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POTENTIAL HYBRIDIZATION OF BIOREMEDIATION AND DESALINATION: A PRELIMINARY STUDY FOR SALT-TOLERANT BACTERIA

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Abstract: Microbes and minerals interact in nature. The compatibility of microbial metabolisms with reactive mineral treatments may allow useful environmental processes to be further engineered for the removal of multiple contaminants. We have focused on hybridizing bioremediation and desalination processes to remove hydrocarbons, sodium and chloride from contaminated oilfield produced water, oilfield wastes and impacted soils and groundwater. The main remedial agents in this combined remediation strategy are native salt-tolerant hydrocarbon-degrading bacteria (hydrocarbon biodegradation) and functionalized mineral amendments (desalination). Some indigenous hydrocarbon degraders in oilfield soils are halotolerant and can be metabolically active in both the absence and presence of salts (NaCl), unlike halophiles, which require salt to grow. These halotolerant populations are potentially beneficial when combining bioremediation and desalination, as the salt concentration varies. Our preliminary live cell image analysis indicated the significant survival of representative salt-tolerant hydrocarbon degraders subjected to over 5% salinity stresses in the presence of hydrocarbons, which potentially suggests the feasibility of developing hybrid bioremediation and desalination strategies for oilfield site remediation. Our study has continuously advanced to exploring the compatibility of the characterized salt-tolerant hydrocarbon-degraders (bioremediation agent) with the positively and negatively charged minerals for desalination.

1 INTRODUCTION

Extractions of subsurface hydrocarbon energy resources such as heavy oil, oilsands, coalbed methane, and shale gas produce a large volume of saline industrial effluent (e.g., drilling fluid) and processed wastes containing liquid hydrocarbons, salts, and chemical additives (Arocena and Rutherford 2005). It is estimated that about 1.7 billion barrels (0.3 billion m^3/year) of oilfield effluent is disposed of in earthen storage facilities such as drilling sumps, tailing ponds, and abandoned flare pits, in association with oil and gas productions in Alberta, Canada (Hum et al. 2006). The salinity of oilfield brine or produced water largely depends on geological formations, and thus varies widely (Collins 1975). Processed soils and wastes contaminated with petroleum hydrocarbons at oilfield waste management areas are commonly additionally contaminated by salts due to the co-disposal of oilfield brine that often contains large quantities of salts (Riis et al. 2003). Excess discharges of highly saline oilfield effluent adversely impacts water quality, soil health, plants and aquatic organisms. Regulations for the treatment and disposal of oilfield produced water and drilling fluid have been established to protect land, water resources, and ecosystems surrounding oil extraction and waste management areas.

However, the accidental spillage, leakage and disposal of untreated oilfield produced water occurs during the production, processing and transportation of oil and gas in Canada (Ulrich et al. 2009). Soils surrounding oil production sites and oilfield waste management areas can be impacted by hydrocarbons and salts (Princz et al. 2012). Physical, chemical and biological remediation technologies have been in demand for

dealing with saline oilfield effluent and soils contaminated with petroleum hydrocarbons and salts. Bioremediation has often been considered a cost-effective, feasible, and minimally destructive remediation technology for petroleum hydrocarbon-contaminated soils and groundwater (Camarillo and Stringfellow 2018), and bioremediation of petroleum hydrocarbon-contaminated soils under environmentally stressed conditions (e.g., low temperature and high salinity) have been extensively studied (Chang et al. 2018, Kim et al. 2018).

On the other hand, we have reported the effectiveness of an engineered dual-mineral adsorbent treatment that uses a combination of calcined layered double hydroxides (LDH) and activated zeolites for the removal of sodium and chloride from brine-impacted groundwater (Gibb et al. 2017). The chemical modification of clay materials can be used to produce adsorbents with improved material properties, including surface chemistry, interlayer spacing, surface areas, and pore spaces, as shown in a large number of publications (Paul et al. 2017). Various chemical modifications to increase the number of reaction sites and improve clay surface reactivity have been developed, including acid activation, biopolymer-based activation and plasma treatment (Gibb et al. 2018). Furthermore, combinations of physical, chemical and biological remediation technologies have attempted to improve the treatability of oilfield wastes and areas impacted by multiple contaminants. We are currently focusing on hybridizing bioremediation and mineral-based desalination for saline contaminated oilfield soils and water. The two key elements of the hybrid treatment are salt-tolerant bacteria capable of degrading hydrocarbons during desalination and minerals that can rapidly incorporate salt cations (e.g., Na^+) and anions (e.g., Cl^-) into their mineral structure, through adsorption, ion exchange, mineral reconstruction, and/or stabilization.

In terms of bioremediation, the co-presence of salts along with hydrocarbons generally represents an additional environmental stress and may hinder hydrocarbon biodegradation activity. However, many oil reservoirs and oilfield waste are saline, and indigenous halotolerant and halophilic hydrocarbon-utilizing microorganisms have been identified in oilfield environments (Fathepure 2014). Halotolerant bacteria can be active in absence and presence of high salinity stresses and are different from halophilic bacteria, which require salts to grow, (Fathepure 2014). Diverse salt-tolerant hydrocarbon-degrading bacteria obtained from various natural and engineered saline environments, including oilfields and mine sites, have been reported in literature (Margesin and Schinner 2001). Halotolerant microorganisms and halophiles have received significant attention for developing environmental remediation strategies and technologies (Margesin and Schinner 2001). Briefly, several diverse genera have been documented as halotolerant or halophilic, including *Halomonas*, *Alcanivorax*, *Marinobacter*, *Dietzia*, *Oleiphilus*, *Oleispira*, *Bacillus*, *Geobacillus* and *Streptomyces* (McGenity and Timmis 2010).

These salt-tolerant hydrocarbon-degrading bacteria capable of degrading hydrocarbons during desalination are key elements of the combined bioremediation and desalination process. Using a live cell imager, this preliminary study aims to assess the potential survival, in the co-presence of salt and hydrocarbons, of hydrocarbon-degrading *Dietzia maris* FTI08 isolated from saline mine tailings. We also tested whether oil droplets inhibit live cell image analyses, which is critical for live cell counting in presence of hydrocarbon liquids. Hydrocarbon-degrading *Dietzia maris* FTI08 is adapted to high salinity and may be useful for treating oilfield-impacted saline soils and groundwater.

2 MATERIALS AND METHODS

2.1 Hydrocarbon-degrading bacteria and culturing

We isolated *Dietzia maris* FTI08 from potash mine tailings, which were highly saline (>10% w/v) (Harris 2017). JS-P2 was isolated from heavy oil-impacted soils. These bacterial isolates grow well in liquid Bushnell Hass (BH) media spiked with 1% diesel (v/v), indicating that these bacteria are hydrocarbon-degraders. The BH nutrient media was adopted for further microbial cell experiments (Section 2.2).

2.2 Hydrocarbon liquid droplet test

The potential negative effect of oil droplets on the live cell imaging and counting were examined. Three cell-counting sets to test the effect of oil droplets: (1) abiotic BH nutrient medium without diesel or bacteria; (2)

abiotic BH nutrient medium with 1% (v/v) diesel and without bacteria; and (3) *D. maris* FTI08-inoculated BH nutrient media without diesel. The solutions collected from the three sets were used for the live cell analysis described in Section 2.3.

2.3 Microbial cell experiments and live cell analysis

The microbial cell experiment was conducted in 250-mL Erlenmeyer flasks containing 100 mL of liquid BH medium with 1% diesel (v/v) and 0.217 M of NaCl (Na^+ concentration of 5000 mg/L and Cl^- concentration of 7711 mg/L). Bacterial cells were inoculated into each of the flasks by adding 1 mL of each of the sub-cultures of the two bacterial isolates, *D. maris* FTI08 and JS-P2. The flasks were incubated at room temperature and agitated at 150 rpm for fourteen days. The number live cells in each flask was counted using a live/dead cell-counting analyzer, JuLI™ (JuLI™, NanoEnTek Inc., Seoul, Korea). During the incubation of the shaking bacterial isolates, 10 µL of the cell cultures were homogenously extracted from the flasks and injected into the counting slide of the cell-counting instrument. Live cell numbers were expressed per mL. The JuLI™ analyzer uses two modes: Bright (Br) mode to asses cell viability using trypan blue staining and Fluorescence (FL).

3 RESULTS AND DISCUSSION

3.1 Effect of hydrocarbon droplets on cell images and counts

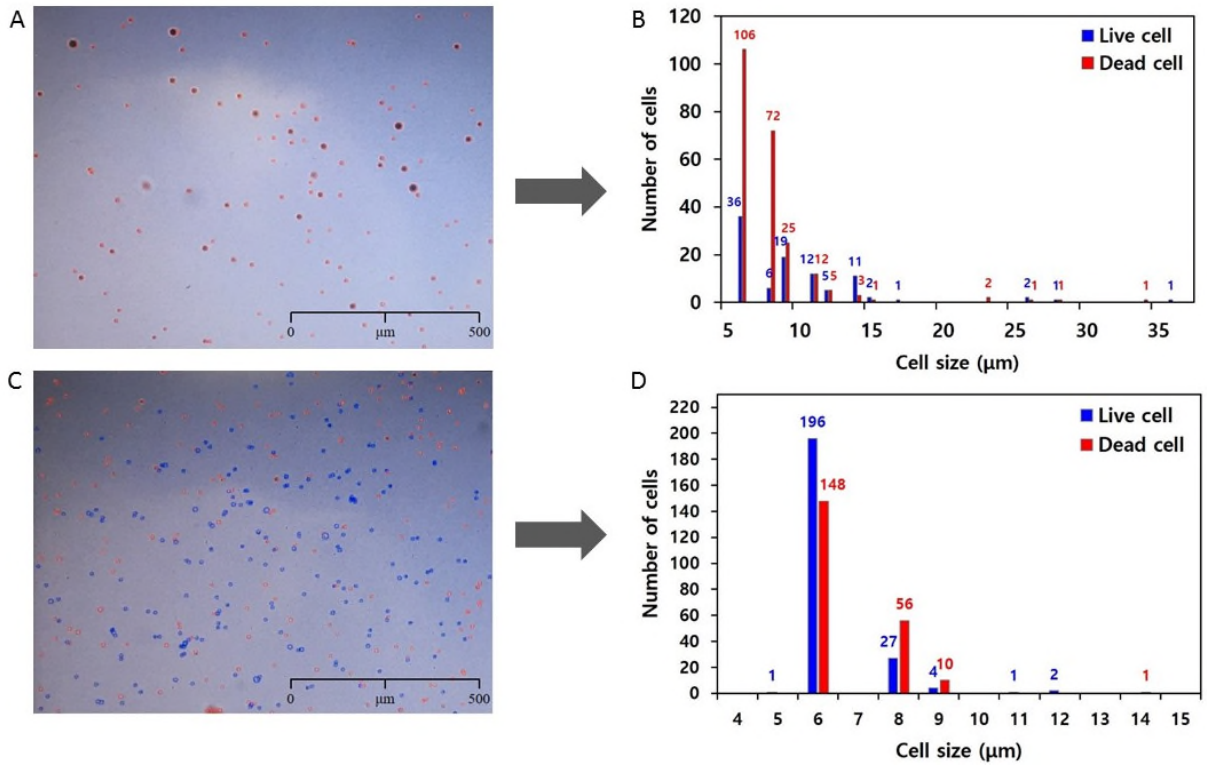

Figure 1: The effect of hydrocarbon droplets on the live cell image analyses. (A) The cell images from abiotic BH nutrient medium with 1% (v/v) diesel and (B) the corresponding cell count. (C) The cell images from *D. maris* FTI08-inoculated BH nutrient media without diesel and (D) the corresponding cell count.

Stable or quasi-stable hydrocarbon droplets likely form after rigorous shaking or vortexing during microbial culturing and microbial cell-related testing, and the presence of hydrocarbon droplets in the aqueous microbial suspension may cause errors when counting or observing cell numbers using microscopic cell

imaging analyses, including bacterial adhesion tests (Zoueki et al. 2010). The significant zeta potential at non-polar hydrocarbon liquid-water interfaces has been measured in previous studies (Clasohm et al. 2007). It has been speculated that the adsorption onto hydrocarbon droplets of hydroxyl ions and co-existing anions in the aqueous solution, and weak van der Waals forces between droplets, may contribute to the stability of oil droplets in aqueous solutions (Marinova et al. 1996). Moreover, microbial colloids may cause the stabilization of hydrocarbon droplets in aqueous solutions. Therefore, we have examined the effect of hydrocarbon droplets on the live cell images and counting.

Figure 1 presents the effect of hydrocarbon droplets on the cell images and counting. Hydrocarbon droplets are most likely observed as red stains, which is expressed as the dead cell colour in the cell image analyses (Fig. 1A and 1B). Thus, hydrocarbon droplets did not interfere with the number live cells, which were indicated as round trypan blue strains (Fig. 1C and 1D). Therefore, within our current scope, our live cell analysis and corresponding set-up could successfully visualize and count viable cells in the BH nutrient solution. The calibrated live cell analysis will eventually act as a rapid assessment tool for the viability of salt-adapted hydrocarbon degraders for developing the hybrid bioremediation and desalination process. However, live cell analyses for more diverse hydrocarbon degraders, as well as the corresponding calibration and setup, under various experimental conditions should be further examined.

3.2 Viability of salt-tolerant hydrocarbon-degrading bacteria

Using the calibrated cell analyzer, cell concentrations under the controlled culturing condition were quantified in the range of 10^4 to 10^7 cells/mL. The sizes of the cells were measured and in the range of 5 to 60 µm. The cell analysis was conducted using the aqueous cultures/samples extracted on the 6th day of incubation. In the *Dietzia maris* FTI08-inoculated BH media spiked with 1% (v/v) diesel and 0.217 M, the salinity level is resembles that of salinized groundwater in North America. Given the saline experimental conditions, it appeared that significant numbers of *D. maris* FTI08 were alive and detectable across all cell imaging options: JuLI-Br (Fig. 2A), JuLI-FL (Fig. 2B) and live/dead cell count (Fig. 2D). Majority of the live-cell sizes were between 5 and 10 µm (Fig. 3). Larger live cells near 35 µm were detected. The confluence that covers live cells on the image was estimated at 0.10% through the live-cell analyzer (Fig. 2C). Based on the live-cell image, cell count and cell sizes, *Dietzia maris* FTI08 were tolerant to the high salinity and hydrocarbon liquids.

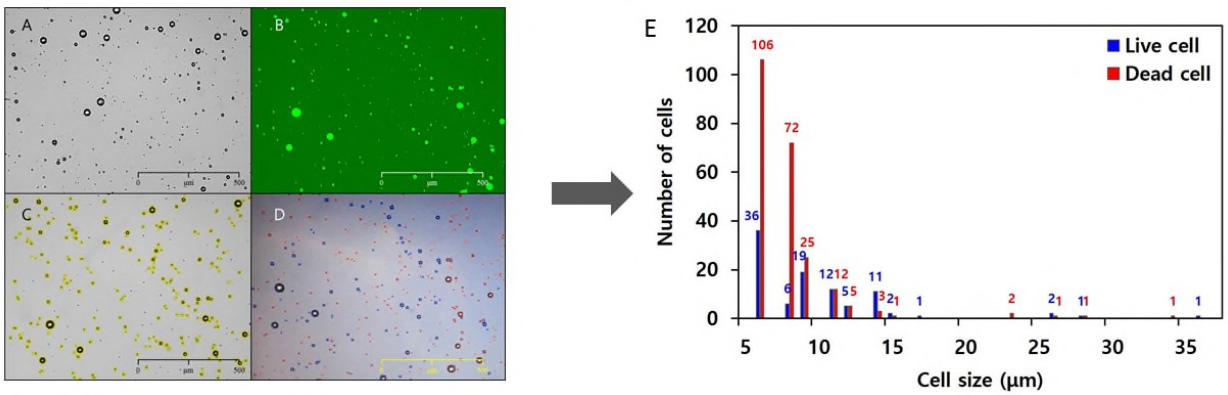

Figure 2: The result of live- and dead-cell analyses of *D. maris* FTI08 on the 6th day of incubation using JuLI-Br/FL. (A) JuLI-Br, (B) JuLI-FL, (C) confluence (0.10%), (D) live and dead cell counting by JuLI-Br (Blue: Live cell, Red: Dead cell), and (E) the cell counts of live and dead cells.

Conversely, JS-P2 cells (hydrocarbon degrader) from the same BH medium spiked with 1% (v/v) diesel under the same highly saline conditions, were not detected, suggesting that JS-P2 are not tolerant to high salinity (Fig. 3). Table 1 compares the cell concentrations and cell numbers of *Dietzia maris* FTI08 and JS-P2 under the saline conditions. The number of live JS-P2 cells (4 cells) was much smaller than the number of live *Dietzia maris* FTI08 (96 cells). Total cell numbers (live cell + dead cells) of *Dietzia maris* FTI08 and JS-P2 cells were 325 and 21, respectively. The results suggest that *Dietzia maris* FTI08 may be worth considering as a bioremediation agent for the proposed hybrid bioremediation and desalination.

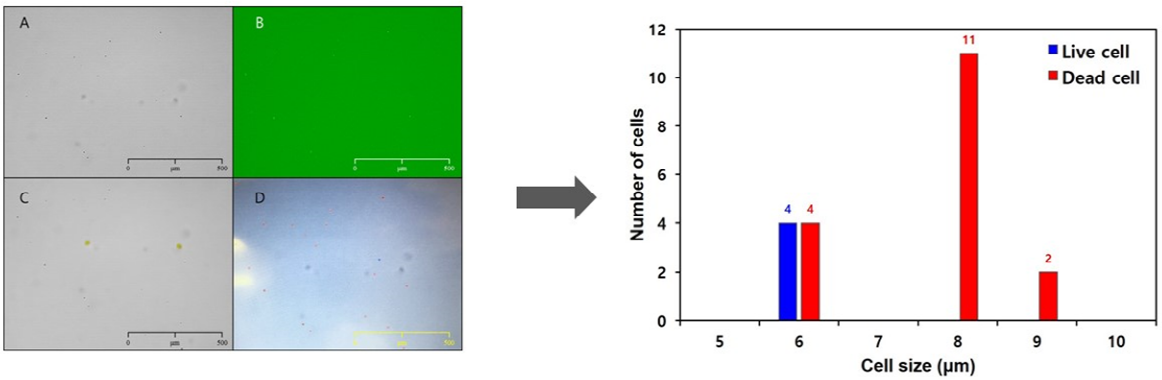

Figure 3: The results of live- and dead-cell analyses of JS-PS2 on the 6th day of incubation using JuLI-Br/FL. (A) JuLI-Br, (B) JuLI-FL, (C) confluence (0.10%), (D) live and dead cell counting by JuLI-Br (Blue: Live cell, Red: Dead cell), and (E) the cell counts of live and dead cells.

Table 1: Comparison of *Dietzia maris* FT108 and JS-P2 on the 6th day of incubation (JuLI-Br)

	Dietzia maris FTI08			JS-P2		
	Concentration	% Cells	Cell count	Concentration	% Cells	Cell count
Total cells	5.4 x 10^6/mL		325	1.7 x 10^5/mL		21
Live cells	1.6 x 10^6/mL	29.5	96	3.3 x1 0^4/mL	19.1	4
Dead cells	3.8 x 10^6/mL	70.5	229	1.4 x 10^5/mL	81.0	17
Avg. cell size	8.89 μm			6.82 μm		

Table 2: *Dietzia maris* FT108 on the 15th day of incubation (JuLI-Br)*

	Dietzia maris FTI08		
	Concentration	% cells	Cell numbers
Total cells	4.7 x 10^6/mL	100	283
Live cells	1.6 x 10^6/mL	33.2	94
Dead cells	3.1 x 10^6/mL	66.8	189
Avg. cell size	7.18 μm		

*The cell counting was obtained with a dilution of 1/10.

Table 2 presents the cell analysis of *Dietzia maris* FTI08 of the 15th day of incubation, indicating the consistent viability of *Dietzia maris* FTI08 under the highly saline conditions. In the literature, *Dietzia maris* is a versatile halotolerant hydrocarbon degrader. For example, *Dietzia maris* NW WC 4 isolated from a Canadian oilfield in the Northwest Territories exhibits significant halotolerance from low to high salinities (>5%) in the presence of hydrocarbon liquids (Chang et al. 2018). *Dietzia maris* strains 7824 A and 4-3 grow optimally in the absence of NaCl and at relatively lower salinities, below 2% NaCl (Borzenkov et al. 2006). Halotolerant oil-degrading *Dietzia maris* strain DSM 43672^T grows well between 0 and 8% NaCl (Nazina et al. 2015). Halophilic oil-degrading *Dietzia* sp. strain 2610-K grows optimally at the high salinity of 12% NaCl, but grows minimally at 5% NaCl and requires NaCl for growth (Plakunov et al. 2008). In addition, some halotolerant hydrocarbon degraders such as *Dietzia maris, Marinobacter hydrocarbonoclasticus*, *Alcanivorax borkumensis* and *Bacillus* sp. are capable of producing biosurfactant and thus emulsifying oil in saline conditions (Fernandez-Linares et al. 1996, Yakimov et al. 1998, Kumar et al. 2007, Nakano et al. 2011). The aqueous solubility of hydrocarbons decreases with increasing salinity and decreasing temperatures (Whitehouse 1984). Therefore, producing biosurfactant may be advantageous for halotolerant hydrocarbon degrader for enhancing the bioavailability/abundance of hydrocarbons in the aqueous phase.

4 CONCLUSIONS

Our preliminary live cell analysis indicated the significant survival and flexibility of representative halotolerant hydrocarbon degraders subjected to high salinity stresses in the presence of hydrocarbon liquids. We indicated that *Dietzia maris* FTI08 isolated from hypersaline mine tailings is viable in highly saline water (Na^+ concentration of 5000 mg/L; and Cl^- concentration of 7711 mg/L) in the presence of hydrocarbons (1% diesel, v/v). The reference microbial cells (JS-PS2), isolated from heavy oil-contaminated soils, did not exhibit salt tolerance under the same culturing conditions. The well controlled live-cell analysis can be used as a rapid tool for measuring the viability of *Dietzia maris* FTI08 in intact aqueous samples collected from microbial culturing systems. This will be very useful in further optimizing the metabolic activity of *Dietzia maris* FTI08 as a biological agent for the hybrid bioremediation and desalination process. We are currently continuing our research to explore the compatibility between the biological and mineral remedial agents. We are investigating how *Dietzia maris* FTI08 behaves during the sequential removal of chloride and sodium ions from saline water using the positively and negatively charged mineral adsorbents, and are producing promising results toward a combined bioremediation and desalination process for the removal of hydrocarbons, Na^+ and Cl^-.

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References

Arocena, J. M., and P. M. Rutherford. 2005. Properties of Hydrocarbon- and Salt-Contaminated Flare Pit Soils in Northeastern British Columbia (Canada). *Chemosphere* **60**: 567-575.

Borzenkov, I. A., E. I. Milekhina, M. T. Gotoeva, E. P. Rozanova, and S. S. Belyaev. 2006. The Properties of Hydrocarbon-Oxidizing Bacteria Isolated from the Oilfields of Tatarstan, Western Siberia, and Vietnam. *Microbiology* (Moscow) **75**: 66-72.

Camarillo, M. K., and W. T. Stringfellow. 2018. Biological Treatment of Oil and Gas Produced Water: a Review and Meta-Analysis. *Clean Technologies and Environmental Policy* **20**: 1127-1146.

Chang, W., A. Akbari, C. A. David, and S. Ghoshal. 2018. Selective Biostimulation of Cold- and Salt-Tolerant Hydrocarbon-Degrading *Dietzia maris* in Petroleum-Contaminated sub-Arctic Soils with High salinity. *Journal of Chemical Technology & Biotechnology* **93**: 294-304.

Clasohm, L. Y., I. U. Vakarelski, R. R. Dagastine, D. Y. C. Chan, G. W. Stevens, and F. Grieser. 2007. Anomalous pH Dependent Stability Behavior of Surfactant-Free Nonpolar Oil Drops in Aqueous Electrolyte Solutions. *Langmuir* **23**: 9335-9340.

Collins, A. G. 1975. *Geochemistry of Oilfield Waters*. American Elsevier Publishing Compancy, Inc., New York, NY, USA.

Fathepure, B. Z. 2014. Recent Studies in Microbial Degradation of Petroleum Hydrocarbons in Hypersaline Environments. *Frontiers in Microbiology* **5**.

Fernandez-Linares, L., M. Acquaviva, J.-C. Bertrand, and M. Gauthier. 1996. Effect of Sodium Chloride Concentration on Growth and Degradation of Eicosane by the Marine Halotolerant Bacterium *Marinobacter hydrocarbonoclasticus*. *Systematic and Applied Microbiology* **19**: 113-121.

Gibb, N. P., J. J. Dynes, and W. Chang. 2017. Synergistic Desalination of Potash Brine-Impacted Groundwater Using a Dual Adsorbent. *Science of The Total Environment* **593-594**: 99-108.

Gibb, N. P., J. J. Dynes, and W. Chang. 2018. A Recyclable Adsorbent for Salinized Groundwater: Dual-Adsorbent Desalination and Potassium-Exchanged Zeolite Production. *Chemosphere* **209**: 721-729.

Harris, N. 2017. Hypersaline Potash Mine Tailings and Brine: Microbial Communities and Metal Biosorption Applications. M.Sc. Thesis, University of Saskatchewan.

Hum, F., P. Tsang, T. Harding, and A. Kantzas. 2006. *Review of Produced Water Recycle and Beneficial Reuse*. Paper No. 19 of the Alberta Energy Futures Project. The Institute for Sustainable Energy, Environment and Economy. Calgary, AB, Canada.

Kim, J., A. H. Lee, and W. Chang. 2018. Enhanced Bioremediation of Nutrient-Amended, Petroleum Hydrocarbon-Contaminated Soils over a Cold-Climate Winter: The Rate and Extent of Hydrocarbon Biodegradation and Microbial Response in a Pilot-Scale Biopile Subjected to Natural Seasonal Freeze-Thaw Temperatures. *Science of The Total Environment* **612**: 903-913.

Kumar, M., V. León, A. De Sisto Materano, and O. A. Ilzins. 2007. A Halotolerant and Thermotolerant *Bacillus* sp. Degrades Hydrocarbons and Produces Tensio-Active Emulsifying Agent. *World Journal of Microbiology and Biotechnology* **23**: 211-220.

Margesin, R., and F. Schinner. 2001. Potential of Halotolerant and Halophilic Microorganisms for Biotechnology. *Extremophiles* **5**: 73-83.

Marinova, K. G., R. G. Alargova, N. D. Denkov, O. D. Velev, D. N. Petsev, I. B. Ivanov, and R. P. Borwankar. 1996. Charging of Oil−Water Interfaces Due to Spontaneous Adsorption of Hydroxyl Ions. *Langmuir* **12**: 2045-2051.

McGenity, T. J., and K. N. Timmis. 2010. *Halophilic Hydrocarbon Degraders*. Pages 1939-1951 *in* T. KN, Editor. Handbook of Hydrocarbon and Lipid Microbiology. Springer, Berlin, Germany.

Nakano, M., M. Kihara, S. Iehata, R. Tanaka, H. Maeda, and T. Yoshikawa. 2011. Wax Ester-Like Compounds as Biosurfactants Produced by *Dietzia maris* from n-Alkane as a Sole Carbon Source. *Journal of Basic Microbiology* **51**: 490-498.

Nazina, T. N., E. S. Shumkova, D. S. Sokolova, T. L. Babich, M. V. Zhurina, Y.-F. Xue, G. A. Osipov, A. B. Poltaraus, and T. P. Tourova. 2015. Identification of Hydrocarbon-Oxidizing *Dietzia* Bacteria from Petroleum Reservoirs Based On Phenotypic Properties and Analysis of the 16S rRNA and gyrB Genes. *Microbiology* **84**: 377-388.

Paul, B., J. J. Dynes, and W. Chang. 2017. Modified Zeolite Adsorbents for the Remediation of Potash Brine-Impacted Groundwater: Built-In Dual Functions for Desalination and pH Neutralization. *Desalination* **419**: 141-151.

Plakunov, V. K., M. V. Zhurina, and S. S. Belyaev. 2008. Resistance of the Oil-Oxidizing Microorganism *Dietzia* sp. to Hyperosmotic Shock in Reconstituted Biofilms. *Microbiology* (Moscow) **77**: 515-522.

Princz, J. I., M. Moody, C. Fraser, L. Van der Vliet, H. Lemieux, R. Scroggins, and S. D. Siciliano. 2012. Evaluation of a New Battery of Toxicity Tests for Boreal Forest Soils: Assessment of the Impact of Hydrocarbons and Salts. *Environmental Toxicology and Chemistry* **31**: 766-777.

Riis, V., S. Kleinsteuber, and W. Babel. 2003. Influence of High Salinities on the Degradation of Diesel Fuel by Bacterial Consortia. *Canadian Journal of Microbiology* **49**: 713-721.

Ulrich, A., S. Guigard, J. Foght, K. Semple, K. Pooley, J. Armstrong, and K. Biggar. 2009. Effect of Salt on Aerobic Biodegradation of Petroleum Hydrocarbons in Contaminated Groundwater. *Biodegradation* **20**: 27-38.

Whitehouse, B. G. 1984. The Effects of Temperature and Salinity on the Aqueous Solubility of Polynuclear Aromatic Hydrocarbons. *Marine Chemistry* **14**: 319-332.

Yakimov, M. M., P. N. Golyshin, S. Lang, E. R. B. Moore, W.-R. Abraham, H. Lunsdorf, and K. N. Timmis. 1998. *Alcanivorax borkumensis* gen. nov., sp. nov., a New, Hydrocarbon-Degrading and Surfactant-Producing Marine Bacterium. *International Journal of Systematic Bacteriology* **48**: 339-348.

Zoueki, C. W., N. Tufenkji, and S. Ghoshal. 2010. A Modified Microbial Adhesion to Hydrocarbons Assay to Account for the Presence of Hydrocarbon Droplets. *Journal of Colloid and Interface Science* **344**: 492-496.