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Technical Notes

Methods for Recovery of Microorganisms and Intact Microbial Polar Lipids from Oil−Water Mixtures: Laboratory Experiments and Natural Well-Head Fluids

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Most of the world's remaining petroleum resource has been altered by in-reservoir biodegradation which adversely impacts oil quality and production, ultimately making heavy oil. Analysis of the microorganisms in produced reservoir fluid samples is a route to characterization of subsurface biomes and a better understanding of the resident and living microorganisms in petroleum reservoirs. The major challenges of sample contamination with surface biota, low abundances of microorganisms in subsurface samples, and viscous emulsions produced from biodegraded heavy oil reservoirs are addressed here in a new analytical method for intact polar lipids (IPL) as taxonomic indicators in petroleum reservoirs. We have evaluated the extent to which microbial cells are removed from the free water phase during reservoir fluid phase separation by analysis of model reservoir fluids spiked with microbial cells and have used the resultant methodologies to analyze natural well-head fluids from the Western Canada Sedimentary Basin (WCSB). Analysis of intact polar membrane lipids of microorganisms using liquid chromatography−mass spectrometry (LC−MS) techniques revealed that more than half of the total number of microorganisms can be recovered from oil−water mixtures. A newly developed oil/water separator allowed for filtering of large volumes of water quickly while in the field, which reduced the chances of contamination and alterations to the composition of the subsurface microbial community after sample collection. This method makes the analysis of IPLs (or indirectly microorganisms) from well-head fluids collected in remote field settings possible and reliable. To the best of our knowledge this is the first time that IPLs have been detected in well-head oil−water mixtures.

Biodegraded oils dominate the world petroleum resources with the largest reserves found in Canada and Venezuela.[1] A significant proportion of petroleum in conventional oil reservoirs is biodegraded, and future oil discoveries are also expected to have been altered by microorganisms.[2] Hydrocarbon biodegradation proceeds in any petroleum reservoir that has a water leg and has not been heated to temperatures over 80 °C and takes place predominantly at the oil−water transition zone.[3,4] While microbial activity in deep subsurface reservoirs has been known for over 80 years,[5] relatively little is known about microbial populations responsible for hydrocarbon degradation. A better understanding of the microbial ecology of these environments, their physiological properties, and the reaction systematics of biodegradation will aid both oil exploration and production.

Although many studies have characterized microbial communities in petroleum reservoirs using both cultivation and culture-independent methods,[2,6−12] reservoir contamination and low abundances of microorganisms in subsurface environments,

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especially petroleum reservoirs, are major obstacles to studying these organisms. Sampling petroleum reservoirs for subsurface microbial populations is prone to contamination with nonreservoir microbes from drilling mud, injected water, and/or biocide application during production. In an uncontaminated setting, reservoir core samples from the oil−water transition zone of biodegraded petroleum reservoirs have yielded higher levels of metabolites characteristic of anaerobic hydrocarbon biodegradation[13] than from the oil-saturated reservoir rock. However, culturing microbes can be challenging as in commercial operations in biodegraded petroleum systems like the Alberta tar sands core samples are often frozen immediately upon sampling, or core is not available from biodegradation zones. Analysis of formation water is a viable alternative; however, highly biodegraded oil reservoirs often yield produced fluids that are foamy water−oil emulsions. These mixtures typically separate at surface, although some can take days or high temperatures to separate, during which the microbial communities may be altered or destroyed. The natural abundance of microorganisms in deep oil reservoirs is very low. Polymerase chain reaction (PCR) amplification is one highly sensitive method which can be used to detect microorganisms in samples with low biomass such as petroleum reservoir samples. The most advanced method for the detection of very low abundance microbes is capillary electrophoresis (CE) with single-cell detection recently reported.[14] Recent developments in liquid chromatography−mass spectrometry (LC−MS) techniques now allow us to detect relatively low concentrations of biomass as intact polar lipids (IPL). The limit of detection for IPL is 2 pg for a single compound (see below), whereas the dry weight of cell membranes of an average 500 nm diameter bacterium is 1.5 fg.[15] Considering that the most abundant single IPL compound is approximately less than 10% of the cell membrane constituents at 100% extraction yield and none is lost during separation the minimum cell number needed is estimated to be still above 10 000. Even though this number is much higher compared to the very low detection limits known for CE, IPL analysis is a fast method for detecting the most abundant and therefore most active microbes, and in some cases IPLs are easier to detect and determine than the DNA, e.g., ladderane lipids in anaerobic ammonium-oxidizing bacteria (anammox). In addition, when biomass levels are low the amount of DNA that can be extracted from samples is also low. For this reason when highly sensitive PCR assays are conducted using primers that target sequences present in the majority of bacteria, low levels of DNA present in the reagents used can often lead to false positives and difficulties in reliably identifying the organisms present in the original samples.[16]

Here we describe methods to quickly concentrate microbes in the field at the well-heads and to evaluate the partitioning of microbial cells between oil and water after phase separation from experimental oil−water mixtures. In addition, we discuss the distribution of IPLs in natural heavy oilfield waters, including highly viscous oil−water emulsions from well-heads of heavily biodegraded oil reservoirs in Western Canada.

METHODS

Microorganisms. An oil field produced-water isolate, *Desulfovibrio* sp. strain Lac6 [U46522 ("clone DVIB57")][17] was grown in saline Postgate's medium C[18,19] and used to inoculate fluid mixture experiments.

Sampling. Well-head fluid samples were collected from several wells from two oilsand fields (Peace River area and north of Fort McMurray, both in Alberta, Canada).

To minimize common contamination issues, all samples collected had to pass the following criteria: (a) The well had been continually on production for a long time before sampling, (b) no water or chemicals had been injected into the reservoirs, (c) production was from a single reservoir interval (no commingled oil), (d) samples were only taken from well-heads, i.e., no separator fluids were taken, and (e) only wells with water cuts (percentage of water in the sampled fluid) higher than 70% were considered. For each well, the well-head valves were flushed prior to sampling. The well-head fluids from the Peace River area were collected into custom-designed 27 L sterile separator cans. These cans were specially designed to allow the well-head samples to separate and to subsample from the water phase to obtain ca. 20 L of water. The water was subsequently filtered through a 0.2 μm pore size sterile PTFE filter using a N_2-pressured "hazardous waste filtration system" from Millipore Corporation, U.S.A., and the filters were stored in a freezer prior to freeze-drying. The procedure to extract the IPLs from the microbial cells is the same as described below for the laboratory experiments.

Natural well-head fluid samples were collected from the Peace River area, Alberta, Canada (Figure 1A) from wells producing highly biodegraded oil where oil chemistry showed microbial action. A previous study[20] indicated that oils in the Peace River area can be geographically divided into a highly biodegraded oil—east of a NW−SE line (Figure 1A), coincident with the 80 °C maximum burial isotherm in the Bluesky Formation reservoir. The sharp decline in level of oil biodegradation to the west was interpreted as an indication of reservoir pasteurization shortly after reservoir charging, due to deeper burial of the reservoirs to the west of 80 °C the maximum burial isotherm, which might have limited biodegradation of the western oils. A selection of oils from

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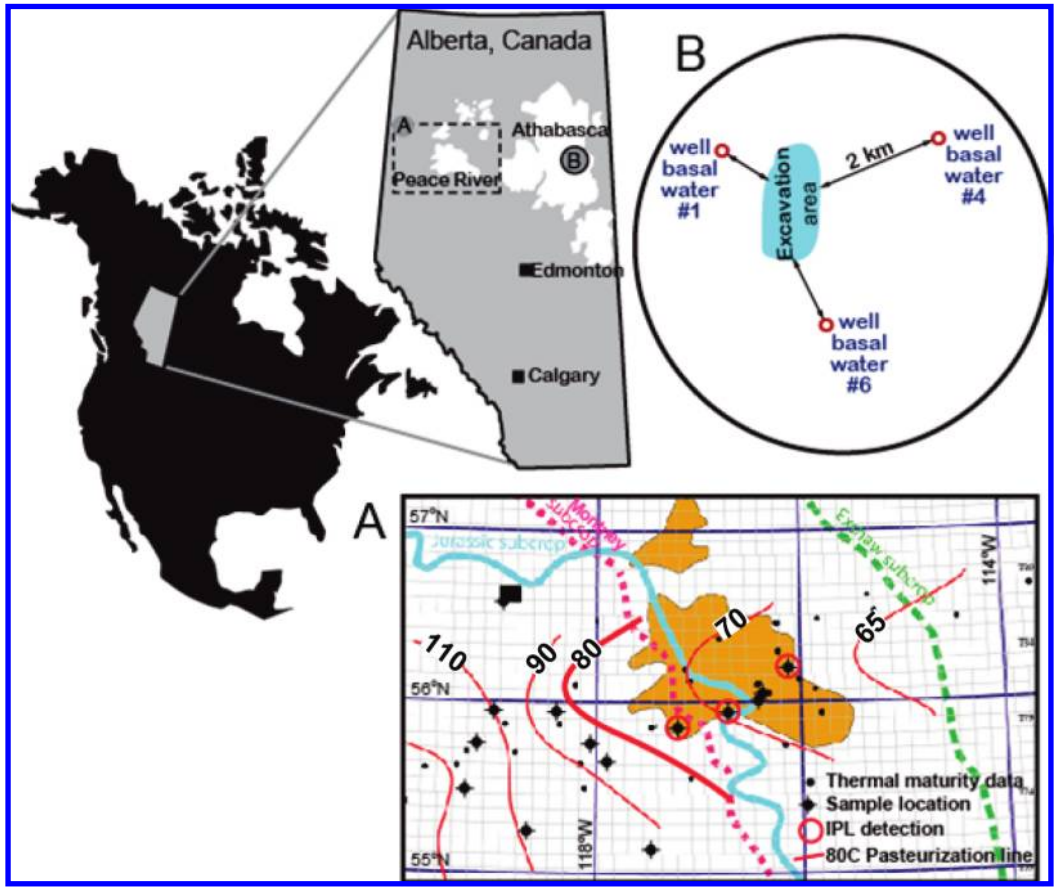

Figure 1. Maps illustrating the sampling areas of (A) wells from the Peace River area and (B) wells from basal aquifers underneath the Athabasca oilsands north of Fort McMurray, both in Alberta, Canada.

Table 1. Conditions and Results of Experiments Made for the Recovery of Microorganisms/IPLs from Oil−Water Mixtures

experiment	water/oil ratio	conditions	all PG + PE [% recovery rel to external standard]	single dominant PG + PE [% recovery rel to external standard]
external standard		culture	100	100
shaker	50/50	170 rpm; 4 h, 30 °C	74.2	74.2
shaker	70/30	170 rpm; 4 h, 30 °C	83.4	83.4
blender	50/50	5 min 20−22K rpm only oil + 5 min 16−18K rpm incl. microorganisms; 50 °C	58.6	58.7
blender	70/30	5 min 20−22K rpm only oil + 5 min 16−18K rpm incl microorganisms; 50 °C	61.6	61.6
ultrasonic probe	70/30	pulse mode 50%; approx 35 W; 8 min, 25 °C	80.8	80.8

both sides of the 80 °C maximum burial isotherm were sampled to investigate the effect of maximum reservoir temperature on microbial populations in the oil reservoirs.

Another set of water samples was obtained from basal aquifers beneath the Athabasca oilsands within 0.5−2.0 km of an excavation area at the Muskeg River oilsands, north of Fort McMurray, Alberta, Canada (Figure 1B). The overlying oilsands are protecting the basal waters from fresh surface water contamination. As the basal waters are in direct contact with the oilsands they can be considered as reservoir oil−water contact (OWC) samples. These water samples did not require any separation. The samples were collected in autoclaved brown bottles without headspace and filtered as described above.

Experimental Approach. To determine into which phase the IPL partition after separation of oil−water mixtures, artificial mixtures of microorganisms and oilfield fluids were created with final concentrations of microorganisms similar to amounts typically measured in the subsurface. Approximately 1.7×10^8 cells (equivalent to 10 000 cells/mL in 17 L of water)[6] were diluted in 100 mL of NaCl containing phosphate buffer and added to oils to produce six two-phase samples with a range of water to oil ratios (Table 1). To simulate emulsions the two-phase samples were treated using three different mixing techniques, i.e., blending, sonicating, and shaking (Table 1). The three techniques cover the full range of mixing intensity from soft two-phase contact mixing (shaking) to vigorous emulsification by high-speed blending. The temperatures of the fluids never exceeded 50 °C during the experiments. The soft mixing experiments were carried out using a shaker for 4 h at 30 °C and 170 rpm. An ultrasonic probe sonicated the samples with a pulse mode of 50% and 35 W for 8

min at 25 °C which was the best compromise between complete mixing without destroying the microorganisms. The high-speed stirring experiments were performed with a blender (Waring, New Hartford, CT). To produce good emulsions, the oil was first blended alone for 5 min at approximately 20 000 rpm. After adding the microorganisms-spiked water, the mixture was stirred for another 5 min at approximately 17 000 rpm and 50 °C until only one foamy phase remained. Of all the methods used, the emulsions generated by blending most closely resembled natural well-head fluids, but destruction of microbial cells during blending cannot be ruled out. The fluid mixtures were allowed to separate for 4 h at 4 °C to minimize microbial growth during phase separation. An external standard (microorganisms-spiked water) was treated in the same way as all water phases after the experiments and used to estimate cell recovery for each of the experimental treatments.

Extraction and Separation. The water phase was separated from the overlying oil in a separating funnel. The water phase was centrifuged at 13 200*g* for 15 min to pelletize the microbial cells. The supernatant was decanted. Residual water associated with the pelleted cells in the centrifuge tubes was removed by freeze-drying.

The freeze-dried microbial cells were spiked with an internal standard (1-*O*-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine; PAF; 20 μL). Pre-extracted organic-free sea sand (4 g) was added to the cell preparations to crush the cells during sonication. Mixtures of methanol, dichloromethane, and a buffer (phosphate buffer (0.05 M) at pH 7.4 or trichloroacetic acid (TCA) buffer (0.3 M) at pH 4.5, respectively) (2:1:0.8 v/v) were added to the cells to extract the IPLs in an ultrasonic bath using a modified Bligh and Dyer method.[21,22] The extraction was repeated three times with each buffer. After sonication for 15 min and centrifugation at 13 200*g* for 15 min after each extraction, the supernatants were collected in a separating funnel. Further dichloromethane and sterile water were added to the mixture to achieve a final methanol/dichloromethane/buffer ratio of 1:1:0.8 to force phase separation. The organic phase was collected, and the overlaying water phase was washed three times with dichloromethane. After removing the organic solvent in a rotary evaporator, the total lipid extracts were separated into a nonpolar fraction and a "glycolipid−phospholipid" fraction. The extracts were loaded on a solid-phase extraction (SPE) column prepacked with 1 g of silica gel (Biotage, Charlottesville, VA). The SPE columns were washed with methanol and dichloromethane and dried overnight prior to use. The nonpolar fraction was eluted with 5 mL of dichloromethane followed by elution of the "glycolipid−phospholipid" fraction with 5 mL of acetone followed by 15 mL of methanol. The "glycolipid−phospholipid" fractions were analyzed for intact membrane lipids by LC−MS.

All organic solvents and deionized, sterile water used were HPLC grade purchased from Fisher Scientific International (Pittsburgh, PA).

LC−MS Analysis. Chromatographic separation of IPLs by high-performance liquid chromatography (HPLC) was done using an adapted normal-phase chromatography procedure described elsewhere.[23−25] This procedure primarily separates lipid mixtures according to their polar headgroups. A LiChrospher Diol column (150 mm, 2.1 mm, 5 μm; Alltech Associates Inc., Deerfield, IL) was fitted with a 7.5 mm × 4 mm guard column of the same packing material and used at 30 °C in a column oven using a ThermoFinnigan Surveyor HPLC system. A linear solvent gradient was used with a flow rate of 0.2 mL min^{-1} as described elsewhere.[25] The gradient program comprised 100% A to 35% A/65% B over 45 min. The solvent composition was held at 35% A/65% B for 20 min. To re-equilibrate the column prior to the next run 100% solvent A was passed through the column for 1 h. Solvent A comprised 79:20:0.12:0.04 (v/v) of hexane/2-propanol/formic acid/14.8 M NH_4(aq), and solvent B comprised 88:10:0.12:0.04 of 2-propanol/water/formic acid/14.8 M NH_4(aq).

MS^n experiments were performed using a ThermoFinnigan LTQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) with an electrospray ionization (ESI) interface. ESI settings were as follows: capillary temperature 275 °C, capillary voltage 42 V, sheath gas flow 8 (arbitrary units), source voltage was set at 5 kV, while other parameters were determined by manual tuning using a solution of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine in solvent A directly infused into the ESI source at a flow rate of 0.2 mL min^{-1} via a T-piece. During routine analyses, the mass spectrometer was configured to run "data-dependent ion tree" experiments where the base peak from each full scan (typically 500−2000 m/z) was fragmented up to MS^3. The "normalized collision energy" (NCE) was typically set to 100%. All samples were analyzed by LC−ESI-MS^n in separate experiments for positive and negative ion modes as this provided complementary structural information. Standards of IPLs (Sigma-Aldrich Inc.) were analyzed to systematize the ionization and fragmentation properties of compound classes of interest. Due to the lack of authentic standards all data treatment remains semiquantitative relative to the internal standard PAF. The percentage recovery of lipids from the mixing experiments was determined relative to the sum of all phosphoethanolamine (PE) and phosphoglycerol (PG) lipids and for the major compound of both compound groups relative to the external standard (same amount of microbial cells in water) which was set to 100% (Figure 2). The coefficient of variation (%CV) of all experiments carried out on the LTQ which includes intra- and interday reproducibility tests was better than 12%. Matrix effects are considered not to be significant due to the precleaning of the "glycolipid−phospholipid" fraction by SPE prior to the separation of the phospholipids by headgroups using HPLC. The linear dynamic range was more than 5 orders of magnitude for standards such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (3 fmol to 1 nmol) with correlation coefficients of 0.995. The limit of detection (LOD) determined at signal-to-noise ratios (S/N) greater than three for phospholipid standard compounds was 2 pg. This is also considered to be the limit of quantitation (LOQ).

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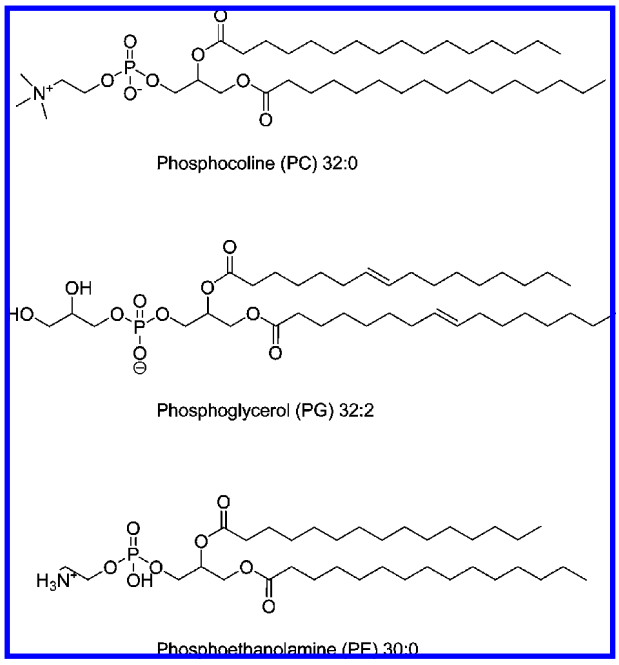

Figure 2. Examples of structures of the dominant IPLs detected in laboratory experiments, well-head fluids, and basal aquifers from petroleum reservoirs from northern Alberta, Canada.

RESULTS AND DISCUSSION

Microorganisms/IPL Recovery Experiments. The fluid mixtures/emulsions separated into two phases within 4 h of mixing for all experiments. The recovery of the water phase was >95% by volume for all experiments indicating that no stable emulsions were created during the experiments. Thus, the analysis of the microorganisms/IPLs from the water phase was not restricted by water (microorganisms) trapped in the oil phase (water-in-oil emulsions). The recovery of IPLs from the oil−water mixtures was between 58% and 84% relative to the aqueous cell suspension. The recovery of IPL decreased strongly with increasing mixing intensity. Whereas the soft mixing experiments with the shaker showed recovery of IPLs relative to the control (external standard) of >80%, the high-intensity mixing experiments with the blender resulted in the detection of approximately half of the IPLs from the added microbial cells (Table 1).

A comparison of the different water to oil ratios in the experiments (Table 1) reveals that the higher the proportion of water relative to the oil, the higher the recovery of microorganisms/IPLs. Although these fluid mixtures were not real oil-in-water or water-in-oil emulsions as commonly produced from some well-heads, these results demonstrate that at least half of the indigenous microbial cells can be expected to be recovered from the water phase of natural fluid mixtures where phase separation is possible.

Microorganisms/IPL Recovery from Natural Well-Head Fluids. *Peace River Oil Fields.* A total of 16 well-head fluids were analyzed. Eleven samples from west and 5 samples from east of the 80 °C maximum burial isotherm in the Bluesky Formation (reservoir) (see Figure 1A) were collected from the Peace River area, Alberta, Canada and analyzed for indications of microbial life in the oil fields. Intact membrane lipids were detected in 3 of the 16 well-head fluid samples. All 11 samples taken from reservoirs with a maximum burial temperature higher than 80 °C (west of pasteurization line; see Figure 1A[20]) did not contain any IPLs. This is consistent with previous observations[3] that basins which experience temperatures higher than 80 °C are sterilized and that petroleum-mineralizing microorganisms do not survive in those environments or recolonize the reservoirs even after uplift to shallower depths and lower temperatures. Three of the five samples to the east of the pasteurization line contained IPLs (Table 2). The two samples with the lowest reservoir temperature (65 °C) contain PE, whereas the well-head fluid at 70 °C contains PG and phosphocholines (PC). No lipids from archaea such as methanogens could be detected which indicates that bacteria rather than archaea dominate in these petroleum reservoirs. Additional DNA analysis is necessary to identify the dominant microorganisms as the knowledge of the diversity and taxonomy of IPL in microorganisms is insufficient. The two additional samples from the eastern part of the Peace River area that yielded no IPLs were highly biodegraded oils, produced as very stable emulsions. These emulsions broke only partly so that the volume of water released from the emulsion was insufficient for IPL analysis. We believe that these two well-head fluids also contained microorganisms, but further method development for breaking strong emulsions is needed to identify microorganisms in those very stable emulsions sometimes found in highly biodegraded fluids.

Nevertheless, this study demonstrates that it is possible to detect membrane lipids of microorganisms from well-head fluids and that IPL analysis is a useful tool to verify the occurrence of microorganisms in petroleum reservoirs. In addition, this study supports the paleopasteurization theory[3] of high-temperate reservoirs (maximum burial temperature >80 °C) in the Peace River area.[20] The reason for nondetection of microbial lipids from deeply

Table 2. Intact Polar Lipids Identified in Basal Aquifers underneath the Athabasca Oilsands and Well-Head Fluids from the Peace River Area, Northern Alberta, Canada[a]

	IPL (core lipid composition)		
samples	PE	PG	PC
basal water no. 4	30:0		
	30:1		
	30:2		
	32:1	32:1	
	32:2	32:2	
	34:2	34:2	
	36:2	36:2	
basal water no. 1	30:0		
	30:1		
	32:1		
	32:2		
	34:2	34:2	
basal water no. 6	30:0		
	30:1		
	32:1	32:1	
	32:2	32:2	
	34:2	34:2	
		36:2	
Peace River (70 °C)		18:2 (lyso)	
			29:0
			30:0
			31:0
		32:0	32:0
		33:1	
		34:2	
Peace River (65 °C)	33:1		
Peace River (65 °C)	33:1		

[a] PE, phosphoethanolamine; PG, phosphoglycerol; PC, phosphocholine. For structures of IPLs see Figure 2.

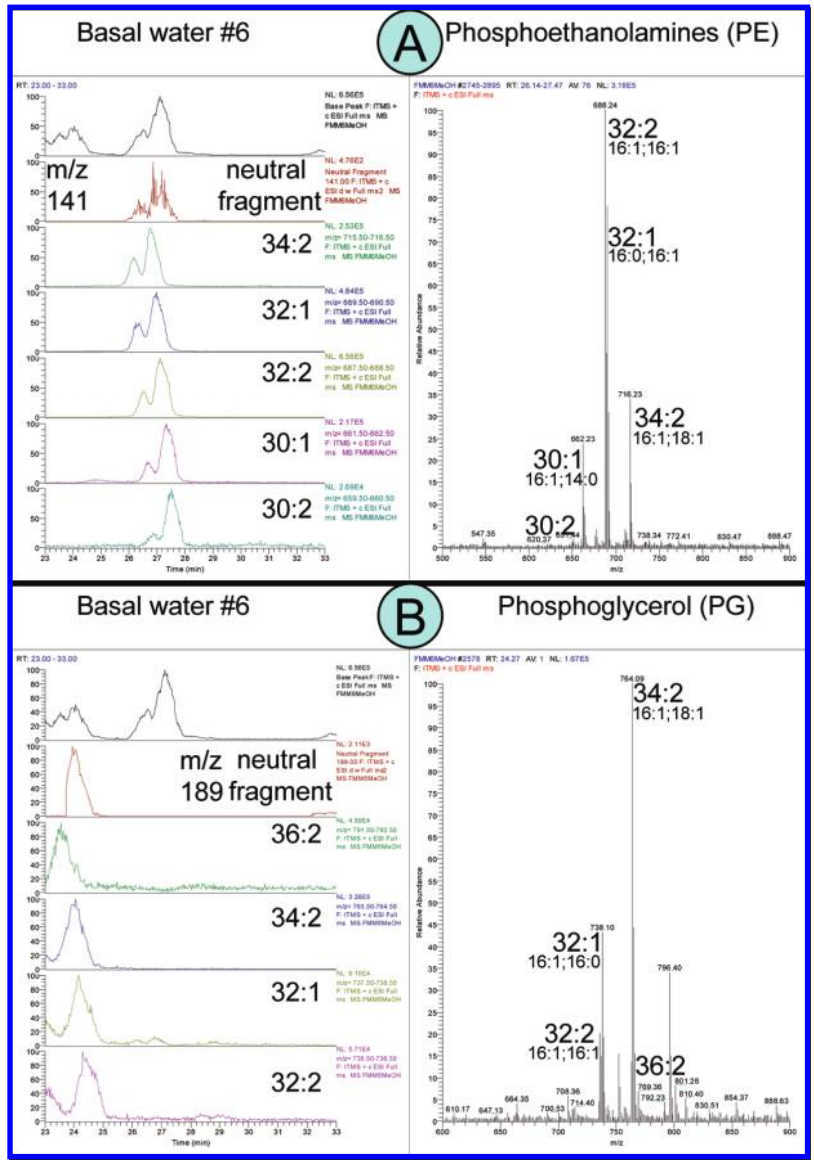

Figure 3. Example of the intact polar lipid distribution of microorganisms detected in the basal aquifers underneath the Athabasca oilsands. The figure illustrates the trace chromatograms and summed mass spectra of IPLs of sample "basal water no. 6".

buried petroleum reservoirs in the western part of the Peace River area is likely due to reservoir pasteurization.

To the best of our knowledge this is the first time that IPLs have been detected from well-head fluids. These studies on natural fluid mixture samples demonstrate that IPL analysis can be used as a fast identification tool of microbial activity for challenging samples such as well-head fluids.

Basal Waters beneath the Athabasca Oil Sands. Three basal water samples were collected 0.5−2 km away from an excavation area in the Athabasca oilsands north of Fort McMurray, Alberta, Canada (Figure 1B). These basal aquifers are situated underneath an 80 m thick oilsand reservoir. All three basal waters contained the same intact polar lipid compounds (phosphatidylethanolamines (PE) and PG) (Figures 2 and 3 and Table 2) indicating that these basal aquifers are connected or the environmental conditions are similar. Archaeal lipids could not be detected which indicates a dominance of bacteria relative to archaea in these waters.

CONCLUSIONS

Experiments to assess the recovery of intact membrane lipid/microorganisms from oil−water mixtures revealed that in produced oil−water mixtures more than half of the sampled indigenous microbial community can be expected to be detected from the water phase after phase separation. The higher the water to oil ratio and the more unstable the produced emulsion, the higher the percentage of microorganisms recovered after phase separation. IPLs were identified in basal waters from below the Athabasca oilsands and reservoir well-head fluids from the Peace River area. Extreme emulsions are still a challenge for detection of microbial membrane lipids as was the case for two highly biodegraded oils produced as stable emulsions from Peace River oil fields.

This intact polar membrane lipid analysis on natural well-head fluids has developed a method to identify microorganisms occurring in oil reservoirs at the oil−water contact zone, to distinguish between archaeal and bacterial microorganisms and their relative quantity. Due to the lack of an extended database of IPL content

of a taxonomically representative range of organisms, and the innately limited taxonomic resolution offered by IPL analysis, IPL analysis only provides a first-order indication of the microorganisms present. However, as the inventory of IPL data from different organisms grows this approach will be able to offer more detailed information about the composition of natural microbial communities.

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