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The effect of oil sands process-affected water and naphthenic acids on the germination and development of Arabidopsis

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HIGHLIGHTS

- OSPW, AEO and NA treatments inhibited the development of Arabidopsis seedlings.
- Only high concentrations of surrogate and model NAs blocked Arabidopsis germination.
- The pH of the growth medium affected OSPW and NA toxicity.
- Low concentrations of NA appeared to have a hormone-mimicking effect.

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ABSTRACT

Oil sands mining in the Athabasca region of northern Alberta results in the production of large volumes of oil sands process-affected water (OSPW). We have evaluated the effects of OSPW, the acid extractable organic (AEO) fraction of OSPW, and individual naphthenic acids (NAs) on the germination and development of the model plant, *Arabidopsis thaliana* (Arabidopsis). The surrogate NAs that were selected for this study were petroleum NAs that have been used in previous toxicology studies and may not represent OSPW NAs. A tricyclic diamondoid NA that was recently identified as a component of OSPW served as a model NA in this study. Germination of Arabidopsis seeds was not inhibited when grown on medium containing up to 75% OSPW or by 50 mg L^{-1} AEO. However, simultaneous exposure to three simple, single-ringed surrogate NAs or a double-ringed surrogate NA had an inhibitory effect on germination at a concentration of 10 mg L^{-1}, whereas inhibition of germination by the diamondoid model NA was observed only at 50 mg L^{-1}. Seedling root growth was impaired by treatment with low concentrations of OSPW, and exposure to higher concentrations of OSPW resulted in increased growth inhibition of roots and primary leaves, and caused bleaching of cotyledons. Treatment with single- or double-ringed surrogate NAs at 10 mg L^{-1} severely impaired seedling growth. AEO or diamondoid NA treatment was less toxic, but resulted in severely impaired growth at 50 mg L^{-1}. At low NA concentrations there was occasionally a stimulatory effect on root and shoot growth, possibly owing to the broad structural similarity of some NAs to known plant growth regulators such as auxins. This report provides a foundation for future studies aimed at using Arabidopsis as a biosensor for toxicity and to identify genes with possible roles in NA phytoremediation.

1. Introduction

The oil sands deposits in the Athabasca region of Alberta, Canada contain an estimated 170 billion barrels of recoverable oil. This represents the third largest oil reserve in the world (Kannel and Gan, 2012). Surface mining is the dominant form of bitumen extraction from the oil sands, and involves an alkaline hot water extraction process to separate bitumen from the sand particles. This process results in the accumulation of large volumes (approximately 720 million m^3 total in 2009) of oil sands process-affected water (OSPW) (ERCB, 2009). OSPW contains residual bitumen, sand and clay particles, as well as inorganic and organic compounds. The organic fraction of OSPW contains a complex mixture of molecules that include the so-called naphthenic acids (NAs) (Grewer et al., 2010). NAs have been classically defined as alkyl-substituted alicyclic carboxylic acids that have the general formula $C_nH_{2n+z}O_2$, where n is the carbon number and z is an even, negative integer that indicates the loss of hydrogen atoms due to cyclization (Whitby, 2010). Recently, it has been shown that less

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than 50% of the acid extractable organic (AEO) fraction of OSPW (the fraction commonly referred to as the NA fraction) fits the classical NA formula, even when it is expanded to include oxy-naphthenic acids (compounds that contain three to five oxygen atoms) (Grewer et al., 2010). Other compounds that contain *N* and *S* atoms are also present in OSPW. Specific organic acids have recently been positively identified in OSPW, thereby providing insight into the complexity of OSPW when compared to commercially available petroleum NA mixtures. OSPW associated NAs include tricyclic (diamondoid), tetracyclic and pentacyclic acids (Rowland et al., 2011a,b); in addition, OSPW has been shown to harbor a substantial aromatic fraction (Jones et al., 2012). NA toxicity studies that were published prior to the identification of specific NAs in OSPW often used simple petroleum 'surrogate' NAs as model compounds in their experiments (Herman et al., 1993, 1994; Lai et al., 1996; Holowenko et al., 2001; Peng et al., 2002; Del Rio et al., 2006; Frank et al., 2008; Han et al., 2009; Quesnel et al., 2011). Although these simple NA compounds may be components of OSPW, they are not likely a major component of the NA profile, as these compounds are rapidly degraded by microbes (Lai et al., 1996; Del Rio et al., 2006). The term "NA" will be used throughout this present report, but it should be emphasized that the "NA fraction" of OSPW includes organic acids with structural features beyond that of the classical definition ($C_nH_{2n+z}O_2$).

NAs are associated with acute and chronic toxicity in many different aquatic organisms (Whitby, 2010). Although the exact mode of toxicity has not yet been determined, NAs are thought to contribute to the cytotoxicity of OSPW by disrupting cell membranes (Quagraine et al., 2005; Frank et al., 2008). Numerous groups of organisms are negatively affected by NAs. For instance, certain phytoplankton were shown to be vulnerable to NA concentrations of 20 mg L^{-1} (Leung et al., 2003), and freshwater fish species had 60-d LC_{50} values ranging from 2 to 14 mg L^{-1} of commercial NAs (Dokholyan and Magomedov, 1983). Studies using native plant species such as aspen, jack pine and the aquatic cattail have identified the effects of OSPW and commercial NAs on water relations and/or gas exchange (Crowe et al., 2001; Kamaluddin and Zwiazek, 2002; Apostol et al., 2004). Cattail showed acute toxicity when exposed to commercial NAs and AEO NAs at 60 mg L^{-1} (Armstrong et al., 2008). Since NAs are present at concentrations up to 120 mg L^{-1} in OSPW (Holowenko et al., 2000), the high NA concentration and evidence of wide-ranging toxicity represents a significant concern for the re-development of healthy ecological communities after the mining process is complete. The continued accumulation of OSPW at oil sands surface mining sites has necessitated a need to identify methods to determine the biological effects of NAs on aquatic and terrestrial life and to reduce the concentration and toxicity of NAs in the environment in general.

Phytoremediation is a plant-based approach used to sequester or degrade toxic compounds in the environment and represents an attractive means to reduce the toxicity of NAs in OSPW. Past phytoremediation successes related to the petroleum industry include a 42–50% reduction of petroleum hydrocarbons at a crude oil spill site using rye grass and St. Augustine grass in only 21 months (Nedunuri et al., 2000). Studies aimed at determining the phytotoxicity and loss of NAs from solution using cattail, common reed grass, and hard stem bulrush demonstrated a change in the NA distribution and reduction of NA toxicity when these plants were grown in NA solutions (Armstrong et al., 2008, 2009). This indicates that plant metabolic pathways have the ability to degrade NAs. Recent evidence has also revealed the potential for an algal-based NA remediation approach. The green alga *Dunaliella tertiolecta* was able to survive on high concentrations of five simple surrogate NA species, and was shown to degrade four of these NAs (Quesnel et al., 2011). Plants demonstrate unparalleled metabolic plasticity and possess unique biochemical pathways that are involved in the synthesis and degradation of thousands of complex compounds (Meagher, 2000). These metabolic characteristics of plants could provide them with the unique ability to degrade certain NA species. Despite these and other studies that demonstrate the potential ability of photosynthetic organisms to degrade NAs, a detailed understanding of the underlying metabolic pathways involved in NA degradation is lacking.

The model plant *Arabidopsis thaliana* (Arabidopsis) has a fully sequenced genome and has been a valuable resource for the identification of genes involved in phytoremediation (Cobbett and Meagher, 2002). Arabidopsis is a widely distributed flowering plant, and is tractable to genetic and molecular biology studies because of its small size, short generation time, and small genome (Meinke et al., 1998). The general metabolism of Arabidopsis is well characterized and transcript expression profile data from plants exposed to a variety of environmental stresses is available. Arabidopsis has not yet been utilized as a model system for NA phytoremediation. However, this model plant has immense potential to advance the future of oil sands NA biosensing and remediation research, as the available genetic and genomic resources are vast and the results obtained from Arabidopsis studies can be correlated to other plant species.

Here we describe experiments aimed at determining the effect of OSPW, the AEO fraction of OSPW, and individual NA treatment on Arabidopsis seed germination and seedling growth. Increasing concentrations of OSPW as well as all NA treatments had significant qualitative and quantitative effects on Arabidopsis seedling growth. All treatments resulted in reduced root growth rate that continued in a concentration-dependent manner. Treatments involving high concentrations of all NA types resulted in inhibition of seed germination. This study establishes Arabidopsis as a model plant species for the study of OSPW and NA toxicity, and could contribute to the isolation of genes that are important in the remediation of NAs.

2. Materials and methods

2.1. Sources of OSPW and NAs

Experiments were conducted with OSPW, the AEO fraction of OSPW, and with individual NA compounds associated with petroleum acids or OSPW. The OSPW was collected from Suncor Energy Pond 6. The AEO fraction was purified from OSPW using a liquid–liquid extraction procedure using dichloromethane (DCM, Sigma–Aldrich, St. Louis, MO) (Holowenko et al., 2000). An equal volume of DCM was mixed with OSPW acidified to pH 2 (using 5.2 M HCl), and the DCM phase that contained the AEO fraction was isolated into a pear-shaped flask using a 1L separatory funnel. The DCM was then removed using a rotary evaporator (Rotavapor RII, BÜCHI, Switzerland) and the resulting AEO fraction was placed in a 42 °C oven to dry overnight. AEO yield was determined by measuring the weight of the pear-shaped flask before and after the extraction. Yields ranged from 22 to 32 mg AEO/L of OSPW (measured on a Mettler Toledo PL303 balance). The purified AEO was suspended in dimethyl sulfoxide (DMSO) to a final stock concentration of 100 mg mL^{-1}. A surrogate NA mixture of cyclohexane pentanoic acid (3-cyclohexylpentanoic acid), cyclohexane butyric acid (4-cyclohexylbutanoic acid), and cyclohexane propionic acid (3-cyclohexylpropanoic acid) was suspended in DMSO and mixed together in equal amounts prior to use (Sigma–Aldrich, St. Louis, MO). The surrogate NA decahydro-2-naphthoic acid (decahydronaphthalene-2-carboxylic acid, DHNA), and the model OSPW NA adamantane carboxylic acid (1-adamantanecarboxylic acid, AdCA) (Sigma–Aldrich, St. Louis, MO) were suspended in DMSO prior to use. DHNA was synthesized as described previously (Dauben and

Hoerger, 1951). Gas chromatography-mass spectrometry was used to analyze the AEO fraction. For GC–MS analysis, the sample was extracted from OSPW as above, transferred to a 1.5 mL autosampler vial (Agilent Technologies, Santa Clara, CA), and derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (Thermo Scientific, Bellefonte, PA). The AEO profile was measured using an Agilent 7890A gas chromatograph equipped with an Agilent HP-5MS 30 m × 0.25 mm × 0.25 μm column and an Agilent 5975C mass selective detector (Agilent Technologies, Santa Clara, CA). Total ion chromatogram integration and analysis of extracted compounds were performed using Enhanced MSD ChemStation E.02.00.493 RTE integrator software (Agilent Technologies, Santa Clara, CA).

2.2. Growth medium preparation and seedling growth conditions

For seed germination assays, solid growth medium was prepared with OSPW, water, and 0.7% phytoagar (Caisson Laboratories Inc.) at concentrations of 0%, 10%, 25%, 50%, and 75% OSPW (vol/vol). After autoclaving, 20 mL volumes were poured into 8.5 cm diameter culture plates. For seedling growth assays, filter-sterilized (Stericup bottle filter unit, 0.22 μm pore size, Millipore) OSPW was added to a warm autoclaved solution of half-strength minimal Murishige and Skoog (MS) medium (pH 5.7, Sigma–Aldrich) containing 0.7% phytoagar (Caisson Laboratories Inc.) to a total volume of 20 mL per plate. For the preparation of AEO- and NA-containing growth plates, a solution containing AEO or NAs in half-strength MS at pH 5.0 or 7.8 was filter-sterilized (0.45 μm pore size, Acrodisc syringe filter) and mixed with an equal volume warm autoclaved water and 0.7% phytoagar of the corresponding pH. For the 10 mg L^{-1} and 50 mg L^{-1} DHNA and AdCA solutions, water was heated to 60 °C before adding the compound to enhance solubility. In place of NAs, an appropriate amount of DMSO was added to control plates. For the AEO and NA assays, plates contained a final medium volume of 30 mL.

Wild-type *Arabidopsis thaliana* (isotype Col-0) seedlings were used for all assays. Seeds were surface sterilized with successive 70% ethanol washes of 10 min and 5 min in length on an orbital shaker rotating at 150 rpm. The seeds were then dried on filter paper in a laminar flow hood, and individually placed on culture plates using sterile forceps. For germination assays, 50 seeds were placed equidistantly on each plate, and radicle (embryonic root) emergence through the seed coat was scored as an indicator of germination. For root growth assays, fifteen seeds were placed in a horizontal line 3–4 mm apart, 1.5 cm above the middle of the plate. The plates were then placed at 4 °C in the dark for 3–4 d to promote consistent a germination rate. Plates used to measure seed germination and root length were grown horizontally and vertically, respectively, at random locations on a growth rack under fluorescent light conditions (21 °C, 24 h photoperiod).

2.3. Root measurements and statistical analysis

Primary root lengths obtained from photographs of seedlings were measured using ImageJ software (version 1.46). Statistics for the experimental assays were calculated using R software (version 2.15.2). For each of the experimental assays, a one-way analysis of variances (ANOVA) test was applied to determine statistical significance among treatments ($p < 0.5$). The Tukey's HSD (honestly significant difference) test was then performed to determine which of the treatments were statistically different from one another.

3. Results

3.1. The effect of OSPW on Arabidopsis seed germination and seedling development

Radicle emergence assays demonstrated that there were no statistically significant differences in mean percent germination of Arabidopsis seeds in OSPW treatments at concentrations up to 75% (vol/vol, Fig. 1A). Primary root length was chosen as an indicator of toxicity, as roots were in direct contact with the medium and root length was easily quantified. The effect of OSPW treatment (0–20% concentration) on root length showed a significant effect after 12 d of growth (Fig. 1B). Treatment with 5% OSPW resulted in an approximately 50% decrease in root growth, whereas at 20% OSPW root growth was essentially eliminated. The reduction in root growth was evident within the first 4 d of treatment (Fig. 1C). An OSPW concentration of 20% contains roughly 4–8 mg L^{-1} of purified AEO based on an estimated AEO concentration of 20–40 mg L^{-1} in OSPW. Qualitative observations identified several phenotypic changes in seedlings grown on increasing concentrations of OSPW. As OSPW concentration increased, the roots displayed a slight wavy growth pattern, the cotyledons were curled downward, and primary leaf growth was reduced (Fig. 1D). In addition, cotyledon bleaching was often observed in plants grown on OSPW concentrations of 15% or higher. Interestingly, when seedlings were grown on 5% OSPW, cotyledon size was consistently larger when compared to the untreated controls and seedlings treated with higher OSPW concentrations (Fig. 1D).

3.2. Effect of OSPW acid extractable organic (AEO) fraction treatment on Arabidopsis seed germination and seedling development

OSPW contains high levels of salt, metals, and other compounds that could influence plant growth (Renault et al., 2000). Therefore, we determined the direct effect of seedling germination and growth resulting from treatment with the AEO fraction of OSPW. In order to provide insight into the complexity of the NAs contained in the AEO fraction, this fraction was analyzed by GC–MS. A wide diversity of NA species was present in the AEO fraction. A large number of peaks was observed in the range of 12–18 carbons, particularly of the acyclic, two, and three-ringed families (Fig. 2A). In a previous study, AEO was shown to have more toxic effects on plant growth in their non-ionized form when equilibrated at low pH (5.0) compared to their ionized form when equilibrated at high pH (7.8) (Armstrong et al., 2009). Thus, we compared the growth pattern of Arabidopsis seedlings exposed to the AEO fraction in both pH 5.0 and pH 7.8 medium. AEO treatments of 10 mg L^{-1} and 50 mg L^{-1} resulted in a statistically significant decrease in primary root growth at pH 5.0 and 7.8 (Fig. 2B). Seedlings grown on plates supplemented with 50 mg L^{-1} of AEO in pH 5.0 medium showed the greatest decrease in root length compared to control seedlings (Fig. 2B). The phenotypic responses to AEO were similar to those described in the OSPW assays. These plants showed decreased primary root length, increased frequency of wavy roots, and downward curling cotyledons (Fig. 2C). Mild chlorosis was also observed at high AEO concentrations (Fig. 2C). Similar to the observations of plant growth on a low percentage of whole OSPW (Fig. 1D), cotyledons and leaves of plants subjected to AEO at a concentration of 0.1 mg L^{-1} in pH 5.0 medium consistently appeared larger than those of the control or any of the other treatment concentrations (Fig. 2C). This increase in leaf size was not accompanied by a decrease in root length, as was observed in the low OSPW treatments.

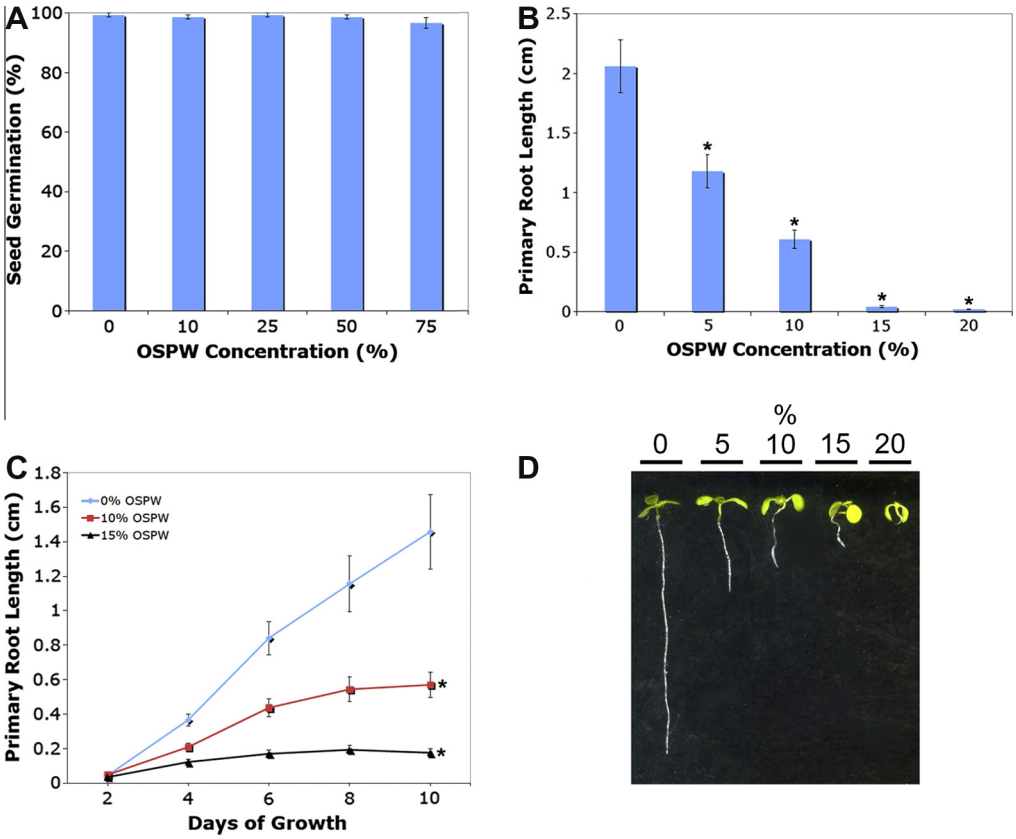

Fig. 1. The effect of OSPW treatment on seed germination, primary root length, and phenotype in Arabidopsis. (A) The percentage of seed germination was determined after 10 d of incubation in the light at 21 °C on culture plates. Mean percent seed germination (±SEM, $n = 50$) is shown using data from three separate experiments. (B) Seedlings were grown at room temperature in the light on vertically positioned culture plates containing increasing concentrations of OSPW. Root measurements were taken after 12 d of growth. Mean primary root length (±SEM, $n = 15$) is shown using data from two separate experiments. (C) Time course experiment demonstrating the effect of OSPW concentration on primary root length. Measurements were taken at two-d intervals over a 10 d period. Mean primary root length (±SEM, $n = 15$) is indicated using data from two separate experiments. (D) Phenotype of seedlings after 10 d of vertical growth on increasing OSPW concentrations. (* = significant difference from control, $p < 0.05$).

3.3. Effect of surrogate and model NAs on Arabidopsis seed germination and seedling development

NAs that are purified from OSPW contain a wide range of structural variants, and the precise composition of the NA fraction is largely unknown. We determined the effect of exposure of Arabidopsis to three single-ringed surrogate NAs (cyclohexane propionic acid, cyclohexane butyric acid and cyclohexane pentanoic acid) two of which were previously identified petroleum NAs (Rowland et al., 2011b). Also used were a double-ringed surrogate petroleum NA (DHNA), and an OSPW diamondoid NA (AdCA).

Seedlings were treated on plates that contained one of the three NA types (single-ring, two-ring, or diamondoid) at total concentrations ranging between 0 mg L^{-1} and 50 mg L^{-1} in medium with a pH value of 5.0 or 7.8. The single-ringed NA treatment consisted of an equal mixture of cyclohexane propionic acid, cyclohexane butyric acid and cyclohexane pentanoic acid at total concentrations shown in Fig. 3A. Primary roots showed a significant decrease in length at concentrations of 1 mg L^{-1} and above on pH 5.0 medium (Fig. 3A and B). At pH 7.8, primary roots demonstrated a significant increase in length at 1 mg L^{-1} compared to the control, whereas root length dramatically decreased at a concentration of 10 mg L^{-1} (Fig. 3A). Additionally, the 1 mg L^{-1} pH 7.8 treatment often resulted in enhanced leaf growth (data not shown). The two-ringed NA (DHNA) treatment resulted in a significant decrease in primary root length at concentrations of 10 mg L^{-1} and above at pH values of 5.0 and 7.8 (Fig. 3C and D). For both of the petroleum surrogate NA treatments (single-ringed and DHNA), inhibition of germination was occasionally observed at 10 mg L^{-1}, whereas seed germination was not observed at 50 mg L^{-1} at pH 5.0 (Fig. 3C and D). For the diamondoid NA (AdCA) treatments, a significant decrease in primary root length occurred at 50 mg L^{-1} the pH 5.0 and pH 7.8 treatments (Fig. 3E). In addition, seed germination was inhibited only at 50 mg L^{-1} and pH 5.0. For all NA treatments, growth was inhibited to a greater extent on pH 5.0 medium. No chlorosis or wavy roots were seen in any of the surrogate or model NA treatments, however downward curling cotyledons were observed at higher concentrations.

4. Discussion

This is the first report to describe the effect of OSPW, AEO and NA treatments on germination and development of the model plant Arabidopsis. AEO toxicity assays were performed to provide a more direct measure of the effects of the acid extractable organic acid component of OSPW (i.e., "naphthenic acids") on plant growth, since OSPW consists of other constituents, including salts, heavy metals and residual bitumen. Specific NAs were also used in these toxicity studies. These consisted of simple surrogate NAs that have been used in other NA toxicity studies, as well as a model diamondoid NA (AdCA) that was recently identified as a known organic acid in OSPW (Rowland et al., 2011a). Simple single-ringed petroleum NAs have been utilized in several studies to determine their toxicity and degradation rates (Herman et al., 1993; Herman et al., 1994; Holowenko et al., 2001; Del Rio et al., 2006; Frank et al., 2008; Quesnel et al., 2011). However, these compounds have not been used previously in plant toxicity studies. Recognizing that

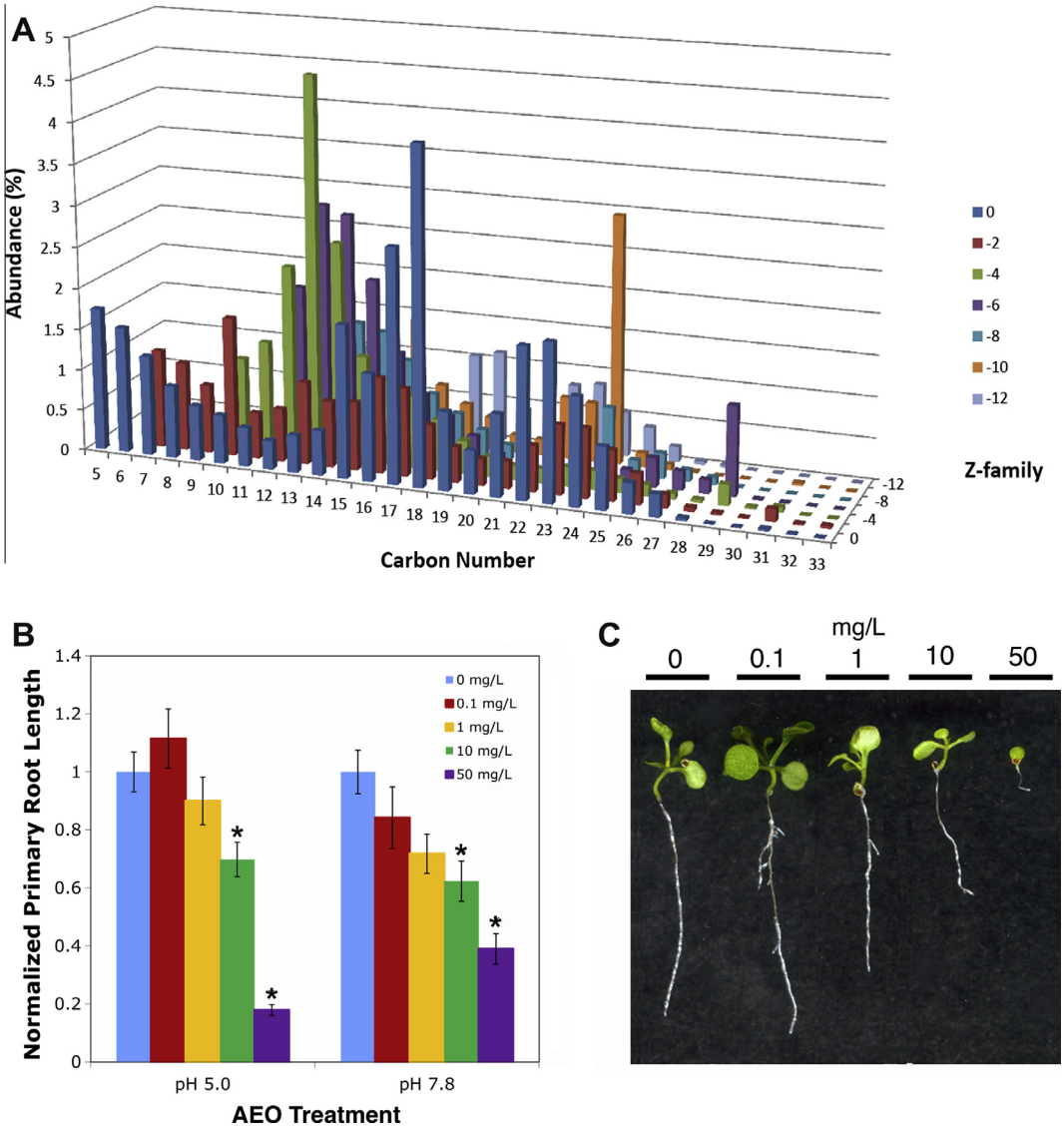

Fig. 2. The effect of AEO treatment on Arabidopsis growth. (A) Three-dimensional plot of the distribution of NAs from AEO fitting the molecular formula $C_nH_{2n+z}O_2$. Data were normalized so that the sum of all bars equals 100%. (B) The effect of medium pH and AEO concentration on primary root length in Arabidopsis. Measurements were taken after 9 d of growth under light-grown conditions on vertically positioned culture plates. Mean primary root length (±SEM, $n = 15$) is shown based on data collected from four replicated experiments. (*significant difference from control, $p < 0.05$). (C) Phenotypes of seedlings after 17 d of growth on increasing concentrations of AEO on pH 5.0 medium.

they may not be significant constituents of OSPW, it was appropriate to test these compounds in plant toxicity studies to provide insight into the general features of NA toxicity on various groups of organisms and to compare with previous studies. DHNA has also been used as a surrogate NA in the past for biodegradation studies (Lai et al., 1996; Del Rio et al., 2006). Although DHNA is a petroleum NA that is simple in structure, it represents a class of double-ringed NA ($Z = -4$) that are among the most abundant of the classical NAs found in OSPW (Grewer et al., 2010). It has been shown that single-ringed compounds (specifically cyclohexane carboxylic acid) as well as DHNA are metabolic by-products of anaerobic aromatic hydrocarbon breakdown by microbes (Zhang et al., 2000; Elshahed et al., 2001). Aromatic hydrocarbons are present in OSPW as a result of the use of low molecular weight hydrocarbon diluents in the oil sands extraction process, and have been shown to be susceptible to anaerobic degradation by tailings pond microbial communities (Siddique et al., 2007) Thus cyclohexane carboxylic acid and DHNA may be components of OSPW. Although several NAs have recently been identified in OSPW, the heterogeneous nature of OSPW will require that further testing be performed to determine the NA composition present in different OSPW samples (Rowland et al., 2011b). Treatment of Arabidopsis seedlings with AdCA was also performed. AdCA is a diamondoid NA that has been identified in OSPW (Rowland et al., 2011a), thus providing a relevant example of an OSPW model NA in this study.

Arabidopsis seed germination was not affected by treatment of seeds with OSPW or AEO, whereas treatment with NAs at 50 mg L^{-1} was effective in preventing germination. In contrast, treatment with OSPW, AEO or NAs each significantly resulted in reduced root growth (Figs. 1–3). We also observed impaired primary leaf growth and chlorosis at high treatment concentrations of OSPW and AEO (Figs. 1 and 2). The observation that OSPW and AEO treatments showed no effect on seed germination, but had a significant effect on seedling development is consistent with previous observations using boreal plant species (Renault et al., 2000; Li et al., 2005). Thus, in this regard, Arabidopsis effectively mimics plant species that are found naturally in the oil sands region. As shown in other studies, the exposure of various plant species to

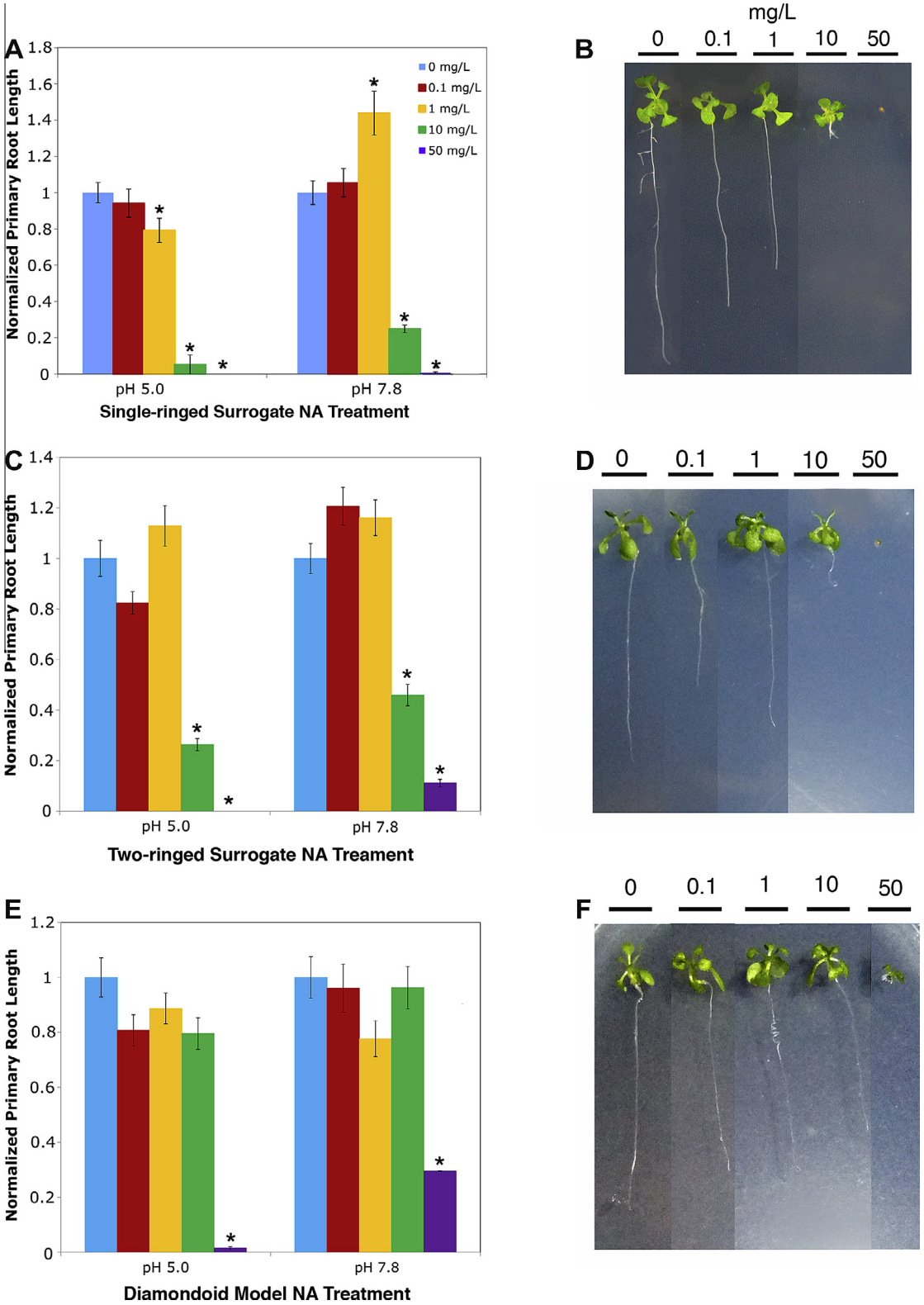

Fig. 3. The effect of NA treatments on Arabidopsis growth. (A) Primary root length of seedlings grown on increasing concentrations of three single-ringed surrogate NAs at medium pH levels of 5.0 or 7.8. The single-ringed NA treatment consisted of an equal mixture of cyclohexane propionic acid, cyclohexane butyric acid and cyclohexane pentanoic acid at total concentrations of 0.1, 1, 10 or 50 mg L^{-1}. (B) Phenotypes of seedlings after 17 d of incubation on increasing concentrations of single-ringed surrogate NAs on pH 5.0 medium. (C) Primary root length of seedlings grown on increasing concentrations of a two-ringed surrogate NA (DHNA) at medium pH levels of 5.0 or 7.8. (D) Phenotypes of seedlings after 17 d of incubation on increasing concentrations of DHNA on pH 5.0 medium. (E) Primary root length of seedlings grown on increasing concentrations of a diamondoid model NA (AdCA) at medium pH levels of 5.0 or 7.8. (F) Phenotypes of seedlings after 17 d of incubation on increasing concentrations of AdCA on pH 5.0 medium. Root measurements were taken after 9 d of growth under lighted conditions on vertically positioned culture plates. Mean primary root length (±SEM) is indicated. (* = significant difference from control, $p < 0.05$).

increasing concentrations of OSPW, fine tailings, and petroleum NAs resulted in reduced growth rates, photosynthetic rates, leaf expansion ratios and O_2 uptake rates of seedling root systems (Bendell-Young et al., 2000; Renault et al., 2000; Kamaluddin and Zwiazek, 2002). These effects could contribute to the OSPW, AEO and NA induced growth phenotypes observed in this study, such as shortened and wavy root growth, chlorosis, and curled cotyledons. In studies performed by Armstrong et al. (2008, 2009), commercial NA and AEO concentrations of 60 mg L^{-1} were acutely toxic to cattail, whereas minimal negative effects were observed at 30 mg L^{-1}. By contrast, our study showed reduced growth at NA and AEO concentrations lower than 30 mg L^{-1}. It may be that NA sensitivity is more pronounced in young seedlings grown on the sterile, solid medium that were used in our study, than the more mature plants that were grown hydroponically (Armstrong et al., 2008). Alternatively, metabolic pathways may differ between aquatic and terrestrial plant species, which may be a key determinant to NA tolerance.

The observed effects of OSPW on Arabidopsis development (Fig. 1) could, in part, be due to compounds other than NAs. Potentially toxic concentrations of ions such as sodium and chloride, as well as sulfate, heavy metals, and other non-acid extractable compounds are present in OSPW (Allen, 2008). Previous studies demonstrated that OSPW sampled from tailings ponds at various depths had sodium concentrations ranging from 0.31 to 0.55 g L^{-1} (MacKinnon and Sethi, 1993; Renault et al., 2000). Arabidopsis plants begin to display negative growth effects at concentrations of 2.9 g L^{-1} (50 mM) NaCl (Xiong and Zhu, 2002). This suggests that the amount of salt in tailings ponds is not enough to be acutely toxic to the plants, but could act additively or synergistically with other compounds to affect sensitivity. Indeed, NAs in particular have been proposed to aggravate salt stress in plants (Kamaluddin and Zwiazek, 2002; Apostol et al., 2004). A possible additive or synergistic effect could be seen in our results by comparing the reduction in root length of the 5% OSPW treatment (Fig. 1B and 1D) to the 1 mg L^{-1} AEO treatment (Fig. 2B and C). OSPW at 5% is roughly equal to 1 to 2 mg L^{-1} of AEO (based on 20–40 mg L^{-1} total NA concentration of our samples), and yet there is a greater phenotypic effect observed in the OSPW treatment.

The pH of the plant growth environment has an effect on the level of NA toxicity (Armstrong et al., 2009). We demonstrated that in the AEO and NA treatments (Figs. 2 and 3), Arabidopsis plants grown on pH = 5.0 medium had more reduced primary root growth compared to those grown on pH = 7.8 medium. The pK_a of NAs was previously reported to range from 5.0 to 6.0 (Headley and McMartin, 2004). Since the AEO fraction was shown to be complex (Fig. 2A), the pK_a values probably extend over a large range. However, at pH levels greater than 6.0, most NAs are likely to be in their ionized naphthenate salt form, and should be predominantly non-ionized at pH levels less than 5.0. In their non-ionized form, NAs are thought to more readily enter cell membranes and cause changes in membrane properties (Seward and Schultz, 1999), resulting in toxicity. The negative growth effect of Arabidopsis seedlings in AEO treatments at pH 5.0 compared to that at pH 7.8 is consistent with this and previous observations (Armstrong et al., 2009). The NAs used in this study have pK_a values slightly below 5.0. However, their higher toxicity at pH 5.0 may be explained by the fact that a proportion of the NAs would be in the ionized form at a pH only slightly above the pK_a value. The majority of oil sands tailings pond water is alkaline at a pH ranging from 7.7 to 8.5. Thus, NAs in OSPW would be expected to be primarily in their ionized form. However, soils in the Athabasca region are often acidic (Biryukova et al., 2007). Thus, NA-containing soils are likely enriched in the more toxic, non-ionized form of NA.

We showed that the NA treatments resulted in a similar trend to the OSPW and AEO treatments in terms of causing reduced root growth in response to increasing treatment concentrations (Fig. 3A). However, the surrogate petroleum NAs were generally more toxic than AEO at higher concentrations (compare Figs. 2B, 3A and C). OSPW presumably contains thousands of NA types. Thus, particular NA species in OSPW that potentially have higher toxicity to plants would be present at lower overall concentrations, in contrast to the much higher concentrations of the individual surrogate NAs used here. Alternatively, this increase in toxicity could be because the simpler, lower molecular weight surrogate NAs are smaller and more readily taken up by the plant roots and, therefore, may accumulate to a toxic level at lower concentrations. Armstrong et al. (2008) suggested that lower molecular weight NAs may be more toxic than higher molecular weight NAs. This is consistent with our results, as the higher molecular weight NAs (DHNA and AdCA) were less toxic than the lower molecular weight, single-ringed NAs. However, Frank et al. (2008) examined the toxicity of different surrogate NAs (including cyclohexane pentanoic acid) on the water flea, *Daphnia magna*, a common toxicity indicator. They found that exposure to higher molecular weight NAs led to increased toxicity. This difference may be explained by a different toxicity mechanism between plants and other organisms. Armstrong et al. (2009) found that the pH of an AEO-containing medium affected plant toxicity but had no effect on *D. magna* toxicity. In addition, the recent analysis of NAs extracted from tailings pond water showed that the AEO fraction contained a substantial number of compounds other than classical NAs (Grewer et al., 2010), which would also contribute to observed differences in toxicity between the NAs and the AEO treatments used in the present study.

In addition to negative phenotypic effects, we frequently observed that Arabidopsis plants subjected to low concentrations of OSPW, AEO or NA (i.e. 5% OSPW and 0.1–1 mg L^{-1} NA) often had larger leaves than those of the control (Figs. 1D, 2C, 3). OSPW and NAs have been associated with stimulatory effects in the algal and plant species, *Pseudokirchneriella subcapitata* and *Phaseolus vulgaris*, respectively (Wort et al., 1973; Nix and Martin, 1992). The stimulatory effect of NAs on plants could be due to the structural similarity between two-ringed NAs and naphthalene acetic acid (NAA), a member of the auxin group of plant hormones (Armstrong, 2008). Two-ringed NAs have been shown to be a dominant class of the NAs present in OSPW (Grewer et al. 2010). The single-ringed NA cyclohexane carboxylic acid is also a well-documented plant hormone (Padmanabhan and Wort, 1977). If auxin receptors recognize some similarly structured NAs, they may be stimulatory in low concentrations and toxic in higher doses, as appears to be the case in this experiment.

Understanding the response of Arabidopsis to NA exposure is a necessary first step toward using this species as a biosensor for NA toxicity and for identifying plant metabolic pathways involved in NA degradation. Arabidopsis shows a comparable phenotypic response to OSPW, AEO and NA treatment as has been observed for species that are native to the Athabasca oil sands region. The unparalleled genetic tools that are available for this model plant species will allow for in depth genetic analysis of Arabidopsis in response to NAs. The availability of Arabidopsis genotypes that are tolerant to various components of OSPW, such as salts and heavy metals, would provide a means to circumvent the toxicity effects from other OSPW-containing compounds in NA-focused studies. Also, there is potential to use Arabidopsis to identify genes that are involved in the uptake and degradation of NAs. Arabidopsis has been used previously to identify genes that encode proteins that are involved in the degradation of organic contaminants, such as cytochrome P450s, oxygenases, dioxygenases, and peroxidases (Cobbett and Meagher, 2002). Plants have extensive root systems, and coupled with their complex and unique biochemical pathways, have the potential to more effectively take up and degrade toxic

compounds than microbes (Cobbett and Meagher, 2002). Once NA detoxifying genes have been identified, indigenous plant species, such as aspen, can be screened for elevated levels of a specific gene to identify natural genotypes with enhanced NA remediation ability. Alternatively, a molecular biology approach could be used to overexpress a specific gene of interest to enhance a plant species ability to degrade NAs.

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