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SHIFTS IN ROOT-ASSOCIATED MICROBIAL COMMUNITIES OF *TYPHA LATIFOLIA* GROWING IN NAPHTHENIC ACIDS AND RELATIONSHIP TO PLANT HEALTH

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Naphthenic acids (NAs) are a complex mixture of organic acid compounds released during the extraction of crude oil from oil sands operations. The accumulation of toxic NAs in tailings pond water (TPW) is of significant environmental concern, and phytoremediation using constructed wetlands is one remediation option being assessed. Since root-associated microorganisms are an important factor during phytoremediation of organic compounds, this study investigated the impact of NAs on the microbial communities associated with the macrophyte Typha latifolia (cattail). Denaturing gradient gel electrophoresis revealed that the impact of NAs on microbial communities was niche dependent, with endophytic communities being the most stable and bulk water communities being the least stable. The type of NA used was significant to microbial response, with commercial NAs causing greater adverse changes than TPW NAs. In general, plant beneficial bacteria such as diazotrophs were favoured in cattails grown in TPW NAs, while potentially deleterious bacteria such as denitrifying Dechlorospirillum species increased in commercial NA treatments. These findings suggest that NAs may affect plant health by impacting root-associated microbial communities. A better understanding of these impacts may allow researchers to optimize those microbial communities that support plant health, and thus further optimize wetland treatment systems.

KEY WORDS: endophytic bacteria, macrophytes, microbial community analysis, naphthenic acids, oil sands, phytoremediation

INTRODUCTION

Naphthenic acids (NAs) are naturally occurring carboxylic acids found in hydrocarbon deposits, including the oil sands deposits near Fort McMurray, Alberta, Canada. During the caustic hot water extraction process required to separate crude oil from oil sands, NAs

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are released from bitumen and subsequently become concentrated in the process water (Clemente and Fedorak, 2005; Headley and McMartin, 2004). As each cubic meter of mined oil sand requires approximately three cubic meters of water for processing, large volumes of NA contaminated process water are produced (Holowenko *et al.*, 2002). This process water is stored in onsite tailings ponds, where the NA concentration may reach as high as 120 mg L^{-1} (Quagraine *et al.*, 2005). Reclamation of steadily accumulating tailings pond water (TPW) and associated sediment represents one of the largest environmental challenges facing companies operating in the Athabasca oil sands region.

TPW contains other compounds such as salts and metals that create challenges for remediation methodologies, but it is NAs which pose perhaps the greatest concern (for review see Clemente and Fedorak, 2005; Headley and McMartin, 2004; Quagraine *et al.*, 2005). As a group, NAs are classified as organic acids and are comprised predominately of alkyl-substituted cycloaliphatic carboxylic acids and to a lesser extent of acyclic aliphatic acids. Although ionized forms of NAs are soluble in TPW, the surfactant nature of some components allows for micelle formation and adsorption on sediments, thus limiting their bioaccessibility and increasing overall recalcitrance (Headley and McMartin, 2004). NAs cause significant acute and chronic toxic effects to aquatic biota, including phytoplankton (Leung *et al.*, 2003), fish (van den Heuvel *et al.*, 2000), and amphibians (Pollet and Bendell-Young, 2000), as well as to birds (Gentes *et al.*, 2007) and mammals (Rogers *et al.*, 2002). A primary reclamation option feasible for large volumes of TPW is the use of constructed wetlands (Gschöbl *et al.*, 1998; Kanagy *et al.*, 2008), therefore strategies to minimize or mitigate the toxic effects of NAs are required.

Studies have shown that bacterial communities from a variety of sources are able to degrade NAs. Bacterial enrichment cultures from TPW have been shown to degrade both commercial NA mixtures (Kodak Chemicals and Merichem Chemicals) and mixtures of organic acids extracted from TPW, with a concomitant decrease in overall toxicity as measured by Microtox assays (Clemente *et al.*, 2004; Herman *et al.*, 1994). Bacterial sediment communities from natural and artificial wetlands that had been exposed to differing amounts of process water, from off-site non-impacted to highly impacted wetlands, were also able to effectively degrade monocyclic NAs (Del Rio *et al.*, 2006). The same study found that enrichment cultures derived from the sediments of a natural wetland impacted by relatively small amounts of TPW seepage were able to degrade up to 95% of a Kodak NA mixture. Similarly, enrichment cultures derived from rhizosphere soil of plants harvested in the vicinity of oil sands operations reduced the concentration of spiked Merichem NAs by up to 90% (Biryukova *et al.*, 2007). Because it is well known that the activity and diversity of microorganisms are increased in the rhizosphere of plants in dry-land phytoremediation systems, the incorporation of specific plants into constructed wetlands may be one technique to stimulate bacterial communities capable of degrading NAs.

Previous studies have demonstrated that the incorporation of aquatic plants, or macrophytes, facilitates the remediation of petroleum industry wastes, including PAHs and methylated PAHs (Machate *et al.*, 1999), trichloroethylene (Bankston *et al.*, 2002), gasoline and diesel range organics (Gessner *et al.*, 2005), and aliphatic and aromatic total hydrocarbons (Salmon *et al.*, 1998). Although less information is available on the feasibility of using macrophytes to remediate NA contaminated water, work by Crowe *et al.*, (2001, 2002) suggests that macrophytes such as *Typha latifolia* (cattail) are able to adapt to TPW contaminated conditions with little or no toxic effects. These results were corroborated during a recent study by our group, which also found that NAs from TPW exerted no significant toxic effects on cattails (Armstrong *et al.*, 2008). In contrast, commercial NAs (Fluka

Chemicals) did exert a toxic effect on the cattail plants used in the study, which may have been due to the greater bioavailability of the increased proportion of low molecular weight NAs. It is also possible however, that NAs had an indirect effect on plant health by impacting root-associated microbial communities, and that these changes contributed to the observed toxicity.

In order for wetland phytoremediation systems to be optimized as an effective treatment option for TPW, both plant and bacterial communities may need to be specifically stimulated. Before this stage is reached however, more information is required on the effects that NAs have on the plant-bacterial relationships. In this study, we expanded on the work of Armstrong *et al.*, (2008) by examining the composition of bacterial communities associated with cattails in the presence and absence of NAs, and investigated whether specific changes in microbial community composition might impact plant health as measured by plant growth.

METHODS

Hydroponic Experiments and Plant Sampling

Cattails were sampled from a study on the efficacy of using different macrophytes to enhance the degradation of NA (previously published: Armstrong *et al.*, 2008). To briefly summarize, cattail root cuttings from a native wetland plant nursery (Bearberry Creek Water Gardens, Sundre, AB, Canada) were acclimatized to growth chamber conditions in modified Hoagland's nutrient medium for at least three weeks and then single plants were transferred to individual hydroponic systems containing sterile Hoagland's nutrient medium. Cattails were then grown in the hydroponic systems for two weeks prior to NA addition. Oil sands NA were extracted from tailings pond water (Fort McMurray, AB, Canada) using an adapted liquid-liquid extraction method (see Armstrong *et al.*, 2008) and Fluka commercial NA mixture was obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada).

Five different NA treatments with three replicates each (0 mg L⁻¹ NAs; 30 mg L⁻¹ Fluka or Oil sands NA; and 60 mg L⁻¹ Fluka or Oil sands NA) were applied. The treatment doses were based on the results of preliminary experiments with cattail and the Fluka NA mixture. A dose of 60 mg L⁻¹ caused acute toxicity in the preliminary trials whereas 30 mg L⁻¹ was the lowest observed adverse effects level (data not shown). Root sub-samples (2 g) and bulk media samples (50 mL) were sampled from each treatment replicate immediately following NA addition (designated time 0) to assess initial diversity. Cattails and associated hydroponic media from all treatment replicates were harvested at the end of the 30-day phytoremediation treatment.

Sample Processing and Assessment of Heterotrophic Communities

Bulk, rhizoplane and endophytic communities were evaluated for each treatment replicate immediately following naphthenic acid addition and at the end of the experiment. For rhizoplane community assessment, roots (2 g at time 0, 6 g at 30 days) were suspended in 5 volumes of monopotassium phosphate (MPP) buffer (0.65 g K₂HPO₄, 0.35 g KH₂PO₄, 0.10 g MgSO₄ in 1L water) in a sterile Erlenmeyer flask, shaken at 200 rpm on a rotary shaker for 1.0 hours, and the resulting slurry was decanted into a sterile Falcon tube. Roots were then rinsed with an additional 5-volume aliquot of MPP buffer and this rinsate was

added to the rhizoplane slurry in the Falcon tubes. For endophytic community assessment, rinsed roots were surface disinfected by sequential washes of 95% ethanol and 5.25% sodium hypochlorite, followed by a minimum of five rinses with sterile water. To assess surface sterility, 100 μ L aliquots of the final rinse water were spread on 1/10th tryptic soy agar (TSA) plates and an additional 1 mL aliquot of the final wash water, boiled to release microbial DNA, was assessed by PCR using the eubacterial primers outlined in the following sections. Roots were stored at 4°C for 24 hours while awaiting results from sterility assessments. Endophytic extracts for heterotrophic analyses were subsequently produced by macerating 1 g of surface-sterile root from each treatment replicate in 9 mL MPP buffer using a sterile mortar and pestle.

Water, rhizoplane sediment and endophytic extracts were serially diluted in MPP buffer and total heterotrophic bacteria were enumerated by plating in triplicate 100 μ L of each dilution (10^{-3} – 10^{-7}) from each treatment replicate on 1/10 TSA plates containing 0.1 g l $^{-1}$ cycloheximide. Plates were incubated at 20°C for 14 days.

The remaining roots, rhizoplane samples, and bulk water from all treatment replicates were archived at –20°C for molecular analysis. Bulk water microbial communities were removed from bulk water by filtration through a sterile 0.45 μ m filter, which was then frozen. Rhizoplane slurries were centrifuged (5 min/7600 rpm, Beckman model TJ-6 centrifuge), the resultant supernatants were filtered through a sterile 0.45 μ m filter, and filters and pelleted rhizoplane samples were combined and frozen. Roots were macerated in MPP buffer, combined with roots from the same treatment replicate used for heterotrophic analysis, and frozen.

Assessment of Microbial Community Structure

Total community DNA was extracted from all treatment replicates of the three niches (bulk water, rhizoplane, and root interior) using a bead-beating protocol as previously outlined in Phillips *et al.*, (2006). This method used a combination of bead-beating, proteinase K, and sodium dodecyl sulphate to lyse cells. Proteins and cellular debris were precipitated using 7.5 M ammonium acetate, and DNA was subsequently precipitated using isopropanol, re-suspended in 100 μ L TE (pH 8.0), and purified using PVPP columns. DNA yield was quantified on ethidium bromide-stained 0.7% agarose gels by comparison with a high DNA mass ladder (Invitrogen).

Community structure and taxonomic diversity were examined by DGGE analysis of PCR-amplified 16S rRNA gene fragments. Total DNA extracts from each treatment replicate were amplified using the universal eubacterial 16S rRNA gene primers U341-GC (5'-CCTACGGGAGGCAGCAGGCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGCGGGGG, Lee *et al.*, 1993) and U758 (5'-CTACCAGGGTATCTAATCC, Rölleke *et al.*, 1996) using the PCR protocol outlined in Phillips *et al.*, (2006). Correct PCR amplification was confirmed on ethidium bromide-stained 1.4% agarose gels. Pooled PCR reactions were precipitated with 0.1 V 3M sodium acetate and 2.5 V 100% ethanol at –20°C overnight and re-suspended in 15 μ L of TE buffer (pH 8.0), and quantified on ethidium bromide-stained 1.4% agarose gels by comparison with a low DNA mass ladder (Invitrogen).

DGGE was performed on a Bio-Rad DCode system (Bio-Rad, Mississauga, Ont.) essentially as described by Lawrence *et al.*, (2004). For each treatment, 300 ng of amplified 16S rRNA gene product was loaded per lane onto an 8% acrylamide gel with a 40–60% urea-formamide denaturing gradient. Electrophoresis was performed for 16 h at 80V and

60°C. The resulting gel was stained with SYBR Green I (Sigma Aldrich) in TAE buffer and visualized using a digital gel documentation system (GelDocMega; BioSystematica, Devon, United Kingdom).

All treatment replicates were initially assessed on separate DGGE gels to determine inter-treatment variability, and replicate DGGE gels produced from separate amplification products were analyzed in order to verify the stability of the final dendrograms.

Assessment of Microbial Community Composition

Well-separated DGGE bands of interest (unique or ubiquitous) were excised from gels using a sterile scalpel and DNA was eluted in sterile deionized water by overnight incubation at 33°C. DNA was re-amplified using the primer set U341 and U758 as described by Juck *et al.*, (2000), with the addition of 6.25 μ g BSA (Amersham Biosciences) to each 50 μ l reaction mixture. Amplification proceeded for 25 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, 1 min extension at 72°C, and a final extension of 3 min at 72°C. Re-amplified DNA was pooled and precipitated as described above, run on a 1.0% agarose gel, and extracted and purified from the gel using the GeneClean gel extraction kit (Qbiogene, Inc., Irvine, CA). Purified 16S rRNA gene fragments were sequenced using the ABI Prism 377 automated fluorescence sequencer (Applied Biosystems, Foster City, CA). Sequences were submitted for comparison to the GenBank databases using the BLAST algorithm (Altschul *et al.*, 1997). Multiple bands with comparable migration were excised from all gels, including initial gels used to assess inter-treatment variability, and sequenced in order to verify identification. Thus, each band of interest was positively identified from a minimum of 2 separate gels.

Assessment of Indigenous Microbes in Naphthenic Acids

In order to determine whether specific microbes were introduced into the hydroponic systems during the addition of NA, the indigenous microbial communities of both naphthenic acids were assessed. Six-hundred microliters of Fluka ($n = 3$) or oil sands NA ($n = 3$) were spiked into separate Erlenmeyer flasks containing 200 mL sterile Hoagland's media, incubated with shaking for two weeks, and then filtered through a sterile 0.45 μ m filter. DNA was extracted from the filters and PCR amplification, DGGE analysis, and band sequencing were performed as described above. System sterility was verified using control Erlenmeyer flasks containing 200 mL sterile Hoagland's media with no NAs.

Statistical Analyses

Total heterotrophic data were examined for overall treatment effects by ANOVA (SPSS 15.0, Chicago, Illinois) followed by a Tukey test to determine whether significant differences occurred between treatments. Homogeneity of variance was assessed using the Levene statistic. Dendrograms were created by cluster analysis of the non-weighted DGGE banding patterns, using the Jaccard similarity coefficient and the UPGMA clustering method (BioNumerics software, Applied Maths). Replicate DGGE gels produced from separate amplification products were analyzed in order to verify the stability of the final dendrograms.

Accession Numbers

All sequences obtained with this study have been deposited in GenBank under accession numbers EU419377-EU419400.

RESULTS

Impact of NA on Plant Growth

Low doses of naphthenic acids did not significantly affect plant growth (Figure 1). However, high doses (60 mg L^{-1} NAs) of the commercial Fluka mixture caused a significant reduction in plant fresh weight gain compared to the control (Figure 1). Although high doses of oil sands NAs also reduced plant fresh weight gain, no significant difference was observed compared to the control group (Figure 1).

Initial Microbial Community Composition

Both heterotrophic community enumeration and DGGE dendrogram analysis indicated that microbial communities were highly similar prior to the addition of NA to the growth medium. No significant differences were observed between heterotrophic community populations within a given niche at the beginning of the experiment. However, differences were observed between the populations of separate niches, with the largest bacterial community populations found on the rhizoplane of plant roots, followed by endophytic and bulk water populations (Table 1). Dendrogram analysis of DGGE banding patterns in each niche revealed a high level of similarity in the community structure between initial treatments (Figure 2), indicating that in the absence of NA exposure plant-specific influences determined bacterial community composition.

Figure 1 Fresh weight gain (g) in cattails ($n = 3$) over a 30 day exposure to Fluka commercial naphthenic acids (Fluka NAs) and oil sands naphthenic acids (Oil Sands NAs). Values are reported as the mean $\pm$ se. Significant differences from the control are indicated with an asterisk (* $p < 0.05$).

Table 1 Total heterotrophic bacteria (log10 colony forming units) present in bulk water, rhizoplane and endophytic niches associated with cattail at time 0 and at final sampling (30 days)

Treatment	Bulk (g ⁻¹ water)	Rhizoplane (g ⁻¹ sediment)	Endophytic (g ⁻¹ wet root)
Time 0*	5.31 (0.31) c	8.49 (0.30) b	7.04 (1.01) a
Planted control	6.12 (0.24) ab	9.64 (0.42) a	7.68 (0.40) a
Oil sands-Low dose	6.45 (0.06) a	10.04 (0.36) a	7.43 (0.60) a
Oil sands-High dose	6.40 (0.08) a	10.10 (0.24) a	7.67 (0.31) a
Fluka-Low dose	6.31 (0.19) a	9.76 (0.38) a	7.18 (0.71) a
Fluka-High dose	5.89 (0.05) b	9.82 (0.41) a	7.53 (0.42) a

*No significant difference was observed between heterotrophic numbers within a given niche at time 0, and averaged values for each niche (n = 15) are presented for comparison with later populations. Data are presented as log 10 transformed means (n = 3) with standard deviation in parentheses. Means in the same column with the same letter are not significantly different (p < 0.05).

Impact of Naphthenic Acid Dose and Type on Overall Microbial Community Structure

Both culturable heterotrophic bacterial numbers and banding patterns exhibited by 16S rRNA gene fragments from each treatment were used to assess the impact of NA on total microbial communities. DGGE analysis was performed on PCR-amplified total

Figure 2 Cluster analysis of non-weighted DGGE banding patterns from (A) bulk water, (B) rhizoplane, and (C) endophytic microbial communities of cattails immediately following naphthenic acid addition to the growth medium. Results are shown for combined replicates (n = 3) of each NA treatment. Control, planted control; low dose, 30 mg L⁻¹; high dose, 60 mg L⁻¹.

community DNA extracted from replicates of each bulk, rhizoplane, and endophytic niche in each treatment. Preliminary DGGE analyses comparing replicates within a treatment were used to assess the stability of microbial community development within a given treatment and niche. The intra-treatment reproducibility of DGGE banding patterns was relatively high ($\geq 83\%$ similarity; endophytic > rhizoplane > bulk water, data not shown), indicating that microbial communities responded consistently and stably to the treatment pressures. For comparative purposes, intra-treatment replicates were then combined to show general shifts in community structure between treatments at the end of the 30-day experiment (Figure 3). Amplified DNA from each niche at time 0 (immediately following naphthenic acid addition; refer to Figure 2) was combined and presented on the same gel to illustrate how the communities changed from the beginning to the end of the experiment.

Endophytic bacterial communities exhibited the highest stability of all communities. Total heterotrophic populations remained consistent with those found at the beginning of the

Figure 3 Representative DGGE of PCR-amplified 16S rRNA gene fragments from (B) bulk water, (R) rhizoplane, and (E) endophytic microbial communities of cattails ($n = 3$) after 30 days of growth in different doses and types of naphthenic acids (NA). C: planted control; O1: low dose (30 mg L^{-1}) oil sands NA; O2: high dose (60 mg L^{-1}) oil sands NA; F1: low dose (30 mg L^{-1}) Fluka NA; F2: high dose (60 mg L^{-1}) Fluka NA; L: molecular ladder. Time 0 lanes represent average patterns seen in all niches immediately following NA addition. Arrows indicate sequenced bands whose closest identities are provided in Table 2.

Figure 4 Cluster analysis of non-weighted DGGE banding patterns from (B) bulk water, (R) rhizoplane, and (E) endophytic microbial communities of cattails ($n = 3$) growing in different doses and types of naphthenic acids (NA). Treatment patterns labelled t0 represent average community structure seen in all niches immediately following NA addition and all other patterns represent community structure seen after 30 days of growth. L: low dose (30 mg L^{-1}); H: high dose (60 mg L^{-1}); Control, planted control.

experiment, at approximately 10^7 bacteria per gram of root (Table 1). Dendrogram analysis of DGGE banding patterns showed that endophytic communities in all treatments retain a high degree of similarity (Figure 4), with endophytes from the control group forming a distinct cluster with those of the NA impacted cattails.

Bulk and rhizoplane communities from control planted treatments (no NA) retained a high degree of similarity with each other and with the initial communities found in these niches. These communities formed two discrete clusters, with the similarity between the bulk and rhizoplane communities increasing from 65 to 70% by the end of the experiment (Figure 4). By the end of the experiment rhizoplane populations had increased by an order of magnitude to approximately 10^{10} bacteria per gram of rhizoplane-sediment in all treatments (Table 1). Although the overall heterotrophic populations were similar in all treatments, DGGE banding patterns indicated that the source of NA had a significant influence on community structure, with rhizosphere communities from oil sands and Fluka NA treatments forming two separate clusters distinct from the control and time 0 communities (Figure 4). These communities were more influenced by type than dose of NA, as banding patterns within the clusters formed by the low and high dose oil sands and Fluka treatments showed 65% and 70% similarity, respectively (Figure 4).

Although all bulk heterotrophic populations also increased by an order of magnitude, high doses of Fluka NA resulted in significantly lower populations than other NA treatments (Table 1). As with rhizoplane populations, DGGE banding patterns showed that type of NA was a dominant influence on community structure in oil sands treatments. In Fluka treatments however, the shift in heterotrophic populations was reflected by a shift in community structure. High dose Fluka treatments did not form a discrete cluster with low dose Fluka treatments. Instead, low dose Fluka bulk populations showed a higher similarity with those from oil sands treatments, which themselves were over 75% similar (Figure 4).

Impact of Naphthenic Acid Dose and Type on Specific Microbial Community Composition

The stability observed in the DGGE banding patterns of endophytic communities was reflected in the relative stability of identified bands (Table 2), indicated by arrows in Figure 3. In general, endophytic bacteria showed similar relative fluxes in all treatments, indicating that plant specific factors were dominant in determining the composition of endophytic communities. Many of these identified endophytic bacteria were stably maintained, or increased equally in relative prevalence, within plant roots in different treatments. These included potentially beneficial bacteria such as *Garrityella koreensis* (band 23, Figure 3), potential plant pathogens such as *Agrobacterium tumefaciens* (band 8, Figure 3), and bacteria of potential interest for bioremediation, such as *Sphingomonas* sp. DEP-AD1 (band 16, Figure 3) capable of degrading aromatic compounds. Some endophytes were, however, impacted by type and dose of naphthenic acid. For example, a potentially harmful *Dechlorospirillum* species (bands 17, Figure 3) increased in endophytic communities in Fluka mixes, while some potentially beneficial bacteria such as the diazotroph *Aquaspirillum* (band 21, Figure 3) increased in control and low dose NA treatments.

Bacteria in the bulk and rhizoplane niches were more susceptible to NAs and few of the identified bacterial species in either niche were maintained at correlative levels in any given treatment. As reflected in DGGE banding patterns (Figure 3), the type of NA present had a significant influence on the prevalence of individual bacterial species. As a general trend, we noted that the presence of Fluka NA, particularly at high doses, favoured potential plant pathogens. For example, a *Dechlorospirillum* species (band 18, Figure 3) was found only in bulk and rhizoplane niches of plants growing in Fluka NAs, while the potentially beneficial diazotroph *Azospirillum* (band 5, Figure 3), increased in relative prevalence in the bulk and rhizoplane niches of control and oil sands impacted cattails, but not in those of Fluka impacted cattails.

Naphthenic Acids as a Source of Microorganisms

DGGE analysis revealed that over eleven separate bacteria were maintained in the concentrated commercial and TPW-extracted NAs and were able to grow using NA as the sole carbon source (Table 3). Six of these bacteria were found in both NAs, and included two *Sphingobacterium* species, three *Methylobacterium* species, and one *Brevundimonas* species. None of these bacteria matched any of the identified sequences found in the cattail treatments.

DISCUSSION

One of the reclamation options being used for the NA contaminated tailings pond water (TPW) produced during oil sands processing is constructed wetlands. Macrophytes are traditionally incorporated into constructed wetlands to increase aeration, nutrient transfer, microbial activity, and degradation, either directly by plant uptake of pollutants or indirectly by facilitating microbial activities. A common plant used in these wetlands, either by design or because they tend to self-volunteer, is the emergent macrophyte *Typha latifolia* (cattail). However, a recently published study by our group (Armstrong *et al.*, 2008) found that while oil sands naphthenic acid (NA) mixtures were less phytotoxic to cattails than commercial NA mixtures, little uptake or degradation of NA occurred. It may be that in order for wetland

Table 2 Phylogenetic affiliation of specific bulk water, rhizoplane and endophytic bacteria associated with cattails growing in naphthenic acids, based on 16S rRNA gene sequences reamplified from DGGE bands

DGGE band	Phylogenetic affiliation	Closest relative	Accession number	Percent similarity	Notes on original source
1	Sphingobacteria	<i>Kaistomonas</i> sp. IMCC1731	DQ664246	99	Freshwater bacteria from an artificial pond
2	ϵ -Proteobacteria	Uncultured <i>Sulfurospirillum</i> sp. clone	AY780560	100	Chlorinated ethene-degrading cultures
3	α -Proteobacteria	<i>Novosphingobium</i> sp. K16	AJ000920	98	Chlorophenol-contaminated boreal ground water
4	Sphingobacteria	<i>Mucilaginibacter paludis</i> strain TPT56	AM490402	96	Acidic sphagnum peat bog
5	α -Proteobacteria	Uncultured <i>Azospirillum</i> sp. clone	AY129807	99	Nutrient-limited cave environment
6	CFB* Group	Uncultured bacterium clone	AJ318147	99	Waste gas-degrading biofilter community
7	α -Proteobacteria	Uncultured α -proteobacterium clone	AY822704	98	Acidic hydrocarbon-seeping outcrop
8	α -Proteobacteria	<i>Agrobacterium tumefaciens</i> strain Ar-4	AB306893	99	Plant endophytic communities
9	α -Proteobacteria	<i>Agrobacterium</i> sp. Strain PNS-1	AY762361	99	Sulphanilic acid degraders from activated sludge
10	α -Proteobacteria	<i>Agrobacterium vitis</i>	AB247621	99	
11	α -Proteobacteria	<i>Sphingomonas</i> sp. HTCC399	AY429693	98	Trichloroethene-contaminated groundwater
12	α -Proteobacteria	Uncultured α -proteobacterium clone	DQ404716	95	Contaminated sediments
13	β -Proteobacteria	Uncultured bacterium	AB205867	97	Activated sludge
14	α -Proteobacteria	Rhizobiales bacterium RR47	AB174816	97	Acidogenic anaerobes of rice plant roots
15	β -Proteobacteria	<i>Polynucleobacter</i> sp. MWH-MoIso1	AJ550671	100	Freshwater habitats
16	α -Proteobacteria	<i>Sphingomonas</i> sp. DEP-AD1	DQ010645	99	Diethyl phthalate degraders from sewage sludge
17	α -Proteobacteria	<i>Dechlorospirillum</i> sp. WD	AF170352	99	Perchlorate-reducing bacteria from sediment
18	α -Proteobacteria	<i>Dechlorospirillum</i> sp. DB	AY530551	100	Various environmental soils and sediments
19	α -Proteobacteria	<i>Devosia ginsengisoli</i>	AB271045	100	Agricultural soils
20	γ -Proteobacteria	<i>Luteimonas</i> sp. TUT1238	AB188220	96	Mesophilic fed-batch garbage composters
21	α -Proteobacteria	<i>Aquaspirillum peregrinum</i> subsp. integrum	AB074521	99	
22	β -Proteobacteria	<i>Derxia gummosa</i> strain IAM14990	AB089481	94	
23	β -Proteobacteria	<i>Garrityella koreensis</i> strain 5YN10-9	DQ665916	97	Wetland
24	γ -Proteobacteria	Uncultured γ -proteobacterium clone	EF420207	98	Oil sands tailings pond in northern Alberta, Canada

*CFB: Cytophaga-Flexibacter-Bacteroides.

Table 3 Phylogenetic affiliation of bacteria found in oil sands and Fluka naphthenic acids, based on 16S rRNA gene sequences reamplified from DGGE bands

NA*	Phylogenetic affiliation	Closest relative	Accession number	Percent similarity	Notes on original source
F, O	Sphingobacteria	<i>Sphingobacterium kitahiroense</i>	AB361248	99	Soil bacteria
F, O	Sphingobacteria	<i>Sphingobacterium</i> sp. N13	EF423371	100	Antarctica
O	Sphingobacteria	<i>Sphingobacterium</i> sp. BM-9.2	AY635870	99	Spent mushroom compost
F, O	α -Proteobacteria	<i>Brevundimonas</i> sp. J22; Uncultured bacterium clone 101–140	EU143355; EF157208	100	Black sand; Rancho la brea tar pits
F	Bacilli	<i>Bacillus firmus</i> strain D1	AJ491843	99	PAH contaminated soils
F	γ -Proteobacteria	<i>Stenotrophomonas maltophilia</i>	EF620448	100	Agricultural soils
F	α -Proteobacteria	<i>Sphingomonas</i> sp. Pd-S-(l)-m-D-3(6)	AB291890	99	Rice endophytic bacteria
F, O	α -Proteobacteria	<i>Methylobacterium brachiatum</i> strain RB603B	AB252207	99	Freshwater
F, O	α -Proteobacteria	<i>Methylobacterium</i> sp. BHF006	AM403498	99	Industrial top furnace sludge
O	α -Proteobacteria	<i>Methylobacterium tardum</i> strain: RB677	AB252208	99	Freshwater
F, O	α -Proteobacteria	<i>Methylobacterium</i> sp. MG28	AJ746085	100	Haemodialysis water

*Naphthenic acid enrichment culture from which bacteria was identified. F: Fluka commercial mixture; O: Oil sands extract.

phytoremediation systems to be optimized as an effective treatment option for TPW, both plant and bacterial communities must be specifically stimulated. Currently however, little is known about the inter-relationship between cattail and associated microbial communities. Thus, the goals of this study were to provide information on the indigenous microbial communities associated with cattail, determine how these communities change when impacted by NA and, finally, determine whether these changes might be detrimental to plant health.

A wide range of bacteria in the bulk water, rhizoplane and endophytic niches of cattails were identified by sequence analysis (Table 2), with the majority belonging to the α - and β -subdivisions of the Proteobacteria. Although no comparative studies have been done on the microbial communities associated with cattails, the diversity that we found was similar to that found in another aquatic monocot, the rice plant. Lu *et al.*, (2006) found that up to 66% of all bacteria associated with the rhizosphere and roots of rice belonged to Proteobacteria and that the majority of those, as found in our study, belonged to the α - and β -subdivisions. Identified bacteria in our study most closely matched bacteria that came from three main environments; plant associations, petroleum by-product impacted situations, or freshwater habitats. Numerous sequences matched bacteria that came from habitats that were an overlap of the latter two environments, such as a *Sphingomonas* species previously isolated from trichloroethene contaminated groundwater (AY429693, Table 2). All but two of the identified bacteria (bands 12 and 24; Table 2, Figure 3) were present on the rhizoplane or in the interior of the cattail roots at the beginning of the

experiment. To determine if the bacterium, previously identified in an oil sands tailing pond as a γ -proteobacterium (EF420207), originated from the NA inoculum itself, the bacteria present in the crude naphthenic acids were characterized (Table 3). There was no correlation between the two groups of sequences, indicating that the inoculum did not significantly contribute to the bacterial community composition. Instead, cattails with no prior exposure to either NAs or other petroleum industry by-products had, as part of their normal indigenous microbial communities, bacteria that may prove capable of degrading NAs.

We compared the impact of two NA mixes (Fluka commercial NA mix and NAs extracted from TPW) at two doses (30 and 60 mg L⁻¹) on microbial community composition and structure. The stability of cattail associated microbial communities when impacted by NAs was niche dependent. Endophytic communities in all NA treatments retained a high degree of similarity with endophytic communities from the control group, forming a distinct cluster exhibiting greater than 70% similarity at the end of the experiment (Figure 4). Although these community patterns shifted from those observed at the start of the experiment, they still exhibit a substantially higher degree of similarity than the communities found in the bulk water and rhizoplane niches at the end of the experiment, revealing that the dominant influence on these communities is the plant itself. Moreover, endophytes appeared to be stably maintained within the plants even after a month of plant growth in NA (Figure 3).

In the absence of NA, both bulk and rhizoplane communities retained a high degree of similarity (Figure 4) and formed a sub-cluster with those of the initial communities, indicating that the plant effect extended to the bulk water surrounding the roots. In the presence of NA however, these communities diverged and formed discrete clusters, separating based on type of NA and on niche. A previous study (Hadwin *et al.*, 2005) on the impact of oil sands on sediment microbial communities found that exposure to any level of process water was the dominant factor in determining community structure. With regards to plant effects, one study concluded that microbial community structure was more influenced by sediment type and location than macrophyte type (Franco *et al.*, 2005), while another found that cattail does exert a stabilizing influence on rhizosphere bacteria (Biesboer, 1984). Angeloni *et al.*, (2006) found that natural sediment bacterial communities became more diverse when invaded by cattails, but that this change was more likely correlated with changes to sediment organic matter content, water temperature, pH, and nutrient concentrations. In our study, we found that while type of NA was a significant factor in determining community composition of rhizoplane niches (Figure 4), the cattail did exert some influence on rhizoplane community structure, as evidenced by the specific clustering according to the rhizoplane niche.

When the cattail plants in this study were exposed to NA, it was found that commercial NAs (Fluka Chemicals) were more phytotoxic than oils sands NAs extracted from TPW (Figure 1). The Fluka mixture NAs have a lower mass range of components (157–297 m/z) than the oil sands NAs (195–325 m/z) (Armstrong *et al.*, 2008) and differences in the molecular weight of the two NA groups may have played a role in determining phytotoxicity, possibly by impacting water transport (Kamaluddin and Zwiazek, 2002). Specific changes to the microbial communities may also have influenced the increased phytotoxicity seen in Fluka treatments. Specific bacteria beneficial to plant growth, such as the diazotroph *Azospirillum* (Table 2) increased in relative intensity in the bulk water and rhizoplane of control and oils sands NA treatments (Figure 3). *Azospirillum* species are known to stimulate the growth and yields of numerous plant species, including aquatic plants, through the release of phytohormones and the fixation of atmospheric nitrogen (for

a comprehensive review refers to Bashan *et al.*, 2004). In contrast, some potentially harmful bacteria such as a *Dechlorospirillum* WD species (Table 2) increased in endophytic and rhizoplane communities in Fluka treatments (Figure 3). *Dechlorospirillum* species are known to reduce nitrate (Bardiya and Bae, 2008) and could thus compete with the plant itself for this essential nutrient. Further, since both *Azospirillum* and *Dechlorospirillum* would utilize the same anoxic niche to fix nitrogen or denitrify, respectively, then increased populations of *Dechlorospirillum* could competitively exclude *Azospirillum*. An observable relative decrease in the presence of a *Sulfurospirillum* species (Table 2, Figure 3) in the endophytic niche of all NA treatments, but not in control plants, supports the hypothesis that nitrate becomes a limiting factor, as this bacterium is known to become less dominant under low nitrate conditions (Hubert and Voordouw, 2007). Although *Dechlorospirillum* appears to be a natural component of aquatic plant microbial communities (Lu *et al.*, 2006) the increased levels found in Fluka treatments could ultimately have an adverse effect on overall plant health.

These findings support our hypothesis that NAs may impact plant health by impacting root-associated microbial communities. NAs may affect root physiology directly, causing chemical injury, which may facilitate colonization by pathogenic microbes (Hallmann *et al.*, 1997). The greater bioavailability of lower molecular weight Fluka NAs may have allowed for a greater influx of NAs into the root, facilitating phytotoxicity and increasing injury to the root system, and thus leading to the proliferation of pathogenic microbes. Alternately, the low molecular weight NAs adversely impact beneficial root-associated microbes, allowing pathogens to proliferate, and increasing the overall phytotoxic response. Regardless of whether the phytotoxic impact of commercial NAs was direct or indirect, this study highlights the limitations inherent to using contaminant analogues to assess phytoremediation potential.

CONCLUSIONS AND FUTURE PERSPECTIVES

A better understanding of how root associated microbial communities are impacted by NAs is required for optimization of wetland treatment systems. This study showed that changes to both beneficial and detrimental bacterial populations may impact plant growth in NA contaminated water. The stability of endophytic communities and the presence of plant-associated bacteria with the potential to degrade NAs suggest the possibility of using bacterial inoculants to stimulate both plant growth and NA degradation in wetlands for TPW reclamation. The potential of bacterial endophytes to enhance phytoremediation has been the subject of several recent reviews (Newman and Reynolds, 2005; Ryan *et al.*, 2008) and has been successfully demonstrated in at least one study (Barac *et al.*, 2004). Finally, as nitrogen is often a limiting factor in the bioremediation of organic contaminants (Nikolopoulou *et al.*, 2007), the enhanced response of diazotroph bacteria to the oil sands NA treatment is promising for the potential remediation of NAs.

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