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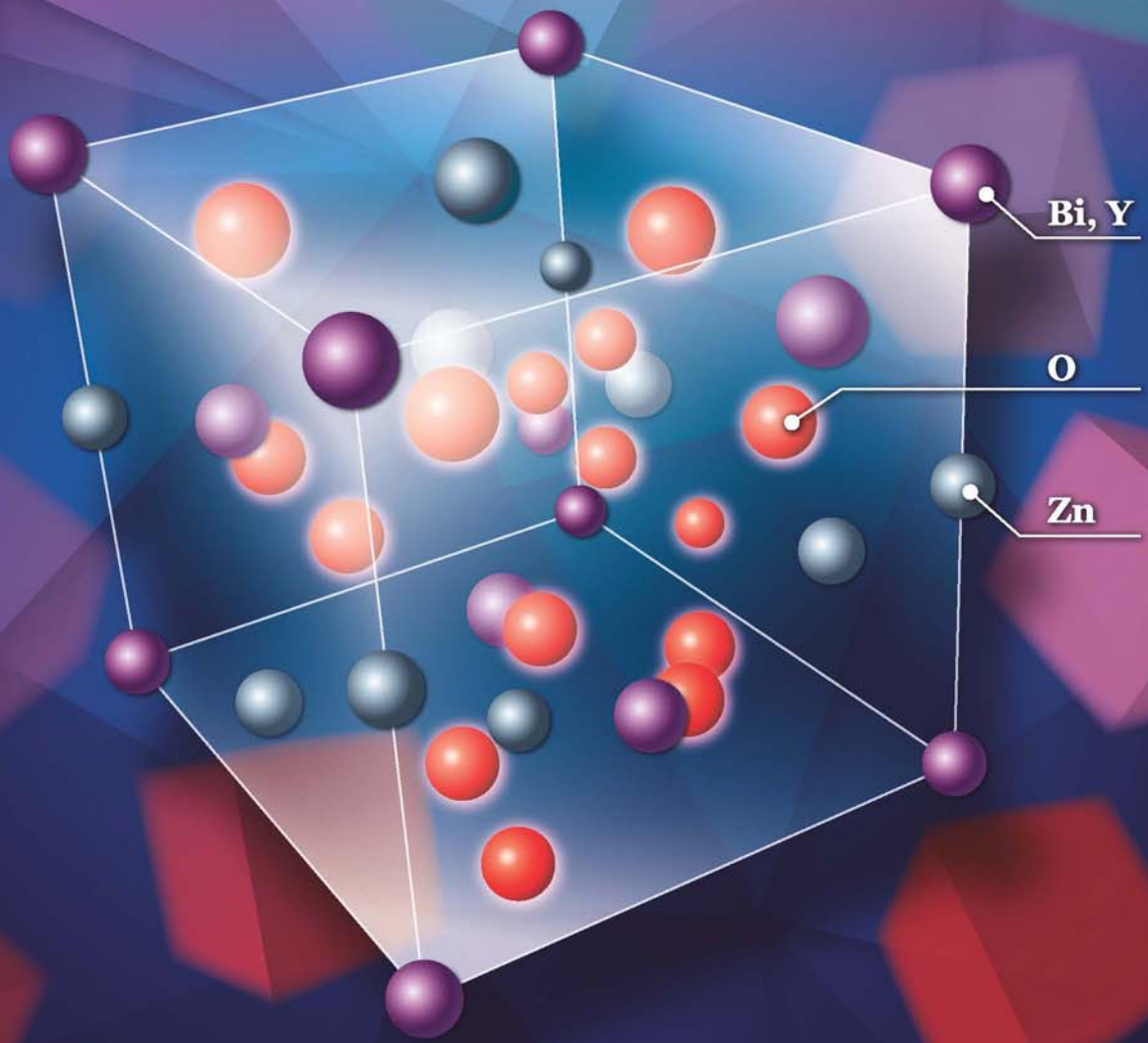

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Soil microbial response to waste potassium silicate drilling fluid

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ABSTRACT

Potassium silicate drilling fluids (PSDF) are a waste product of the oil and gas industry with potential for use in land reclamation. Few studies have examined the influence of PSDF on abundance and composition of soil bacteria and fungi. Soils from three representative locations for PSDF application in Alberta, Canada, with clay loam, loam and sand textures were studied with applications of unused, used once and used twice PSDF. For all three soils, applying ≥40 m^3/ha of used PSDF significantly affected the existing soil microbial flora. No microbiota was detected in unused PSDF without soil. Adding used PSDF to soil significantly increased total fungal and aerobic bacterial colony forming units in dilution plate counts, and anaerobic denitrifying bacteria numbers in serial growth experiments. Used PSDF altered bacterial and fungal colony forming unit ratios of all three soils.

Introduction

Drilling fluid is a lubricant used in drilling oil and gas wells and in exploration drilling rigs. Used drilling fluid properties vary with additives to optimize and improve drilling (Zvomuya et al., 2009). With increased drilling and formation fracturing in the petroleum industry worldwide, unprecedented volumes of used drilling fluids are generated and must be disposed of in an environmentally responsible and cost effective manner. A few studies addressed impacts of used drilling fluids on soil physical and chemical properties and vegetation, but few addressed effects on soil microbial community dynamics. The main effects on soil–water–plant systems are related to high pH, salinity, heavy metals and petroleum hydrocarbons of drilling fluids (McFarland et al., 1992, 1994; Miller et al., 1980; Nelson et al., 1984; Wojtanowicz, 2008; Zvomuya et al., 2008). Soil anaerobic conditions increased as hydraulic conductivity decreased due to high salts and clay particles from drilling fluids (Yao, 2013; Yao et al., 2014; Zvomuya et al., 2009). Some drilling fluid utilizing microorganisms, such as *Alcaligenes* and *Micrococcus*, were isolated in a tropical mangrove swamp oil field (Benka-Coker and Olumagin, 1995). Struchtemeyer et al. (2011) found that adding drilling fluid components to drilling water increased bacterial numbers, culturable aerobic heterotrophs, acid producers and sulfate reducers.

Potassium silicate drilling fluid (PSDF), a water based mud system with organic polymers, high potassium and low sodium content, is becoming popular in major drilling areas (Ghiselin, 2004). Although its use as a soil amendment could negatively affect soil, similar to other drilling fluids, a few studies found no detrimental effects on soil quality and plant growth (Yao, 2013). Whether potentially negative effects are mitigated by natural attenuation is unknown. Adding PSDF to soil increased available potassium and sulfur concentrations that could stimulate indigenous microorganisms, by adding nutrients and oxygen to soil. Organic polymeric substrates and potential petroleum hydrocarbons and metals in PSDF may increase soil organic carbon and energy sources, which highly

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impact soil microbial decomposition processes (Atlas, 1981; Baldi et al., 1996; Lie et al., 1999; Röling et al., 2003). Increasing soil pH, electrical conductivity and sodium adsorption ratio following PSDF application could affect biodegradation efficiency, which can be affected by salinity (Okpokwasili and Odokuma, 1990), electron donors (Achtnich et al., 1995; Chukwuma et al., 2010), electron acceptors (Lovley et al., 1996) and fertilizer (Jobson et al., 1974). Yao et al. (2014) found that PSDF with highly exchangeable potassium and sodium and clay particles decreased soil hydraulic conductivity and increased anaerobic conditions, which in turn affect plant productivity and organic matter and nutrient dynamics (Tiedje et al., 1984). Some anaerobic bacteria have been identified in oil fields, such as sulfate reducing bacteria (Struchtemeyer et al., 2011), nitrate reducing bacteria (Myhr and Torsvik, 2000) and iron reducing bacteria (Greene et al., 1997), but it is not known if these groups were affected by PSDF.

Recycling drilling fluid to drill more than one hole is an important sustainability strategy to reduce waste volume and disposal costs. However, waste volumes increase with the load of rock cuttings and sub-surface water carried to the surface, with limited efficiency of cutting removal during rotary drilling (Wojtanowicz, 2008). These drilling cuttings have potential to change the properties and composition of used drilling fluid. Some research found that drilling fluid preparation and drilling processes introduced exogenous microorganisms into oil and natural gas reservoirs (Struchtemeyer et al., 2011). It is necessary to study soil microbial activity with unused and recycled PSDF to ascertain the influence of recycling PSDF on potential microbial loads.

Research has been conducted on distribution and diversity of microorganisms in remediation of oil based drilling fluid contaminated soil, but not on used PSDF as a soil amendment. We hypothesized that PSDF application could influence soil microbial community dynamics. The soil microbial community plays a major role in processing organic wastes and recycling nutrient constituents in PSDF. To test this hypothesis, we sought to determine whether soil biota (anaerobic and aerobic bacteria and fungi) were impacted by unused PSDF, recycled PSDF or rate of PSDF application; and determine the relationship between microorganism and soil response to PSDF application.

1. Material and methods

1.1. Experimental design

A replicated laboratory experiment was established using a complete randomized design with treatments representing reclamation scenarios. The same soil and drilling fluid were used in previous greenhouse and laboratory experiments (Yao, 2013; Yao et al., 2014). Soils were collected from three locations in Alberta, Canada, where drilling was active. Unused PSDF was provided by the supplier (Marquis Alliance Ltd., Canada) and the same used drilling fluids were collected from active well sites. All fluids were refrigerated until used. Soil and drilling fluid properties (chemical and physical) were determined by a commercial laboratory (Exova Laboratory Group, Canada).

Soil samples were air dried, sieved to remove large particles, then ground to <2 mm. Available nitrate (NO_3^-) and ammonium (NH_4^+) were determined by extraction with 2.0 mol/L KCl (Carter and Gregorich, 2008); available phosphorus and potassium by modified Kelowna extraction (Ashworth and Mrazek, 1995); and available sulfate by extraction with 0.1 mol/L $CaCl_2$ (McKeague, 1978). Cation exchange capacity was determined by exchange with ammonium acetate at pH 7 (McKeague, 1978). Total nitrogen was determined by Kjeldahl digestion distillation (Bremner, 1996). Total carbon was determined by dry combustion and total organic carbon by Walkley–Black wet dichromate oxidation (Nelson and Sommers, 1996). Water soluble cations (sodium, calcium, potassium, magnesium), pH, sodium adsorption ratio and electrical conductivity were determined from saturation paste extracts (Carter and Gregorich, 2008). Sulfate and chloride were determined by ion chromatography with chemical suppression (Clesceri et al., 1992). Hydrocarbon fractions (F1, F2, F3, F4) were from gas chromatographic results with flame ionization (CCME, 2001). Trace metals restricted in concentration by the Canadian Council of Ministers of the Environment (silver, arsenic, barium, beryllium, cadmium, cobalt, chromium, copper, mercury, molybdenum, nickel, lead, antimony, selenium, tin, thallium, uranium, vanadium, zinc) were determined by inductively coupled plasma (ICP) following strong acid digestion (USEPA, 2008) and hot water soluble boron was determined by azomethine-H method (McKeague, 1978). Sand, silt and clay were determined by hydrometer after treatment with calgon (Carter and Gregorich, 2008).

Three soil textures, sand, loam and clay loam, covered a range of soils with potential for reclamation using drilling fluids in Alberta. Three types of PSDF were unused, used once and used twice. Soil and PSDF were mixed at PSDF rates of 0, 40 and 120 m^3/ha (equivalent to 0, 15 and 45 mL PSDF per kg of dry soil, respectively), which were developed around the current regulated maximum disposal rate in summer (40 m^3/ha) for Alberta. Mixed PSDF with soil was stored in polypropylene bags at room temperature (25 to 27°C) for 2 weeks to allow time for soil microorganisms to respond to PSDF. The three types of PSDF without soil were also analyzed.

1.2. Enumeration of sulfate reducing, denitrifying and iron reducing bacteria

Numbers of culturable sulfate reducing, denitrifying and iron reducing bacteria in PSDF treatments were determined using most probable number (MPN) dilutions (Cochran, 1950). All MPN experiments were performed with quintuplicate serial dilutions from 10^{-1} to 10^{-5} by using 16 × 150 mm glass tubes and serially diluting 1.0 mL of sample into 9.0 mL of an appropriate sterile medium. Sulfate reducing bacteria were enumerated using a medium of 1 L distilled water with 0.5 g of K_2HPO_4, 1 g of NH_4Cl, 2.0 g of Na_2SO_4, 1.5 mL of 60% and 1.0 g of yeast extract (BD Difco™, Mississauga, Ontario, Canada) with pH 7.1 to 7.2 (Butlin et al., 1949). Denitrifying bacterial growth media was prepared by dissolving 5 g of KNO_3 and peptone in 1 L distilled water with pH adjusted to 7, excluding agar in the original formula (Aaronson, 1970). Iron reducing bacteria growth media was prepared by dissolving 0.5 g of NH_4SO_4, 0.5 g of Na_2SO_4, 0.1 g of K_2HPO_4, 1.0 g of $MgSO_4{\cdot}7H_2O$, 5 g of ferric ammonium phosphate and 5 g of nutrient broth into 1 L distilled water (Aaronson, 1970).

Replicated dilutions were incubated at 21°C for 7 days before initial assessment. Sulfate reducing bacteria, denitrifying bacteria

and iron reducing bacteria were enumerated in their respective selective culture media. Individual treatment dilution tubes were scored positive if they turned black for sulfate reducers from production of ferrous sulfide, if they produced visible gas bubbles in Hungate tubes for denitrifying bacteria as they produced nitrous oxide, and if they displayed a visible color change for iron reducing bacteria during ferric iron reduction to ferrous iron and precipitation. Observations were repeated every 7 days for 21 days.

1.3. Enrichment and quantification of heterotrophic bacteria and fungi

Changes in aerobic heterotrophic microbial numbers were determined using a serial dilution spread plate count technique. Total heterotrophic aerobic bacteria and actinomycetes were enumerated on plate count agar (Gilman and Abbott, 1957) and fungi on rose bengal malt extract agar (Ottow and Glathe, 1968). Both agars were sterilized in an autoclave at 121°C for 30 min before dispensing into petri plates. All plates were cooled and dried for 24 hr, then refrigerated until used.

Soil inocula were prepared by adding 10 g of each soil, put through a flame sterilized sieve, into a 90 mL phosphate buffer solution blank (10^{-1} dilution) and shaken for 40 min in a laboratory shaker. Shaking time was based on a previous test with soil samples shaken for increasing 10 min periods up to an hour, after which serial dilutions were completed, 0.1 mL samples were plated, and colony forming units (CFUs) of bacteria were counted to verify if numbers were no longer increasing or decreasing due to shaking times. After this step, 10 mL of soil solution was added to another 90 mL phosphate buffer blank (10^{-2} dilution) and then shaken by hand for 15 sec. These 10^{-2} diluted aliquots were serially diluted to 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}, with 15 sec shaking periods at each dilution.

Quadruplicate plates for each dilution of a soil sample were prepared. A 100 μL volume of a given dilution was inoculated onto each plate and uniformly distributed with a sterile glass spreader over the agar surface. Final plated dilutions for plate count agar were 10^{-5}, 10^{-6} and 10^{-7}. Plated dilutions for rose bengal malt extract agar were 10^{-3}, 10^{-4} and 10^{-5}. Replicate plates were placed cover side down in plastic bags to avoid evaporation and incubated in the dark at 21°C for 6 days before assessment. CFUs were cumulatively counted each week over a 4-week period. Colonies were scored by hand using marks on the back side of the plates to accurately count.

A composite subsample from each soil was used to measure soil water content. Subsamples were weighed, oven dried at 80°C for 24 hr and reweighed. A dry weight factor was calculated for each soil and used to estimate number of CFUs per gram of dry soil. Mean number of bacteria and fungi CFUs from each soil sample was multiplied by its dry weight factor. Morphological types of fungi, bacteria and actinomycetes on each replicate plate at the counting dilution were described based on shape (regular, irregular, conical, round), color and texture (glossy, opaque, velvety).

1.4. Identification of bacteria and fungi

Examples of each morphologically distinct colony type from the counted dilution of plate count and rose bengal agar were picked and struck on fresh individual agar slants. Isolates were stored on slants of plate count agar and rose bengal malt extract agar for bacteria and fungi, respectively. A small portion of each fungal colony growth was picked with a sterile needle and placed on separate glass slides containing a drop of lactophenol cotton blue (Gilman and Abbott, 1957). A cover slip was applied and the slide examined under 4 to 40 times power objectives. Fungi were identified to genus based on morphological features and arrangement of spores and fruiting bodies as observed under microscopy following standard determinative schemes (Watanabe, 2010).

Bacterial isolates representing the most abundant members of the mixed population were classified to genus on the basis of the following tests. For gram reaction, a dilute bacterial smear on a glass slide was heat fixed then sequentially stained using standard gram stain protocols (Gram, 1884).

For gram negative bacteria an oxidase test was conducted to determine whether isolates were oxidase positive or negative. Oxidase positive bacteria were inoculated into Oxi/Ferm strips (BD BBL™, Oakville, Ontario, Canada) and oxidase negative into Enterotube (BD BBL™, Oakville, Ontario, Canada) strips. After overnight incubation, color and gas production in strip chambers were observed to indicate positive or negative results for the presence of a particular enzyme. Each positive result was used in generating a five digit number, which identified bacteria based on the Oxi/Ferm or Enterotube code book (Gilardi, 1985; Lennette et al., 1985).

1.5. Statistical analyses

Microbial counts (MPN, CFU) for each soil were logarithmically transformed and all data subjected to analysis of variance (ANOVA). The influence of PSDF recycled times on microbial properties was determined by conducting ANOVA on pooled measurements across rates by generalized linear model (GLM) procedure. Means for PSDF type and rate effects were separated by a least significant difference (LSD) test at the 5% level and planned orthogonal contrasts where appropriate. Regression models were used to test relationships between fungal:bacterial ratios and individual soil parameters. Distribution of the taxonomic composition of the soil microbiota associated with PSDF and soil texture was identified in an ordination using correspondence analysis, a multivariate statistical technique, since taxonomic composition is categorical rather than continuous (Greenacre, 2010). The taxonomic distance among genera was assessed using Hellinger distance calculated from scores of the first two axes (Legendre and Gallagher, 2001). Position of the treatments and genera indicate the relationship between microbiota and environmental variables, such as soil and PSDF. Analyses and graphics were performed with R software (R Development Core Team, 2012).

2. Results

2.1. Soil and PSDF

Selected baseline soil and PSDF chemical properties prior to treatment are presented in Table 1. Only trace elements above the provincial limit for agriculture and natural land use (Alberta

Table 1 – Selected chemical properties of soil and potassium silicate drilling fluids (PSDF).

Property	Soil type			PSDF type		
	Clay loam	Loam	Sand	Unused	Used once	Used twice
Hydrogen ion activity (pH)	7.9	4.8	6.5	11.4	10.8	10.7
Electrical conductivity (dS/m)	2.0	1.4	0.1	14	62	44
Sodium adsorption ratio	9.9	0.1	0.3	7.1	867	436
Potassium (mg/kg)	10.0	12.3	0.3	6033	19,200	9647
Sulfate (mg/kg)	127	5.1	0.5	BDL	3017	2437
Nitrate nitrite (mg/kg)	2.9	83.2	1.6	0.4	7.2	BDL
Total C_6–C_{10} (mg/kg)	BDL	BDL	BDL	ND	90	116
Total C_{11}–C_{40+} (mg/kg)	460	590	13	ND	2677	3828
Boron (mg/kg)	1.0	0.5	0.0	0.1	5.1	9.6
Barium (mg/kg)	203	222	59	0.3	9430	4803

ND: not detected; BDL: below detection limit.

Environment and Sustainable Resource Development, 2010) and the federal guidelines for agricultural, residential and parklands (CCME, 2000, 2007) are shown. Unused PSDF was comprised of 1 m^3 fresh water with 6 pregelatinized starch, 2 polyanionic cellulose, 1 anionic water soluble polymer, 2 xanthan gum, 3 potassium hydroxide and 5.5 raw silicate (all units kg/m^3) (Ma, 2008). Unused PSDF density was 1137 kg/m^3, equivalent to a dry bulk density of 220 kg/m^3, as determined by the manufacturer. Most notably, unused PSDF had high pH and electrical conductivity and did not contain hydrocarbons.

2.2. Sulfate reducing, denitrifying and iron reducing bacteria

Sulfate reducers were not detected in pure PSDF (without soil) except in used twice PSDF (Table 2). The recipient soil and PSDF type had significant impacts on sulfate reducer MPN values. There was no response of sulfate reducing bacteria numbers to PSDF application in sand soil and unused PSDF in all three soils. Between used once and twice PSDF, sulfate reducers had no significant response in clay loam and loam soil.

All three soil substrates had a resident denitrifying population; addition of PSDF to soil significantly stimulated nitrate reducing bacteria, relative to controls without PSDF (Table 2). Small numbers of denitrifying bacteria (18 cells per gram of PSDF) were detected in used twice PSDF without soil (pure PSDF) but not in unused and used once pure PSDF. Relative to pure PSDF, denitrifiers increased 1000 to 10,000 fold per gram of PSDF soil mix. PSDF type variably impacted nitrate reducers. There was no significant difference between unused and used once PSDF dosed soils on denitrifiers, but adding used once PSDF to clay loam and sand soil significantly simulated the denitrifying population relative to that with used twice PSDF and soil mixes. PSDF application rate effects on nitrate reducers were not significant.

In pure PSDF, only used twice PSDF had detectable iron reducers; growth was stimulated when PSDF was mixed with soil (Table 2). PSDF type effects varied with soil. Clay loam soil with unused PSDF had significantly lower iron reducing populations than other used PSDF types and the control. There was no significant difference in loam soil. Mixing used twice PSDF in sand soil, significantly stimulated iron reducer growth over that in other PSDF sand treatments. MPN estimates of iron reducing bacteria were not significantly stimulated with increasing PSDF rate.

2.3. Fungi and bacteria colony forming units

PSDF type significantly affected heterotrophic bacterial abundance (Table 3). Growth of aerobic heterotrophic bacteria was significantly stimulated in soil with used PSDF relative to no

Table 2 – Viable counts of specific anaerobic bacteria groups in PSDF, control soils and soils with drilling fluid (unit: MPN/g).

	Substrate	No PSDF	PSDF type		
			Unused	Used once	Used twice
Sulfate reducers	Pure PSDF		ND	ND	7×10^2
	Clay loam	2×10^1	ND	6×10^1	2×10^1
	Loam	2×10^1	ND	4×10^1	5×10^1
	Sand	ND	ND	ND	ND
Nitrate reducers	Pure PSDF		ND	ND	2×10^1
	Clay loam	$5 \times 10^{2\ c}$	$5 \times 10^{5\ ab}$	$9 \times 10^{5\ a}$	$2 \times 10^{4\ b}$
	Loam	$3 \times 10^{4\ b}$	$2 \times 10^{5\ a}$	$1 \times 10^{6\ a}$	$3 \times 10^{5\ a}$
	Sand	$3 \times 10^{2\ c}$	$9 \times 10^{5\ ab}$	$2 \times 10^{6\ a}$	$4 \times 10^{5\ b}$
Iron reducers	Pure PSDF		ND	ND	2×10^2
	Clay loam	$5 \times 10^{2\ a}$	$5 \times 10^{1\ b}$	$4 \times 10^{2\ a}$	$3 \times 10^{2\ a}$
	Loam	1×10^5	5×10^5	8×10^5	5×10^4
	Sand	$3 \times 10^{3\ b}$	$5 \times 10^{3\ b}$	$6 \times 10^{3\ b}$	$1 \times 10^{4\ a}$

ND: not detected.
Means followed by the letter within a row are significantly different using LSD test at the 5% level.

Table 3 – Colony forming units (CFUs/g) and species of microorganisms in soil with PSDF.

	Substrate	No PSDF	PSDF type		
			Unused	Used once	Used twice
Bacteria	Pure PSDF		ND	$1.1 \times 10^{7\ b}$	$3.9 \times 10^{8\ a}$
	Clay loam	$3.6 \times 10^{8\ b}$	$2.3 \times 10^{8\ b}$	$8.5 \times 10^{8\ a}$	$4.7 \times 10^{8\ ab}$
	Loam	$9.7 \times 10^{7\ c}$	$1.5 \times 10^{8\ bc}$	$2.6 \times 10^{8\ ab}$	$2.7 \times 10^{8\ a}$
	Sand	$6.6 \times 10^{7\ d}$	$5.1 \times 10^{8\ c}$	$1.1 \times 10^{9\ a}$	$7.4 \times 10^{8\ b}$
Fungi	Pure PSDF		ND	2.5×10^{3}	ND
	Clay loam	$2.0 \times 10^{4\ c}$	$2.4 \times 10^{5\ a}$	$2.7 \times 10^{5\ a}$	$6.6 \times 10^{4\ b}$
	Loam	$6.6 \times 10^{4\ b}$	$1.2 \times 10^{6\ a}$	$4.9 \times 10^{5\ a}$	$6.8 \times 10^{5\ a}$
	Sand	$4.3 \times 10^{3\ c}$	$6.4 \times 10^{4\ b}$	$4.4 \times 10^{5\ a}$	$3.8 \times 10^{4\ b}$
Species of microorganisms		Actinomycete *Achromobacter* sp. *Bacillus* sp. *Rhodococci* sp. *Aspergillus* sp. *Ascomycota* sp. *Cephaliophora* sp. *Cephalosporium* sp. *Cladosporium* sp. *Gonatobotrys* sp. *Trichoderma* sp.	Actinomycete, *Achromobacter* sp., *Agrobacterium* sp., *Bacillus* sp., *Providencia* sp., *Pseudomonas* sp., *Rhodococci* sp.		
			Aspergillus sp., *Aureobasidium* sp., *Cephalosporium* sp., *Cladosporium* sp., *Fusarium* sp., Gonatobotrys sp., *Penicillium* sp., Trichoderma sp.		

ND: not detected.
Means followed by the letter within a row are significantly different using LSD test at the 5% level.

PSDF, except clay loam soil with used twice PSDF. Application of unused PSDF to sand soil significantly stimulated aerobic bacterial abundance relative to pure soil. The increased bacterial population with pure PSDF provided evidence that repeated use of drilling fluid significantly increased viable bacteria CFU counts. No aerobic bacteria were detected in pure unused PSDF, and its addition to soil did not significantly increase bacterial growth relative to a control soil except sand soil. Used once PSDF with soil stimulated 10 to 100 fold more aerobic bacterial growth than pure used once PSDF. PSDF application rate had significant stimulation effects on bacterial counts in sand soil (Fig. 1a). Both PSDF application rates had significantly increased bacterial populations relative to a control soil, except clay loam soil with PSDF.

Adding PSDF to soil significantly stimulated a 10 to 100 fold increase in fugal abundance in soil relative to no PSDF (Table 3). Relative to pure PSDF, used once PSDF with soil showed a 100 fold fungal CFU increase. Only pure used once PSDF demonstrated some fungal presence. PSDF application rate impacted fungal abundance significantly (Fig. 1b). Increasing PSDF application rate significantly increased the soil fungal population in clay loam and loam soil. The lower rate of PSDF in sand soil had the highest fungal abundance relative to other rates in sand soil.

2.4. Relationship with soil properties

Bacterial counts were naturally much higher than fungal counts in all treatments when determined as CFUs (Table 3). The fungal to bacterial count ratio calculated using CFUs varied from 0.00001 to 0.003, with a tendency to increase with significantly increasing soil organic carbon ($R^2 = 0.52$, $p < 0.01$; Fig. 2c) and extractable hydrocarbon C_{11}–C_{40+} ($R^2 = 0.54$, $p < 0.01$; Fig. 2d); and to slightly decrease with increasing soil pH ($R^2 = 0.36$, $p < 0.01$; Fig. 2b). There was a positive linear relationship between soil nitrogen availability ($R^2 = 0.32$, $p < 0.01$; Fig. 2a), or soil potassium availability ($R^2 = 0.29$, $p < 0.01$; data not shown) and ratio of fungal and bacterial counts.

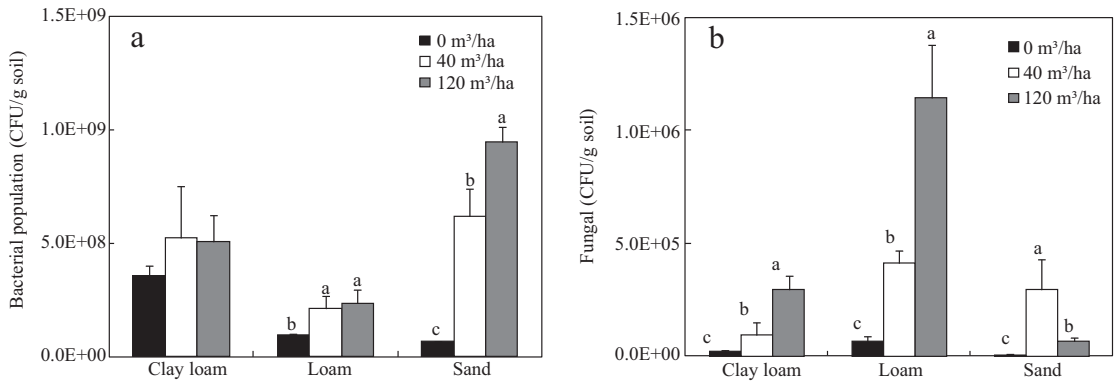

Fig. 1 – Bacterial (a) and fungal (b) populations in soils amended with potassium silicate drilling fluids (PSDF) at three rates. Vertical bars correspond to one standard error. Means are grouped according to the LSD test at $p < 0.05$.

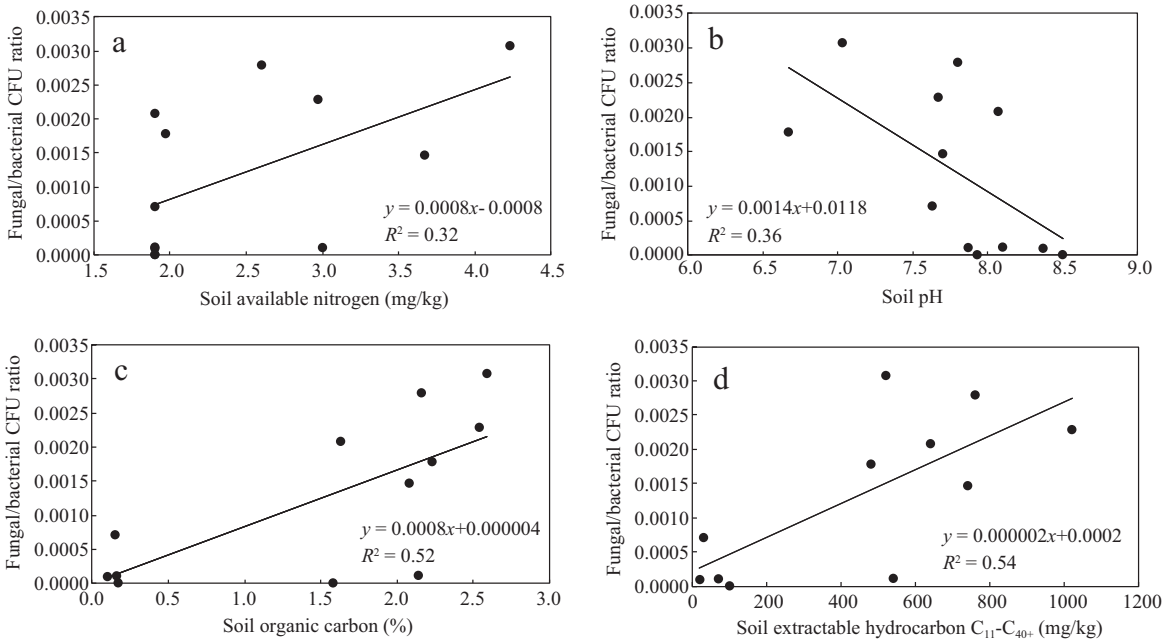

Fig. 2 – Effect of soil available nitrogen (a), pH (b), organic carbon (c) and extractable hydrocarbon (d) in treatments with used PSDF on the relationship between fungal and bacterial colony forming units (CFUs).

2.5. *Analysis of microbial communities in soils mixed with PSDF*

PSDF in soil significantly increased abundance of detected bacterial genera and diversity of fungal genera isolated in soil relative to soils with no PSDF (Table 3). Isolated bacterial genera were identified as *Achromobacter*, *Agrobacterium*, *Bacillus*, *Providencia*, *Pseudomonas* and *Rhodococcus*. Fungi were identified as members of *Aspergillus*, *Ascomycota*, *Aureobasidium*, *Cephaliophora*, *Cephalosporium*, *Cladosporium*, *Fusarium*, *Gonatobotrys*, *Penicillium* and *Trichoderma* genera. These isolates were morphologically distinct colony types picked from the counting dilution of each dilution series and thus represent the most numerous bacterial and fungal isolates in each soil type under controlled conditions and under PSDF influence.

Correspondence analyses showed that microbial communities in soil with PSDF were grouped by soil type (Fig. 3). All groups of treatments, which displayed short gradients on their own, were associated with characteristic microbial genera. No significant separation by PSDF type was observed. *Bacillus* was the most common bacteria genus in PSDF and soil treatments. A total of 30 soil and PSDF treatments were used to generate this ordination, and 82.7% of the microbial genera variation due to soil and PSDF is explained in the first two axes. This analysis indicates that in each soil type, soil microbial composition was similar between the control and with PSDF. Relative to pure PSDF without soil treatments, clay loam soil with used twice PSDF had a similar microbial community composition.

3. Discussion

Drilling had a significant effect on the population and diversity of soil microorganisms by introducing microorganisms into PSDF waste and increasing the population of sulfate reducing, denitrifying, iron reducing and aerobic bacteria. As there were no microorganisms in unused PSDF, microorganisms potentially originated from the drilling process. Struchtemeyer et al. (2011) deduced that drilling mud was responsible for introducing microorganisms into oil and gas reservoirs. In our study, data before and after drilling showed that drilling was the main origin of microorganisms in drilling fluid, and the number of

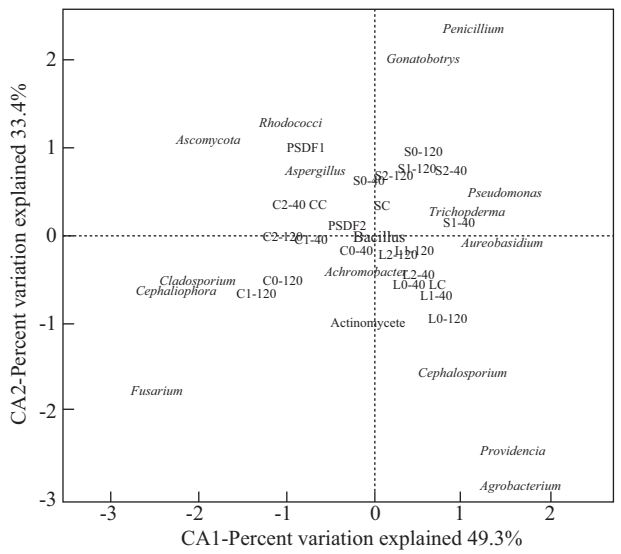

Fig. 3 – Correspondence analysis of microbial communities in PSDF and soil. C, L and S: clay loam, loam and sand soil; 0, 1 and 2: unused, used once and used twice PSDF; 40 and 120: PSDF loading rate at 40 and 120 m^3/ha.

times that the drilling fluid recycled increased the opportunity for introduction of exogenous microorganisms.

This work showed that PSDF type had a significant impact on populations of sulfate reducers in soil mixed with PSDF. Results of MPN analysis showed addition of unused PSDF to soil resulted in no introduction of sulfate reducing bacteria. Barium sulfate could serve as a source of sulfate for bacteria that reduced sulfate to sulfide with PSDF and soil mixing. Unused PSDF has very low barium (0.3 mg/kg) and sulfate (<85 mg/kg) relative to used PSDF barium (4800–9400 mg/kg) and sulfate (2400–3000 mg/kg). Magot (2005) found that biogenic sulfide production by microorganisms in oil fields induced a number of problems, including reservoir plugging, souring and corrosion of metal containing equipment, but microorganism origin and food resource were unclear. Baldi et al. (1996) and Struchtemeyer et al. (2011) found that barite and sulfonates appeared to stimulate sulfide producing bacteria. Thus it was not surprising that sulfate reducers were not detected in sand soil with low barium and sulfate concentrations relative to the other two soils. As drilling fluids generally contain high concentrations of sulfate and sulfate reducers produce organic acids and H_2S which can be harmful to plant productivity, regulations limited sulfate loading rates when land spraying drilling fluids to cultivated land (Saskatchewan Ministry of Energy and Resources, 2011).

Significant stimulation of denitrifier growth with PSDF relative to no PSDF in soil is likely due to organic matter and petroleum hydrocarbons in PSDF. Denitrifying bacteria can grow aerobically without nitrate or use nitrate as an electron acceptor for growth in the absence of oxygen. Since the amount of nitrate carried from PSDF is low, the stimulated number of denitrifying bacteria was not likely due to nitrate in PSDF under aerobic conditions. Previous work showed that clay dispersion by potassium and sodium and clogged pores when mixing PSDF in soil significantly impeded saturated hydraulic conductivity and ponded water on the soil surface (Yao et al., 2014). Land application of PSDF decreases oxygen diffusion into soil, which is necessary for formation of nitrite and nitrous oxide, thus stimulating denitrification. Similarly, large amounts of organic polymers and starch substrates added to PSDF could act as energy and carbon sources. Several groups of microorganisms, including polymer degraders, sugar or monomer metabolizers and volatile fatty acid degrading bacteria degrade organic polymeric substrates (McInerney et al., 1981) and were detected in drilling muds (Struchtemeyer et al., 2011). With increasing PSDF recycling, total petroleum hydrocarbons will increase in used PSDF, which can supply potential carbon resources for all bacteria including denitrifiers. Previous work showed that petroleum hydrocarbons served as potential electron donors as fatty acids, hydrogen and carbon resources for denitrifying bacteria (Altenschmidt and Fuchs, 1991; Ball et al., 1996; Evans et al., 1991; Fries et al., 1994; Rabus and Widdel, 1995; Schocher et al., 1991). However, denitrification caused soil nitrogen availability loss and could have detrimental effects on revegetation in reclamation. Nitrogen is a key nutrient for microbial growth and hence for organic matter decomposition. This stimulation of denitrifying bacteria suggests the necessity for nitrogen fertilizer inputs to enhance soil nitrogen availability for plant growth when utilizing PSDF as a soil amendment.

Depletion of oxygen and nitrate may account for the microbiological ferric reduction. Increased soil anaerobic conditions following PSDF application will lead to ferric reduction. Another possible mechanism for transformation is for iron to function as an electron acceptor in cell respiration, analogous to reduction of nitrate by denitrifying bacteria. Organic media in PSDF could bring a portion of added ferric oxide into solution (Lovley and Lonergan, 1990). Therefore, those factors possibly resulted in a reduced ability to detect significant differences in iron reducers among treatments as the other two anaerobic bacteria MPN results.

Adding used PSDF to soil significantly stimulated population and abundance of total aerobic bacterial and fungal microorganisms due to potential growth substrates in PSDF. With increasing PSDF recycling, total petroleum hydrocarbons will increase; used twice PSDF had increased petroleum hydrocarbon fractions F1 to F4 (C_6–C_{40}) to almost 4000 mg/kg from 0 mg/kg in unused PSDF. Distribution of *n*-alkanes in used PSDF was attributed to their selective utilization by bacteria stimulation. The bacteria species and population detected in used once PSDF without soil and their significant increase when mixed with soil, indicate potential for bioaugmentation with addition of external microbial populations from PSDF to soil (Okoh, 2003).

The relatively higher bacterial CFU counts (10^8–10^9 per gram dry soil) than fungal CFUs (10^4–10^6 per gram dry soil) in all treatments indicated that the microbial community is a bacterial dominated food web with PSDF application. However, the bulk of CFUs appearing on agar is from spore forming species. It is possible that PSDF suppressed fungal sporulation, thereby understating actual fungal activity in this altered soil environment. The negative relationship between soil pH and fungal:bacterial ratio showed that high pH and salinity from PSDF are more favorable for bacteria (Bååth and Anderson, 2003). The occurrence of *Bacillus*, a known alkaliphile, in all used PSDF treatments indicated the increase in pH associated with PSDF stimulated alkaliphilic microorganisms. Other research showed that nitrogen fertilizer is a critical factor for hydrocarbon contaminated soil biodegradation and indigenous microbial activity enhancement (Xu and Obbard, 2003). Organic matter is an important contributor to biodegradation as it provides nutrients and carbon substrate to microorganisms (Bento et al., 2005). The trend of slightly increasing fungal:bacterial count ratio with increasing potassium availability suggests that potassium silicate in PSDF potentially provides more benefit to the soil fungal based food web. Most factors for fungal:bacterial ratio are related to nutrient availability. *Aspergillus* spp. had high solubilization potential for potassium and potassium aluminum silicate (Bin et al., 2008). The occurrence of *Aspergillus* in all used PSDF treatments indicated that the increase in potassium associated with PSDF application potentially stimulated a fungal based soil food web.

Since microbial isolates were morphologically distinct colony types from each counting dilution they, by probability, represent the most numerous bacteria and fungi in each soil type under controlled conditions and under PSDF influence. With used PSDF without soil actinomycetes, *Bacillus*, *Rhodococcus* and *Aspergillus* genera were isolated. The ability to degrade hydrocarbons is widespread in *Rhodococcus* (Warhurst and Fewson, 1994) which play a significant role in the fate of organic pollutants in the environment and could be useful for treatment of contaminated soils or effluents (Bell et al., 1999). In PSDF with soil, genera of *Agrobacterium*, *Providencia*, *Pseudomonas*,

Aureobasidium, *Fusarium* and *Penicillium* were stimulated relative to soils without PSDF. Actinomycete, bacterial genera *Bacillus* and *Pseudomonas* and fungal genera *Fusarium* and *Penicillium* can utilize drilling fluid waste as a carbon source (Chen et al., 2009; Nnubia and Okpokwasili, 1993; Struchtemeyer et al., 2011). The presence of *Fusarium* genera with PSDF in clay loam soil could indicate slight growth in ferric salts without oxygen and acidic soil (Gunner and Alexander, 1964). *Agrobacterium*, a heterotrophic bacteria, could be stimulated by PSDF as an energy source (Bergey and Breed, 1989). *Providencia* are gram negative bacteria, commonly found in soil, water and sewage. *Aureobasidium* is cosmopolitan black yeast like fungus, widely distributed in hypersaline habitats (Nagahama, 2006). Increased pH with PSDF in sand soil (Yao et al., 2014) could stimulate growth of these organisms. These results suggest that bioaugmentation with microbial species from used PSDF may promote degradation and increased plant establishment and development in soil with used PSDF.

Loading rate of PSDF variably impacted colony forming units of microorganisms as soil properties interfered. Soil texture with PSDF was strongly correlated with microbial composition. Spatially diverse soils with the same soil texture had nearly identical bacterial communities, as soil texture is important in determining soil water and nutrient status (Girvan et al., 2003; Lauber et al., 2008). Lauber et al. (2008) found that soils with extractable phosphorus had similar fungal communities. In our study, baseline soil texture, pH and available nutrients may have a significant impact on bacterial and fungal communities in addition to PSDF impact on soil for land reclamation.

Some questions remain regarding reliability of results obtained with cultural methods derived from soil microbiology standards, because the PSDF soil mix matrix is very different from the soil matrix. Cultural methods present some biases due to the capacity of microorganisms to be cultivated and to the specificity of the growing media. Molecular methods like qPCR could avoid these biases. But an efficient DNA extraction can be difficult to obtain in such a solid matrix especially for minor microorganisms. Both methods could be complementary.

4. Conclusions

This initial study indicates that a wide range of soil textural classes with PSDF applied at ≥40 m^3/ha significantly affected the existing microbial flora in the soils. PSDF has potential as a soil amendment for reclamation, especially for low quality soils. Utilizing used PSDF with soil stimulated soil microbial populations and increased diversity of the microbial community relative to treatments without PSDF. It is possible to use PSDF as soil amendments for reclamation and as a disposal option, but substrate properties, PSDF type and rate will need to be considered.

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