WE) are thought to be limited to a few strains. Hydrocarbonoclastic bacteria (HCB), key players in bioremediation of hydrocarbon contaminated ecosystems, are among this group of strains. Hydrocarbon rich wastewaters have been overlooked concerning their potential as raw material for microbial lipids production. In this study, lubricant-based wastewater was fed, as sole carbon source, to two HCB representative wild strains: *Alcanivorax borkumensis* SK2, and *Rhodococcus opacus* PD630. Neutral lipid production was observed with both strains cultivated under uncontrolled conditions of pH and dissolved oxygen. *A. borkumensis* SK2 was further investigated in a pH- and OD-controlled fermenter. Different phases were assessed separately in terms of lipids production and alkanes removal. The maximum TAG production rate occurred during stationary phase (4 mg-TAG/L h). The maximum production rate of WE-like compounds was 15 mg/L h, and was observed during exponential growth phase. Hydrocarbons removal was 97% of the gas chromatography (GC) resolved straight-chain alkanes. The maximum removal rate was observed during exponential growth phase (6 mg-alkanes/L h). This investigation proposes a novel approach for the management of lubricant waste oil, aiming at its conversion into valuable lipids. The feasibility of the concept is demonstrated under low salt (0.3%) and saline (3.3%) conditions, and presents clues for its technological development, since growth associated oil production opens the possibility for establishing continuous fermentation processes.

1. Introduction

Lipids produced by microorganisms are currently of great interest as raw material for biofuels production (Röttig et al., 2010) as well as food applications, cosmetics and oleochemicals (Santala et al., 2011).

Biosynthesis of reserve polymers, such as polyhydroxyalkanoates (PHA), is a characteristic wide spread among bacteria (Koutinas et al., 2014). On the other hand, significant biosynthesis and accumulation of neutral lipids, such as triacylglycerol (TAG) and wax esters (WE) are thought to be limited to a few strains (Wältermann and Steinbüchel, 2006). Hydrocarbonoclastic bacteria (HCB), key players in bioremediation of hydrocarbon contaminated ecosystems, are among this group of strains. HCB are able to produce and accumulate neutral lipids, which production can be maximized up to 76% of cell dry weight, when submitted to growth-limiting conditions (Kosa and Ragauskas, 2011; Manilla-Pérez et al., 2010b). Growth-limiting conditions may encompass stress conditions such as nitrogen scarcity and dissolved oxygen tension (Alvarez et al., 2002; Bredemeier et al., 2003).

Neutral lipids have been reported to be biologically produced from separately fed hydrocarbons (HC) (Kalscheuer et al., 2007; Manilla-Pérez et al., 2010a). However, waste streams rich in HC have been overlooked concerning their potential as C-source for biological production of bacterial lipids (Naether et al., 2013). One example of such potential is the lubricant waste. The intensive usage of crude oil derivatives as lubricants was estimated to be around 30 million tonnes per year, about 1% of the World's total

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List of abbreviations

C-source	carbon source
COD	chemical oxygen demand
FA	fatty acid
FAME	fatty acid methyl esters
GC-FID	gas chromatography and Flame ionization detector
HC	hydrocarbons
HCB	hydrocarbonoclastic bacteria
HPLC	high-performance liquid chromatography
LW	lubricant waste-oil
NIR	near-infrared region
OD_x	optical density at X nm wavelength
SPE	solid phase extraction
TAG	triacylglycerol
TLC	thin layer chromatography
TPH	total petroleum hydrocarbons
WE	wax ester
WS/DGAT	wax ester synthase/acyl coenzyme A (acyl-CoA):diacylglycerol acyltransferase

mineral oil consumption, according United Nations - Industrial Commodity Statistics Database (2010), and is known to originate equivalent volumes of recalcitrant waste rich in HC. Treatment options for spent lubricant oil include incineration and landfill dumping, which disregards the energy, among other resources, that can still be recovered from these waste streams (Koutinas et al., 2014). Lubricant waste (LW) consists of a complex matrix containing a mixture of straight-chain alkanes ranging from C10 to C34. The apolar fraction of LW contains compounds that are unresolvable in regular HC analysis by gas chromatography (GC-FID).

In this research, model organisms were selected as representative of different branches of the hydrocarbonoclastic cluster: *Alcanivorax borkumensis* SK2 and *Rhodococcus opacus* PD630. *A. borkumensis* SK2 is a ubiquitous marine gram-negative bacterium (Manilla-Pérez et al., 2010a; Schneiker et al., 2006; Yakimov et al., 1998), which goes from nearly undetectable to dominant in open sea or coastal waters after HC-based contamination (Atlas, 1981). *R. opacus* PD630 is an actinomycete gram-positive soil bacterium capable of degrading a wide range of carbon sources (Alvarez et al., 1996; Gouda et al., 2008; Kalscheuer et al., 2000).

Combining valuable lipidic compounds production with industrial wastewater treatment can contribute to make waste management more economic and environmentally sustainable. This study aims at assessing the potential of HC-rich wastewaters as cheap raw-material for biological production of valuable neutral lipids.

2. Materials and methods

2.1. Inoculum

Rhodococcus opacus PD630 (DSM number 44 193, Braunschweig, Germany) was pre-cultured at 28 °C in Mineral Salts (MS) Medium according to Schlegel et al. (1961) and *Alcanivorax borkumensis* SK2 (DSM number 11 573) was cultivated in saline medium (ONR7a) according to DMSZ, Braunschweig, Germany.

The biomass was harvested at the end of the exponential phase and washed to remove the remaining carbon source: pyruvate. The culture was centrifuged (20 000 × *g*, 10 min and 10 °C) in a centrifuge Avanti J25 (Beckman Coulter), supernatant was discarded and the pellet was resuspended in NaCl 0.9% (w/w). This procedure was repeated three times. Fresh culture medium was used to resuspend the pellet after the last centrifugation. The inoculum volume was calculated according to the targeted initial concentration for each assay (initial $OD_{600} = 0.1$).

2.2. Carbon source (oily wastewater)

The concentrated emulsified lubricant-based wastewater (LW), used as substrate for lipid production, was collected from a heavy machinery maintenance service unit from Alstom Corporation in Maia, Portugal (2011). This concentrate corresponds to the floating phase of the wastewater generated in the machinery washing process, separated in a gravimetric oil/water separation unit.

2.3. Experimental design

Lubricant-based wastewater (LW) was fed to *A. borkumensis* SK2 and to *R. opacus* PD630 as sole carbon source in non-controlled 50 mL batch experiments. *A. borkumensis* SK2 was also cultivated in controlled conditions: 2 L batch experiments operated at constant pH and dissolved oxygen concentration.

Growth and lipid production were monitored in two different cultivation stages: in the first, referred to as growth-stage, bacteria were grown in adequate medium supplemented with nitrogen source in excess; and in the second stage, the so-called production-stage, the cultures grown in the first stage were fed with the same carbon source (LW) and limited nitrogen source concentration (ammonium chloride).

The transition between stages, in non-controlled shake flask experiments - E1 (*A. borkumensis* SK2) and E2 (*R. opacus* PD630) - was done manually by the transference of bacteria to the new media with three different levels of nitrogen scarcity (see sub-section 2.3.1. Uncontrolled cultures). In controlled batch experiments, where *A. borkumensis* SK2 was further studied (R1 and R2), the transition between the two stages was prompted by growth-related nitrogen depletion (see sub-section 2.3.2. Controlled cultures).

2.3.1. Uncontrolled cultures

A. borkumensis SK2 and *R. opacus* PD630 were cultivated in 250 mL shake flasks with 50 mL of ONR7a medium and MS medium, respectively. The incubation temperature was 28 °C and stirring speed 140 rpm. Each culture was composed of two distinct and consecutive stages. First, bacteria were grown in adequate medium supplemented with LW (3.9 g/L COD) as sole carbon source and excess ammonium chloride (0.3 g/L) as nitrogen source. The grown biomass was washed following the described 3-step centrifugation process (see section 2.1. Inoculum) and inoculated in the corresponding medium for the second stage. The medium for the second stage was supplemented with LW (3.9 g/L COD) and 3 different levels of nitrogen concentration: 29, 15, and 7 mg-N/L, which corresponds to fractions of the recommended for cultivation of both strains. Incubation in nitrogen limiting conditions was performed for 48 and 90 h.

2.3.2. Controlled cultures

A. borkumensis SK2 was further cultivated in controlled batch operation mode. A double-wall water-jacketed CSTR reactor with 2 L working volume was used. The culture was monitored and controlled by using an ez-Control device from Applikon Biotechnology, Inc. (Delft, The Netherlands). The data was compiled with BioXpert v2 software package from Applikon Biotechnology®. Dissolved oxygen (dO_2) and pH were monitored with AppliSens probes, and controlled by automated addition of NaOH (4 M) and

pure oxygen respectively, as response to a proportional-integral-derivative controlling process. Temperature was kept constant at 28 °C by using a thermal bath. Stirring speed was achieved using a metal propeller of 4 cm diameter set to 800 rpm. Absorbance was measured in real-time at the near-infrared region (NIR) by an ASD photometric probe coupled to a Fermenter Control converter from Optek-Danulat, Inc. (Essen, Germany). This measurement corresponds to the detection of undissolved matter, which comprises bacteria, emulsified carbon source and hydrophobic biological products.

Two experiments (R1 and R2) were performed with identical start-up conditions (Table 1). Biomass was grown in LW 1.2% (wt./vol) (3.9 g/L COD) as sole carbon source and limited amount of ammonium chloride. The transition from growth-stage to starvation-stage occurred naturally due to the depletion of nitrogen source.

2.4. Analytical procedures

Optical density at 600 nm wavelength (OD_{600}) was used to monitor the increase in turbidity of pre-cultures, which is assumed to be correspondent to biomass growth only in balanced growth conditions, in a medium containing no hydrophobic compounds (Kalscheuer et al., 2007).

Biomass concentration, as total suspended nitrogen (TSN), was determined using cuvette test LCK238, and the ammonium (NH_4^+) depletion was followed by cuvette test LCK 303 (Hach Lange®, Germany).

COD, TN, nitrates, nitrites were estimated using the commercial kits LCK 914, LCK 338, LCK 340, LCK 341, LCK 303 (Hach-Lange®, Germany). All analyses were done in triplicate, following the procedures recommended in kits protocol.

2.4.1. Extraction and analyses of total hydrocarbons

HC profile was assessed using the standard method ISO 9337-2 for TPH quantification as guide line. Samples were preserved for no longer than 14 days at temperatures below 5 °C and pH below 2. Extracts were obtained by solvent extraction. The extraction solvent was dichloromethane (DCM). Extraction was performed in a 3-step process, at room temperature (19-23 °C). The mixture of sample and DCM (3:1 in volume) was promoted by shaking the separation funnel for 5 min. Extracts were further cleaned with solid phase extraction columns (Florisil 1 g/6 cc, Supelco). Clean extracts were injected in a Varian 3900 gas chromatograph (GC) equipped with a Factor Four™ Capillary Column VF-5 ms (30 m length, 0.25 mm diameter, 0.25 μm film thickness). 1 μL of each sample was injected at 325 °C with 1:3 split ratio. The column temperature was 60 °C for 1 min in the beginning of the 45 min run and increased till 310 °C at a rate of 8 °C/min. The flame ionization detector (GC-FID) operated at 315 °C and acquisition frequency of 20.0 Hz. To determine the extent of degradation of straight-chain alkanes present in LW, the areas of individual peaks (from duplicate samples) were quantified.

Table 1
Start-up conditions for the controlled batch cultivation of *A. borkumensis* SK2 in ONR7a supplemented with lubricant contaminated wastewater (R1 and R2).

Parameter	R1	R2
pH	7.5	7.5
Temperature	28 °C	28 °C
Dissolved oxygen[a] (mg/L)	20% (7)	20% (7)
Absorbance[b] (c.u.)	0.51	0.47
Stirring speed (rpm)	800	800
[N−NH_4Cl] (mg/L)	59	66

[a] Percentage of the medium saturation with pure O_2.
[b] Average of the online acquired values from first 30 min containing inoculum and C-source.

2.4.2. Extraction and analyses of lipids

For each sample the biological matrix was separated from the medium by centrifugation (20 000 × *g*, 10 min, 10 °C) in a centrifuge Avanti J25, Beckman Coulter. The biological matrix encompasses bacterial biomass and insoluble viscous material which density is lower than the medium. The biological matrix was freeze dried and lipids were extracted with Chloroform:Methanol (3:1, volume) for 2 h at 25 °C and stirring speed of 200 rpm. The crude extracts were filtered through a Pasteur pipette packed with glass wool (1.5 cm packing) in order to separate the cell debris and salts (Santala et al., 2011). The clean extract was finally fractionated in classes by polarity using SPE columns (SiOH 1 g/6 cc) from Supelco. Fractionation followed the scheme adapted from Revellame et al. (2012) (Table 2). Each fraction was analysed by thin layer chromatography (TLC). TLC was performed on a 60F254 silica gel plate (Merck) applying n-hexane:diethyl-ether:acetic acid (80:20:1, volume) as a solvent system. The elution area is 8 cm long and 2 cm wide. Lipids were visualized by staining with iodine vapor (Silva et al., 2010). FA (Oleic acid), TAG (glyceryl trihexanoate, glyceryl trilinoleate glyceryl tri-oleate), WE (methyl hexanoate, methyl linoleate, methyl oleate) were used as references.

TAG and WE-like compounds were quantified gravimetrically. Each class of lipids was scraped from the TLC plate, transferred into a Pasteur pipet containing glass wool and eluted 3 times with 0.5 mL of chloroform from the silica (Santala et al., 2011). The extracts containing the isolated lipid class were dried with a gentle flow of nitrogen and their weight was measured daily till constant value.

The isolated lipid classes were submitted to methanolysis in the presence of sulfuric acid (Brandl et al., 1988). Heptanoic acid (C7:0) and pentadecanoic acid (C15:0) were used as internal standards. The mixture containing the sample, 1.5 mL of chloroform, 3 mL of the solution methanol/sulfuric acid 85:15 (%) was kept at 100 °C for 3.5 h 1 mL of ultra-pure water was added and the fatty acid methyl esters (FAME) were collected within the organic phase. The originated FAME were analysed by gas chromatography (Varian 3800) equipped with a flame ionization detector (GC-FID) and a TR-WAX capillary column (CP-Sil 52 CB 30 m × 0.32 mm x 0.25 μm) from Teknokroma. FAME were injected (1 μL) in splitless mode. Injector temperature was set at 220 °C. Helium was the carrier gas at a flow rate of 1.0 mL/min. Initial oven temperature was 50 °C for 2 min with an increase of 10 °C/min till a final temperature of 225 °C. The detector temperature was 250 °C. All solvents used were HPLC grade.

3. Results and discussion

The oily wastewater (LW), fed as sole carbon-source, consisted mainly of straight-chain alkanes dissolved in a complex GC-unresolved mixture, with predominance of non-volatile alkanes from C9 to C12 (Fig. 1). The straight-chain alkanes (C9–C30), analysed peak by peak, correspond to 5.5 ± 0.2 g-alkanes/L in total. In addition to the GC-resolved compounds, it was also detected a substantial elevation of the baseline between retention time 12 and 37 min, corresponding to unresolved complex mixture, which has been reported for lubricant oil (Anderson et al., 2003). This portion of the waste is usually more resistant to biodegradation (Frysinger et al., 2003). The total petroleum hydrocarbons (TPH) present in LW is 308 ± 36 g/L. The COD is in the range of 325 ± 25 g/L. The concentration of bioavailable nitrogen (ammonium) is inferior to 2 mg-N/L.

The presence of the unresolved complex matrix did not seem to hamper significantly the biodegradation capacity. In uncontrolled

Table 2
Elution scheme for lipid class separation by solid phase extraction, adapted from Revellame et al. (2012).

Fraction Label	Solvent system	Volume (mL)
F1	*n*-Hexane/diethyl-ether (94:6, v/v)	1.2
F2		1.5
F3		1.0
F4		1.0
F5		3.5
F6		10.0
F7	*n*-Hexane/diethyl-ether/acetic acid (85:15:2, v/v/v)	15.0
F8	Methanol	5.0

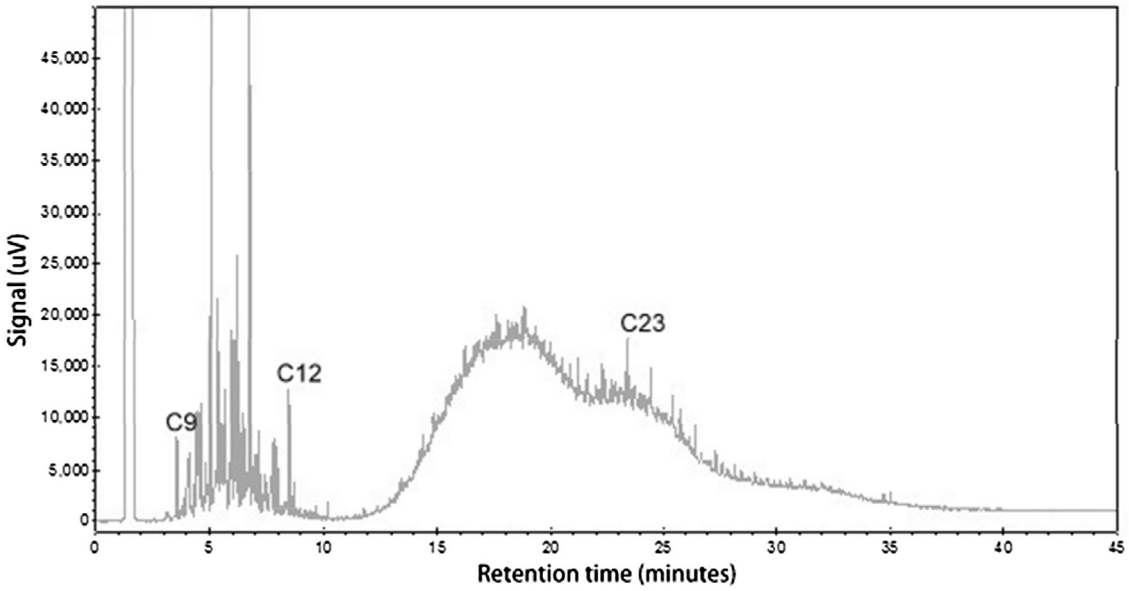

Fig. 1. GC-FID chromatogram of the apolar fraction of the oily wastewater (LW) extracted and cleaned through solid phase extraction column (Florisil 1 g/6 cc, Supelco). C9, C12, and C23 identify the peaks corresponding to nonane, dodecane and tricosane respectively. The signal is given in microvolts (uV).

experiments (shake flasks) the straight-chain alkanes' removal ranged from 20% for *R. opacus* PD630 to 30% for *A. borkumensis* SK2. However, in controlled conditions, the degradation efficiency reached 97% of the GC-resolved n-alkanes (C9 – C30) (Fig. 2-A). Ability for hydrocarbons' degradation is a characteristic of hydrocarbonoclastic bacteria, but has only been reported in these strains, for individual hydrocarbons (Alvarez et al., 1996; Manilla-Pérez et al., 2010a; Naether et al., 2013). Adding to that, the degradation of a complex mixture containing a wide range of n-alkanes is herein reported for saline (3.3% w/w) and low salt conditions (0.3% w/w). The degradation efficiency observed for exponential growth phase (70% removal within 7.5 h) was significantly higher than the removal during the stationary phase (21% of the initial concentration after 16 h) (Fig. 2-A). This may be explained by the differences on carbon demand of bacterial replication and storage lipids synthesis. Exponential growth is significantly more carbon-demanding, than nitrogen-limiting periods, where storage compounds are the main result of carbon uptake (Martins dos Santos et al., 2010).

Production of lipids, such as TAG and WE-like compounds, was observed during the exponential and stationary growth phases. WE-like compounds are apolar products for which the behaviour after extraction and separation resembles the neutral lipids WE. However, the content in fatty acids is significantly low. Neutral lipids production was observed in low salt conditions by *R. opacus* PD630 and saline conditions by *A. borkumensis* SK2. In shake flasks, *R. opacus* PD630 produced comparable amounts of TAG and WE-like, up to 0.15 g/L and 0.21 g/L, respectively, whereas *A. borkumensis* SK2 produced mainly WE-like up to concentrations 10 times higher and nearly insignificant amount of TAG (Table 3). The less effective TAG producer in shake flasks: *Alkanivorax borkumensis* SK2, was further studied in controlled bioreactor experiments, aiming for full assessment of its potential. This strain is adapted to a more restrict environment, when compared with *R. opacus* PD630, which may play a role in contamination control for future biotechnological application. The controlled fermenter, inoculated with *A. borkumensis* SK2, was operated according to the conditions described in Table 1. In such conditions TAG production was, as expected, nitrogen scarcity-linked whilst the production of WE-like compounds was, surprisingly, found to be growth-linked (nitrogen limiting-independent). The production of storage compounds is usually associated to nutrient imbalance between carbon (in excess) and another essential nutrient (in limiting concentration) (Athenstaedt and Daum, 2006; Ratledge and Wynn, 2002; Wältermann and Steinbüchel, 2005). This nitrogen scarcity-linked production was observed for TAG but not for WE-like products (Fig. 2-B). TAG production during the early stationary phase reached 92% of the whole TAG production. Nitrogen scarcity (<2 mg-N/L) was the determinant factor (Fig. 2-A). In contrast, the significant production of the WE-like compounds occurred in all the different cultivation phases (Fig. 2-B). This includes the periods: of growth on hydrocarbons (first 24 h), of nitrogen limiting conditions (between t = 24 h and t = 63 h), and of growth on both hydrocarbons and previously produced TAG (after t = 63 h).

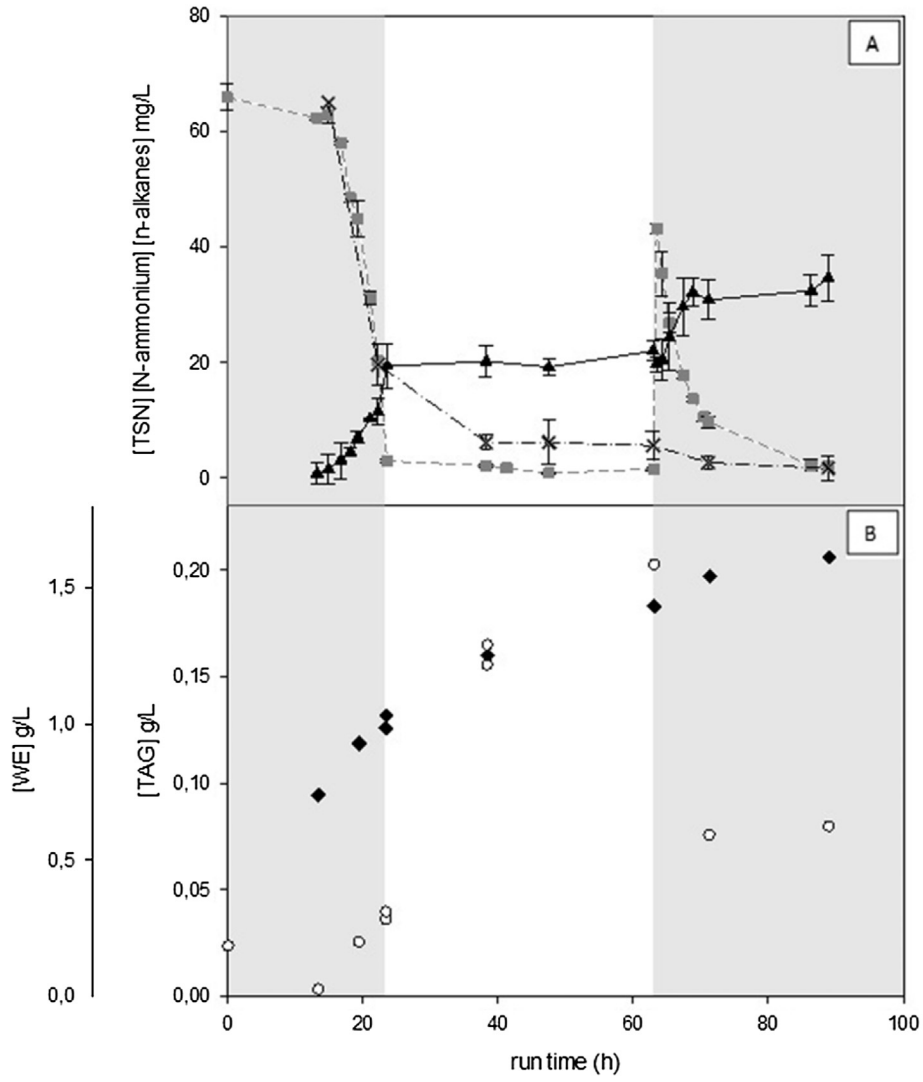

Fig. 2. Time course of nitrogen compounds as ammonium nutrient (A, ■) and biomass concentration (as Total Suspended Nitrogen, TSN - A, ▲) and carbon substrate as GC-resolved alkanes, C930 (A, **X**), and products as WE-like compounds (B, ◆) and TAG (B, ○). *Alcanivorax borkumensis* SK2 was incubated in ONR7a, fed with LW as sole carbon source. Temperature and pH were controlled at 28 °C and 7.5 respectively, and the dissolved oxygen kept at 20% medium saturation with pure O_2. Ammonium was fed in the beginning and after 63 h. The shadowed areas correspond to periods in which nitrogen was bioavailable. The error bars were calculated from triplicates and correspond to 95% confidence.

Table 3
Bacterial neutral lipid production in non-controlled conditions. *Alcanivorax borkumensis* SK2 and *Rhodococcus opacus* PD630 was incubated in ONR7a, fed with LW as sole carbon source. Temperature was set at 28 °C and stirring speed at 140 rpm. Ammonium was fed in 3 different levels aiming at COD/N molar ratios of 134, 260 and 557.

Initial COD/N	Time	*A. borkumensis* SK2		*R. opacus* PD630	
		TAG	WE-like	TAG	WE-like
	h	g/L		g/L	
134	48	0.02	n.d.	0.13	0.06
	90	0.05	2.38	0.14	0.19
260	48	0.02	1.70	0.11	0.18
	90	0.03	1.95	0.14	0.21
557	48	0.04	1.85	0.07	n.d.
	90	0.06	2.08	0.15	n.d.

n.d. – not detected.

Moreover, the highest WE-like compounds production rate (15 mg/L.h) was found in the exponential growth phase instead of in the nitrogen-limiting stationary phase, where production rate was 11 mg/L.h.

The predominant fatty acid (FA) species found in TAG samples, after nitrogen-limiting period are C16:0 (43%) and C18:1 (27%). Although these FA species decrease significantly (80% in wt.) during the second growth stage, they remain the predominant species in TAG produced by *A. borkumensis* SK2 in controlled conditions. In WE-like products, from exponential growth phase, the predominant FA are C17:1 (28%) and C18:0 (19%). This profile does not seem to change during the nitrogen-limiting phase. In contrast, during the second growth phase the FA profile shifted to C16:0 (27%) and C18:1 (32%), which became predominant within the measured FA.

The growth-linked production of WE-like compounds, by *A. borkumensis* SK2, was observed in this study and is herewith reported for the first time. Manilla-Pérez et al. (2010a) reported higher WE production in the least imbalanced cultivation of *A. borkumensis* SK2. However no further discussion on that result has been presented. The commonly used shake flasks culturing method does not allow full monitoring of each cultivation phase. This hinders the isolation and characterization of each growth phase, regarding for instance, lipid production. The exponential

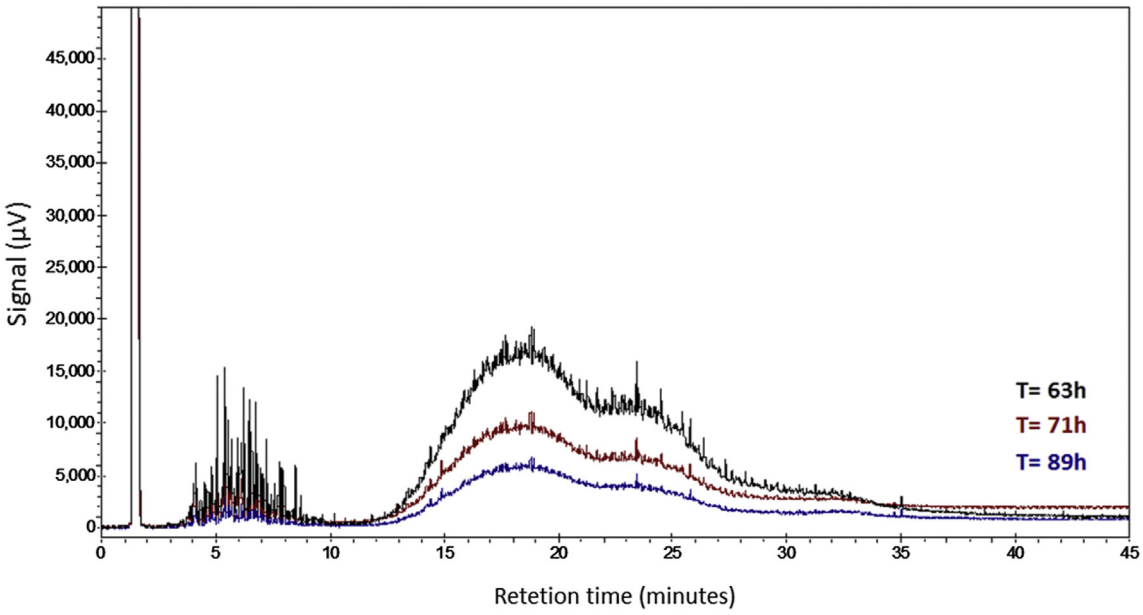

Fig. 3. Chromatogram of lubricant waste oil processed by *Alcanivorax borkumensis* SK2 in controlled batch conditions. The chromatograms depict the profile of apolar or slightly polar compounds during the second growth phase: right before N-source was reestablished (black), 8 h later (red), and when nitrogen concentration was again limiting (blue). *Alcanivorax borkumensis* SK2 was incubated in ONR7a, fed with LW as sole carbon source. Temperature at 28 °C and pH at 7.5 were kept constant. The first peaks (retention time between 1 and 2 min) correspond to the solvent used as samples' matrix: hexane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth phase presented the highest HC removal rate, which coincided with the highest WE-like compounds production rate. The apparent correlation is hereby hypothesized to be related to the carbon uptake pathway. This process strongly depends on the carbon source available (Alvarez et al., 1997; Naether et al., 2013). The conversion of n-alkanes into TAG requires the oxidative production of fatty acids (FA) and glycerol production from scratch (*de novo* synthesis). In contrast, WE production consists of esterification of two intermediates of n-alkanes biodegradation: one fatty acid and one fatty alcohol (Wältermann and Steinbüchel, 2006; Wentzel et al., 2007). Fatty alcohols are the first intermediates from n-alkanes degradation. Expectedly, high HC removal corresponds to high fatty alcohols production. These fatty alcohols can be either readily esterified to WE or further oxidized to FA (Kalscheuer et al., 2007; Wältermann et al., 2007). Fatty alcohols are reported to induce toxicity to *A. borkumensis* SK2 cells (Naether et al., 2013) and neutral lipids have been suggested as depot of potentially harmful metabolic intermediates (Arabolaza et al., 2008; Wältermann and Steinbüchel, 2005). Therefore WE production may serve as a protective mechanism by neutralizing the harmful effect of both fatty alcohols and unusual FA. In opposition to the common pairing classification of TAG and WE as storage lipids (Wältermann and Steinbüchel, 2006), these results suggest divergence between these neutral lipids metabolism.

The highest growth rate achieved for *A. borkumensis* SK2 in this study does not differ significantly from the maximum growth rate reported for separately fed alkanes. The reported average of the doubling time for growth on n-alkanes (C14 – C19) is 2.3 h (Naether et al., 2013) whilst the observed maximum growth on a complex mixture of HC showed, in this work, a doubling time of 2.6 h. The growth of *A. borkumensis* SK2 in a controlled fermentor occurred in 2 different periods. In the first period, the exponential growth was sustained, in terms of carbon, solely by the hydrocarbons present in LW. In the second growth period, where growth was sustained by recently produced TAG and remaining LW constituents, the doubling time was 7.3 h. The second growth phase was a result of nitrogen source addition. Mobilization of neutral lipids, when growth conditions were reestablished, was rather selective to TAG (Fig. 2-B). In contrast, WE-like compounds accumulation rate in the reactor increased again, confirming its correlation with growth. In this study, TAG metabolism matches the storage compounds description, from the previously described nitrogen scarcity-linked production to its usage to sustain growth when nitrogen is available (Manilla-Pérez et al., 2010b). The difference between growth rates of each phase can be explained by the usage of increasingly recalcitrant carbon source along the run. During the period where growth was sustained by TAG and remaining carbon present in LW, the unresolved area of the chromatograms decreases significantly (Fig. 3) which indicates its removal (Nievas et al., 2008). This result reveals the capability of *A. borkumensis* SK2 to access and biodegrade the unresolved complex matrix of LW.

Apparently, decrease on the pH affected negatively growth of both strains *A. borkumensis* SK2 and *R. opacus* PD630. In uncontrolled experiments (shake flask) the dissolved oxygen was not monitored but the pH was observed to decrease from 7.5 to 5, with the growth (doubling times higher than 20 h). The alteration in pH is known to affect negatively fatty acid (FA) production (Janßen et al., 2013). FA production is an intermediate step between the hydrocarbons uptake and either the respiration or the biosynthesis (Wentzel et al., 2007). Thus the inhibition of FA production corresponds to a major limitation to growth on alkanes. This can explain the significant difference, in terms of growth rates, between shake flasks experiments and the controlled fermenter, in which pH was constant.

4. Conclusions

In this work, the production of WE-like compounds associated to the growth of a model hydrocarbonoclastic bacterium (*A. borkumensis* SK2) was demonstrated when using a complex mixture of hydrocarbons present in an industrial wastewater. WE of long-chain FA and long-chain fatty alcohols are of particular

commercial interest (Muller et al., 2014; Wentzel et al., 2007). The growth-linked lipids production, from a waste, broadens its production possibilities and prompts the development of continuous operation mode processes. This is a favorable alternative for both: the lubricant-based wastewater treatment, and oleochemicals biosynthesis. The cost of a downstream separation process must be compensated by the value of the final product. Therefore the full profiling of the biological treatment products is a pre-requirement for further development of the concept.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.05.062.

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