



Removal and biodegradation of naphthenic acids by biochar and attached environmental biofilms in the presence of co-contaminating metals



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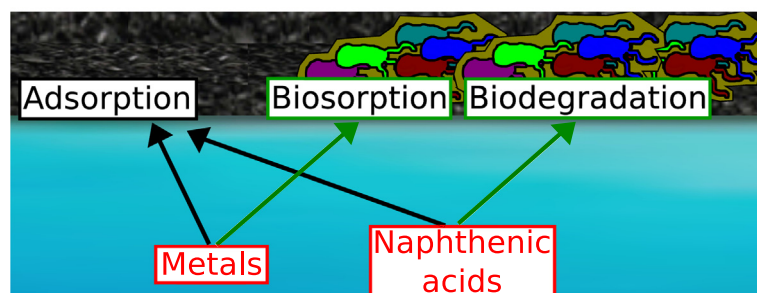
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HIGHLIGHTS

- A combined biofilm-biochar approach for contaminant removal was evaluated.
- Biotic-biochar was more effective at removing naphthenic acids vs. sterile biochar.
- Evidence for enhanced organics removal by biotic-biochar in the presence of metals.
- Greater metal sorption evident with biofilm-attached biochar.
- This combined approach was more effective at removal than either independently.

GRAPHICAL ABSTRACT



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ABSTRACT

This study evaluated the efficacy of using a combined biofilm-biochar approach to remove organic (naphthenic acids (NAs)) and inorganic (metals) contaminants from process water (OSPW) generated by Canada's oil sands mining operations. A microbial community sourced from an OSPW sample was cultured as biofilms on several carbonaceous materials. Two biochar samples, from softwood bark (SB) and Aspen wood (N3), facilitated the most microbial growth (measured by protein assays) and were used for NA removal studies performed with and without biofilms, and in the presence and absence of contaminating metals. Similar NA removal was seen in 6-day sterile N3 and SB assays (>30%), while biodegradation by SB-associated biofilms increased NA removal to 87% in the presence of metals. Metal sorption was also observed, with up to four times more immobilization of Fe, Al, and As on biofilm-associated biochar. These results suggest this combined approach may be a promising treatment for OSPW.

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Abbreviations: AC, activated carbon; OSPW, oil sands process water; NAs, naphthenic acids; ECM, extracellular matrix; CBD, Calgary Biofilm Device; MBBR, moving bed biofilm reactor; BH, Bushnell-Haas media; BH_G, BH amended with 1 g L⁻¹ glucose; BH_{8χ} or 8χNA, BH amended with 200 mg L⁻¹ NAs; HA, hexanoic acid; CPCA, cyclopentane carboxylic acid; mCHCA, 3-methyl-1-cyclohexane carboxylic acid *cis* and *trans* stereoisomers; CHCA, cyclohexane carboxylic acid; CHAA, cyclohexane acetic acid; DA, decanoic acid; CHBA, cyclohexane butyric acid; ACA, 1-adamantane carboxylic acid; BH_{M+} or Model OSPW, 8χNA amended with 17 metals; TSA, tryptic soy agar; BH_{G0}, BH_{8χ0} or BH_{M+0}, experiments inoculated with amended media (i.e. BH_G, BH_{8χ}, or BH_{M+}) and incubated without amended media (i.e. BH); CLSM, confocal laser scanning microscopy; EDX, energy dispersive X-ray spectroscopy; GC-FID, gas chromatography coupled with flame ionization detection; BRE, biological removal efficiency; SEM, standard error of the mean.

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1. Introduction

Historically, adsorbents have been used to treat wastewater challenges. Activated carbon (AC) has a long history being used as an adsorbent, but its cost is restrictive to widespread use. AC can be made from biochar, which are produced by pyrolyzing (i.e. thermal degradation in the absence of O₂) biomass from a wide range of feedstocks (e.g. wood, bark). Biochar, while having lower porosity and surface areas than AC, can effectively adsorb diverse organic and inorganic contaminants from soil and water (Ahmad et al., 2014) for a fraction of the price. One context where such contaminants have become an issue is Canada's oil sands operations. Oil sands surface mining operations are water intensive (Suncor, 2014), generating what is referred to as oil sands process water (OSPW). These liquid tailings have become an issue of environmental concern due to their large volumes and toxicity (Allen, 2008), the latter of which has them subject to a "zero discharge" policy (Brown and Ulrich, 2015). Naphthenic acids (NAs), a broadly defined group of acyclic and cycloaliphatic carboxylic acids, contribute to the acute toxicity associated with OSPW (Lo et al., 2006) and thus have been the focus of OSPW remediation research. Tailings ponds have been reported to contain an average NA concentration of 110 mg L⁻¹, as compared to <1 mg L⁻¹ is found in regional rivers; OSPW reclamation currently relies on natural processes to remove NA contaminants, however, some have NAs proven to be refractory and thus the timeframe for this remediation is lengthy (i.e. decades) and uncertain (Allen, 2008; Brown and Ulrich, 2015). To date, experimental tailings ponds and nutrient enriched simulated wetlands, which utilized the natural attenuation of these tailings ponds, continued to exhibit chronic toxic effects in bioassays after 20 years and 1 year, respectively (Brown and Ulrich, 2015). The purpose of this study was to investigate the potential use of biochar, in concert with environmental microbial populations, as an inexpensive and passive means to expedite reclamation efforts.

Recent biochar research has explored its ability as a microbial inoculum carrier (Hale et al., 2015). To attach and adhere to a surface (e.g. biochar), living cells excrete a variety of polymers used to anchor themselves to the substrate, developing an extracellular matrix (ECM)-enclosed microbial biofilm (Hall-Stoodley et al., 2004). The close proximity biofilm-embedded cells may allow for genetic or metabolite exchanges that can facilitate the degradation of xenobiotic substrates (Singh et al., 2006). Biofilms are also resistant to mechanical stress and retain microbial biomass when attached to solid carriers (Nicolella et al., 2000). Due to these qualities, some researchers have investigated the utilization of biofilms in *ex situ* bioreactors for NA degradation (McKenzie et al., 2014).

Using the Calgary Biofilm Device (CBD), a high throughput method of growing biofilms, microbial communities indigenous to the oil sands tailings environment have been successfully cultured as biofilms. Notably, this method was able to maintain 70–80% of the microbial community from the original tailings sample (Golby et al., 2012), outperforming traditional *in vitro* methods of culturing microbes from an environmental inoculum. Using the same method, the OSPW environmental community has demonstrated the capacity to aerobically degrade model NAs of varied recalcitrance to below detectable limits (Demeter et al., 2015). Furthermore, these bench-scale experiments were translated to wastewater treatment techniques by harnessing OSPW microbial biofilms on moving bed biofilm reactor (MBBR) carriers; reactors inoculated in this manner retained the previously observed ability to degrade select model NAs (Lemire et al., 2015). In response to the oil sands industry's indication of the need for a passive OSPW treatment system (i.e. requiring little to no energy input or maintenance), the work herein aimed to transfer this concept to

a passive approach, using biochar as the biofilm support material. The complexity of the system was increased to include inorganics, to be more reflective of the tailings pond environment (Allen, 2008), as well as to assess the effects of metals on NA degradation by the native OSPW microbiota. While metals are known to inhibit the microbial degradation of organic pollutants (Sandrin and Maier, 2003), the indigenous OSPW microbial community has been shown to be tolerant to metal concentrations multiple orders of magnitude higher than those reported in tailings ponds (Frankel et al., 2016).

Alberta's oil sands were used as a case study to investigate the combined potential of biochar and environmental microbes to facilitate contaminant removal from industrial wastewater. The goal was to evaluate the hypothesis that a combined biofilm-biochar approach would remove organic and inorganic contaminants more effectively than either approach could independently. This approach combines the inherent adsorptive capacity of biochar with the biodegradative ability of native OSPW microbes, and has potential to enhance oil sands and other industrial wastewater treatment operations.

2. Methods and materials

2.1. Carbon support materials

A range of support materials was screened and will be referred to interchangeably as support materials, biochar, or biochar samples. Eight types of biochar were evaluated, all of which derived from woody biomass feedstocks native to northern Alberta. The microbial attachment efficacies of biochar samples were compared to that of two commercially available non-biochar alternatives: ColorSorb G5, a steam activated carbon from Jacobi Carbons AB (Kalmar, Sweden), and Carbon Black (CBb), a solid amorphous carbon produced from natural gas using the CarbonSaver[®] process (Atlantic Hydrogen Inc., Fredericton, NB, Canada). Feedstocks and physical descriptions of support materials can be found in Table 1; refer to Veksha et al. (2014) for methods used for characterization.

2.2. Media

An adapted Bushnell-Haas (BH) minimal salts media (Bushnell and Haas, 1941) (pH 6.6, 1.0 g KH₂PO₄, 1.0 g Na₂HPO₄, 0.5 g NH₄-NO₃, 0.5 g (NH₄)₂SO₄, 0.2 g MgSO₄·7H₂O, 0.02 g CaCl₂·2H₂O, 0.002 g FeCl₃, 0.002 g MnSO₄·2H₂O, per liter of double distilled water (ddH₂O)) was used in all experiments; stocks were made 2X the concentration of working solutions, and stored at room temperature. BH has been used in previous work evaluating the microbial utilization of hydrocarbons by indigenous OSPW microorganisms (Herman et al., 1994; Wyndham and Costerton, 1981). Different amendments were added to BH, depending on the assay. Initial support material screens used 1 g L⁻¹ of glucose (BH_C). NA assays were performed with a synthetic mixture of 8 commercially available model NAs previously described by Demeter et al. (2015), prepared to a total NA working concentration of 200 mg L⁻¹ (vs. 110 mg L⁻¹ reported in average tailings ponds (Brown and Ulrich, 2015)) with 0.1 g L⁻¹ glucose in BH (BH_{8χ} or 8χNA, following previous nomenclature); NA stocks were made as salt naphthenates (Sigma Aldrich) at 10X-working concentration and stored at room temperature. The mixture contains equimolar (1.7 mM) amounts of each of the following NAs: hexanoic acid (HA), cyclopentane carboxylic acid (CPCA), 3-Methyl-1-cyclohexane carboxylic acid *cis* and *trans* stereoisomers (mCHCA), cyclohexane carboxylic acid (CHCA), cyclohexane acetic acid (CHAA), decanoic acid (DA), cyclohexane butyric acid (CHBA),

Table 1
Physical characteristics of carbon support materials screened. Materials denoted with asterisks (*) are commercially produced non-biochar materials: steam activated carbon (G5) and Carbon Black (CBb). For methods used to characterize sample BET surface areas, total and micropore volumes, refer to Veksha et al. (2014).

Name	Feedstock	Particle size [mm]	BET surface area – N ₂ [m ² g ⁻¹]	Micropore volume – N ₂ [ml g ⁻¹]	Total pore volume – N ₂ [ml g ⁻¹]
G5*	Steam activated hardwood	<0.15	973	0.30	0.60
CBb*	Natural gas	0.5–2.0	89	<0.01	0.34
N5	Aspen wood chips	0.3–1.0	425	0.17	0.19
N3	Aspen wood chips	0.3–0.6	4	<0.01	<0.01
H3	Aspen bark ^a	2–10	21	<0.01	0.04
H5	Aspen bark ^a	2–10	107	0.04	0.07
W3	Aspen wood ^a	2–10	5	<0.01	0.10
W5	Aspen wood ^a	2–10	393	0.17	0.12
SB	Softwood (pine/spruce) bark ^b	2–20	189	0.07	0.12
SS	Softwood (pine/spruce) sawdust ^b	2–20	286	0.11	0.15

^a Biomass sourced from northern Alberta sawmills.

^b Biomass sourced from Abri-Tech™ modular construction (QC, Canada).

and 1-adamantane carboxylic acid (ACA). Model OSPW (BH_{M+}) work included the 8 χ NA mixture with the addition of 17 metals, see Table 2; metal mix stocks were made to 100X-working concentration and stored at room temperature. The additional carbon source was included in 8 χ NA media and Model OSPW since previous OSPW community work demonstrated NA degradation benefitted from a 0.001 to 1.0 g L⁻¹ glucose amendment (Demeter et al., 2015); 0.1 g L⁻¹ was chosen because this was the lowest concentration added to 8 χ NA that yielded detectable protein concentrations over 6-day incubations (unpublished data). In the context of an environmental inoculum, it has been suggested that some level of readily available substrate may be necessary to maintain a diverse community with adaptable metabolisms (Demeter et al., 2015). Additionally, microbial degradation of recalcitrant organics have been found to be facilitated by the presence of additional substrates, whether used directly (i.e. growth) or indirectly (e.g. degradative enzyme maintenance), via what has been coined as “co-metabolism” (Nzila, 2013).

2.3. Biofilm growth conditions

A single sample of OSPW taken from an oil sands tailings pond in Northern Alberta, Canada was used to inoculate all experiments; the sample was stored in a sealed container at 4 °C upon receiving them to minimize storage effects. So as to avoid interactions between OSPW contaminants and support materials, OSPW community biofilms were cultured on the CBD prior to experiments. In this, a CBD lid is placed in a standard 96-well microtiter plate

Table 2
Metals included in Model OSPW.

Metal	Concentration (μ g/L)
Li	180
Mg	27,400
Ca	57,000
Sr	196
Ba	60
Al	16,820
Fe	33,400
Cd	160
Mn	340
Co	4
Ni	15
Cu	4
Zn	40
Pb	230
V	320
Mo	630
As	6

with OSPW diluted 1:1 with amended BH; biofilms adhered to CBD polystyrene pegs, which were snapped off with sterile pliers and stored at –80 °C in media with 20% glycerol for later use. Previous OSPW community growth assays suggest that 6 days are required for a robust biofilm to form (unpublished data), thus, CBD seed pegs were inoculated for 6 days, and media replenished every 2 days.

An initial screen of support materials was performed using the ubiquitous soil organism *Pseudomonas fluorescens* (ATCC13526). Once the most efficacious materials for attachment and growth were established, they were then assayed with the OSPW community. OSPW cultures were inoculated with CBD pegs, and *P. fluorescens* with 1:30 dilution of 1.0 McFarland Standard (approx. 3×10^8 - cfu mL⁻¹) suspension. All incubations were placed on a gyratory shaker (150 rpm, model G2, New Brunswick Scientific Co.) at 95% humidity. The OSPW community and *P. fluorescens* biofilm cultures were grown at 25 °C, for 6 and 4 days, respectively. Subcultures and spot plates were performed on tryptic soy agar (TSA) plates.

2.4. Support material screening

Initial microbial-biochar attachment assays were performed using 24-well cell culture plates. Test wells contained 750 μ L BH_C with 30 mg of support material in each well, then inoculated with 750 μ L of a 1:15 dilution of a 1.0 McFarland Standard *P. fluorescens* suspension; sterile controls contained 1.5 mL of media with carbon supports. Any unused rows were filled with 0.9% saline solution to prevent dehydration. After 6 h to allow for planktonic attachment, media with suspended carbon support material were transferred into a sterile 1.5 mL microfuge tube, centrifuged at 10,000 rpm for 2 min, and overlying media discarded. Carbon pellets were resuspended with 1 mL saline solution and aspirated several times, centrifuged, decanted, and resuspended again for another rinse (2X total). At this point, 20 μ L aliquots of media-biochar were taken and spot-plated on TSA to evaluate if there was growth from microbes attached to biochar samples.

2.5. Assessing proliferation on support materials

Support materials that demonstrated attachment were then assayed with both the OSPW consortia and *P. fluorescens* to investigate proliferation. Inoculated plates were incubated in BH_C for 6 h to allow planktonic attachment to carbon supports, then isolated and rinsed as previously described. Next, samples of suspended support material and media (100 μ L) were taken, and the remaining support materials from the inoculation plates were split between two replenishment plates: one with amended media (BH_C), and another with unamended media (BH_{C0}). After 4 and

6 days for *P. fluorescence* and the OSPW community, respectively, another sample was taken. All samples were lysed in a boiling water bath (10 min) and stored (≤ 15 days) at 4 °C for subsequent protein analysis.

Carbon support materials that demonstrated the most proliferation in amended media (vs. unamended BH), were then grown for a 30-day assay in the presence of NAs ($BH_{8\chi}$) and NAs with metals (BH_{M+}); unamended ($BH_{8\chi_0}$ and BH_{M+0}) experiments were also performed in parallel, as with the initial screens. Since biochar is reported to be biologically and chemically recalcitrant within the time-frame of this study (Spokas, 2010), unamended assays were performed to elucidate if microbes could utilize biochar-adsorbed NAs for growth and reproduction.

Under these conditions, samples were taken every 2 or 3 days and twice rinsed every 4 or 6 days for *P. fluorescens* and the OSPW community, respectively. Samples were taken before and after rinsing, so as to evaluate total protein concentration from free swimming and attached cells (unrinsed samples), as well as those solely attached to biochar samples (rinsed samples).

2.6. Protein analysis

The Bio-Rad protein assay (Bio-Rad Laboratories, California, USA) was used to measure protein concentration. As only living cells can produce new proteins, an increase in protein concentration over the incubation period was considered a reflection of microbial growth and reproduction. The manufacturer's instructions were modified for a 96-well microtiter plate.

Optical density was recorded with a spectrophotometric plate reader (EnSpire[®], PerkinElmer) at 595 nm. Protein yields were calculated after comparison with standard curves made from diluted 200 $\mu\text{g mL}^{-1}$ bovine serum albumin solution.

2.7. Confocal laser scanning microscopy (CLSM)

After rinsing, biofilms were fixed on support materials with 5% glutaraldehyde solution. Acridine Orange (0.1% w/v) was used to visualize biofilms under a Leica DM IRE 20X objective lens (Harrison et al., 2006). Images were captured using Leica Confocal Software (LCS, Leica Microsystems). 3D images were compiled and biofilm thickness assessed with Imaris X64 Image Processing Software (Bitplane Scientific Software, South Windsor, CT, USA).

2.8. Scanning electron microscopy

Samples were prepared as described in Harrison et al. (2006) to examine the ECM attached to support materials. In brief, 0.1 M cacodylate buffer (pH 7.2) was used to fix rinsed biofilms to support materials (2 h at room temperature), then air dried for a minimum of 5 days before mounting. Samples were sputter coated with gold-palladium and visualized using an FEI XL30 scanning electron microscope. Elemental analysis was performed using an energy dispersive X-ray (EDX) spectrometer; Genesis V5.2 was used to quantify the relative abundance of elemental spectral peaks identified using NIST DTS-II (Iona 2015-08-27 revision). EDX measurements were taken on areas on biochar samples where biofilm was visually prevalent, as well as absent, for direct sample comparison; for this purpose 1–3 EDX measurements were taken from 10 to 11 different pieces of each biochar. Larger areas (0.24–0.48 mm^2) of biochar, cultured in Model OSPW with and without microbes, were also measured via EDX to evaluate adsorption of elements to biochar pieces as a whole ($n = 7$). Elements in Model OSPW guided EDX peak identification, and only spectra with definitive elemental signatures in samples were included in further analysis. Numbered red boxes in scanning electron micrographs show areas where EDX analyses were performed.

2.9. NA removal analysis

NA analysis was performed before and after 6-day incubations using 8χ NA and Model OSPW from sterile and biofilm-associated biochar cultures. Spent media (1.25 mL) was filtered and collected in sterile 1.5 mL microfuge tubes; an internal standard was added (100 μL 4-phenyl butyric acid at 1.35 g L^{-1}) and acidified to pH = 2 (with 5.2 M HCl). Samples were transferred into two-dram glass vials with Teflon-lids for extraction with two volumes of dichloromethane. Organics separated from media in dichloromethane were analyzed for NAs using gas chromatography coupled with a flame ionization detector (GC-FID, Agilent model 7890) as described by Demeter et al. (2015). Briefly, an Agilent HP-5 30 m column was used, with a 4 μL injection, 2:1 injector split ratio, under the following oven program: 2 min at 70 °C, ramped up (5 °C min^{-1}) to 230 °C and held for 2 min; specific retention times for each component NA can be found in Demeter et al. (2015). Fresh media was used to determine untreated (day 0) NA levels, which was used to normalize the relative abundance of each NA in sterile and biotic-biochar incubations as a fraction of the starting concentration. Due to the nature of the methods used in this study, any observed removal of NA from media was due to change or loss in NA parent structure (Demeter et al., 2015), such as by adsorptive removal or biological modification of NA original structure (e.g. biodegradation via β -oxidation (Quesnel et al., 2011)).

2.10. NA removal and elemental sorption activity calculations

Total NA removal was calculated as the percentage of NAs removed, using the equation:

$$\text{NA Removal (\%)} = \left(1 - \frac{\sum \text{NA}_{\text{treated}}}{\sum \text{NA}_{\text{untreated}}} \right) \times 100 \quad (1)$$

where $\sum \text{NA}_{\text{treated}}$ and $\sum \text{NA}_{\text{untreated}}$ were the sum of the component NA concentrations (normalized to internal standard) before and after treatment with biochar, respectively.

Biological Removal Efficiency (BRE) for each component NA was calculated as the percent change in sterile biochar NA removal due to microbial presence (i.e. biodegradation), using the following equation modified from He et al. (2014):

$$\text{BRE (\%)} = \frac{\frac{\sum_i^{n_{\text{sterile}}} C_{\text{sterile}}}{n_{\text{sterile}}} - \frac{\sum_i^{n_{\text{biotic}}} C_{\text{biotic}}}{n_{\text{biotic}}}}{\frac{\sum_i^{n_{\text{sterile}}} C_{\text{sterile}}}{n_{\text{sterile}}}} \times 100 \quad (2)$$

where n_{sterile} and n_{biotic} were the number of sterile and biotic samples; C_{sterile} and C_{biotic} were the relative NA concentration remaining after 6-day incubations in sterile and biotic biochar cultures, respectively.

2.11. Statistical analysis

Protein assay data from triplicate incubations and GC-FID data from duplicate samples were reported as means with standard error of the mean (SEM). Graphs, unpaired t-tests, linear regression plots, and coefficients of determination (r^2) were obtained using GraphPad Prism version 6.0. Slopes of lines were determined significantly non-zero by P-values of less than 0.05.

3. Results and discussion

3.1. Attachment and proliferation on biochar samples

Initial experiments using the model organism *P. fluorescens* showed that this bacterium had the ability to attach and form biofilms on all the carbon support materials, albeit at different efficacies; this result was further supported by experiments using

OSPW derived cultures. Fig. 1 shows that SB, N3, and SS were the only samples to demonstrate statistically significant increases in protein concentration, as measured by unpaired t tests (P-values of 0.003, 0.028, 0.009 respectively). Subsequent screens with $BH_{8\chi}$ determined SB and N3 to be the only support materials to consistently show significant differences in growth between carbon-amended and unamended cultures and were thus used for the 30-day assays in the presence of naphthenic acids ($BH_{8\chi}$) and metals (BH_{M+}).

30-day $BH_{8\chi}$ assays suggest OSPW community proliferation in the presence of NAs on N3 and SB (Fig. 2). Linear regression analyses of attached as well as total (attached and planktonic free swimming cells) protein vs. time demonstrated significant positive slopes on both biochar N3 and SB (Table 3). N3 had the steepest slope and strongest relationship for total protein from cells ($r^2 = 0.61$, P-value < 0.001), and SB having the strongest relationship with regards to attached protein over the 30 days ($r^2 = 0.45$, P-value = 0.002).

Model OSPW (BH_{M+}) cultures only demonstrated a significant relationship with total protein measurements in N3 incubations (Table 4). While SB protein accumulation (total and attached) did not show significant relationships, their measured protein concentrations were within the same range as sterile 30-day incubations ($1.2\text{--}1.8 \mu\text{g } 20 \mu\text{L}^{-1}$). Analogous protein ranges in biotic and sterile experiments suggests that concentrations (and associated microbial

growth) may have been below the detectable limits of the assay. CLSM measurements of biofilm thickness demonstrate that, while there was no apparent increase in protein, there was indeed an increase in biofilm thickness over the 30-day period (Fig. 3).

Differences in microbial-biochar interactions have been attributed to the physical and chemical properties of materials (Hale et al., 2015), which are known to vary by feedstock and pyrolysis temperature, and contribute to different effects on attached microorganisms (Lehmann et al., 2011) (i.e. attachment and proliferation). As described by others (Xie et al., 2015), there was an increase in biochar porosity and surface area with increased preparation temperature. Some have posited that increased surface area, alone, is not enough for a material to be an effective inoculant carrier (Lehmann et al., 2011). These results suggest this can be expanded to the suitability of a material for microbial proliferation as well, as there was no correlative relationship between biochar surface area (or other physical properties measured) and accumulated biomass as evaluated by final protein concentrations after 6-day incubations in BH_C .

3.2. NA removal

Recent work has shown that model NAs can be both biodegraded by the native OSPW community (Demeter et al., 2015), and adsorbed by biochar (Alessi et al., 2014) in aqueous environments.

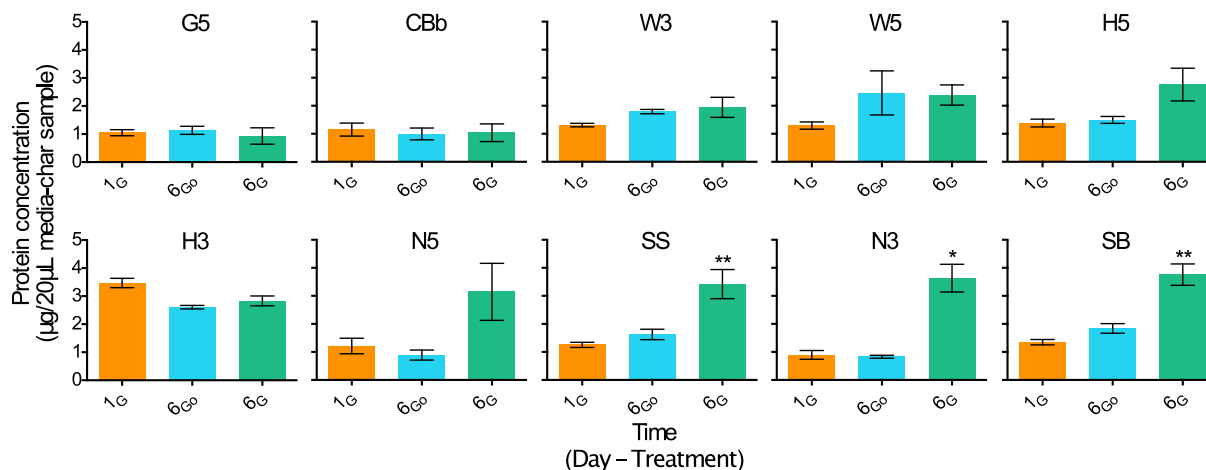


Fig. 1. Protein concentration ($\mu\text{g}/20 \mu\text{L}$ sample) of biochar-OSPW community cultures after inoculation ($1c$) and 6 days of incubation with ($6c$) and without ($6co$) glucose amendments to media. Each bar represents the mean (\pm SEM) of three to nine trials. Asterisked bars indicate significant differences between $6c$ and $6co$ final protein concentrations, as measured by unpaired t-tests with P-values (*) < 0.05 and (**) < 0.01.

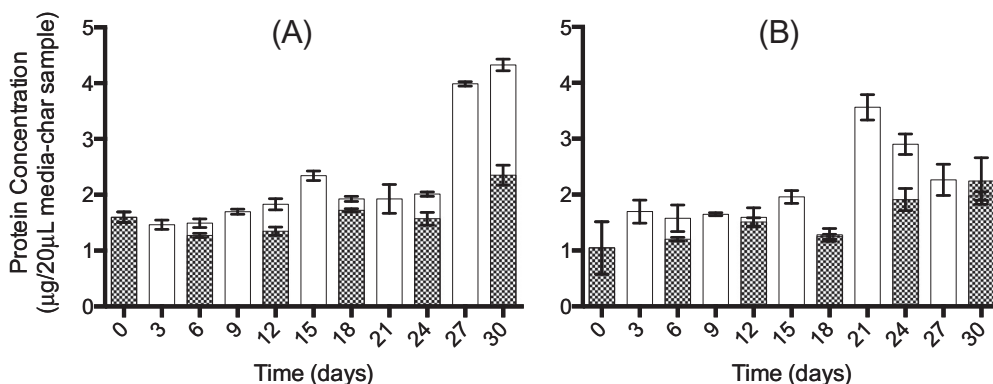


Fig. 2. Protein concentrations ($\mu\text{g}/20 \mu\text{L}$ sample) measured from OSPW-biochar cultures grown in the presence of naphthenic acids ($BH_{8\chi}$), over 30-day incubations for attached (checkered bars) and total (white bars) on (A) biochar N3 and (B) SB. Each bar represents the mean (\pm SEM) of measurements taken in triplicate.

Table 3

Linear regression analysis of protein measurements from 30-day naphthenic acid (BH_{8 χ}) experiments, with biochar N3 and SB. Protein accumulations are the lines of best fit (\pm SEM) from samples taken in triplicate, made every three or six days (for total or attached protein, respectively). BH_{8 χ o} denotes experimental control where, after 6 h of inoculation in BH_{8 χ} , cultures were incubated in unamended BH media.

Treatment	Protein measured	N3			SB		
		Protein accumulation (ng protein/20 μ L sample-day)	r ²	P-value	Protein accumulation (ng protein/20 μ L sample-day)	r ²	P-value
BH _{8χ}	Total	78.4 \pm 11	0.61	<0.001	43.6 \pm 12	0.29	0.001
	Attached	24 \pm 7	0.42	0.004	37.6 \pm 10	0.45	0.002
BH _{8χo}	Total	8.8 \pm 2	0.30	0.001	-6.8 \pm 3	0.12	0.050
	Attached	6.3 \pm 3	0.18	0.079	-8.4 \pm 5	0.18	0.077

Values in bold denote significant correlation ($P < 0.05$).

Table 4

Linear regression analysis of protein measurements from 30-day model OSPW (BH_{M+}) experiments, with biochar N3 and SB. Protein accumulations are the lines of best fit (\pm SEM) from samples taken in triplicate, made every three or six days (for total or attached protein, respectively). BH_{M+o} denotes experimental control where, after 6 h of inoculation in BH_{M+}, cultures were incubated in unamended BH media.

Treatment	Protein measured	N3			SB		
		Protein accumulation (ng protein/20 μ L sample-day)	r ²	P-value	Protein accumulation (ng protein/20 μ L sample-day)	r ²	P-value
BH _{M+}	Total	5.8 \pm 3	0.13	0.038	-3.1 \pm 3	0.03	0.320
	Attached	-3.5 \pm 4	0.04	0.402	-7.2 \pm 4	0.16	0.096
BH _{M+o}	Total	-13.1 \pm 3	0.36	<0.001	-9.5 \pm 5	0.11	0.055
	Attached	-15.8 \pm 3	0.66	<0.001	-8.5 \pm 6	0.10	0.194

Values in bold denote significant correlation ($P < 0.05$).

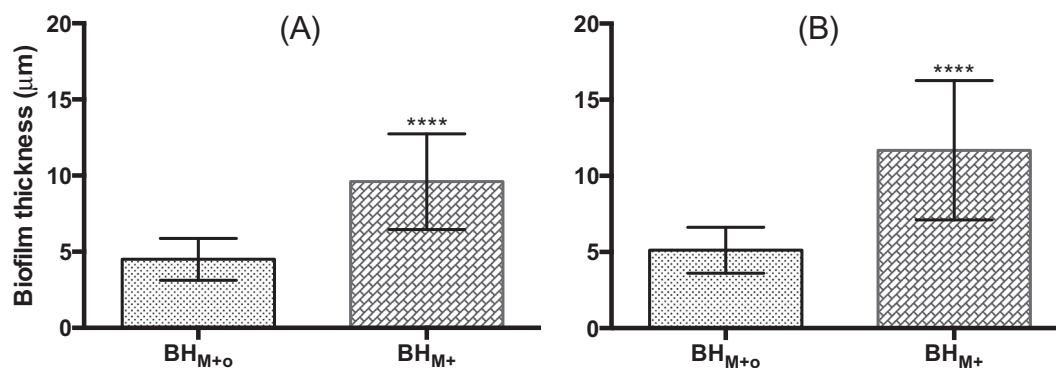


Fig. 3. Measurements of biofilm thickness of OSPW microbial cultures on (A) biochar N3 and (B) SB after 30 days. BH_{M+o} denotes an experimental control where, after 6 h of inoculation in the presence of Model OSPW (BH_{M+}) cultures were incubated in unamended BH media (i.e. no supplemental carbon source); BH_{M+} cultures were inoculated, and then further grown in the presence of Model OSPW for the entirety of the 30-day incubation. Each bar represents the mean (\pm SEM) of triplicate samples. Asterisked bars indicate significant differences between BH_{M+o} and BH_{M+} final biofilm thicknesses, as measured by unpaired t-tests with P-values (****) <0.0001.

As such, the ability of a combined microbial-biochar approach for NA removal was investigated.

GC-FID analysis of media showed that while there was 22–25% total NA removal from the initial 200 mg L⁻¹ concentration by sterile biochar, biofilm-biochar experiments demonstrated greater removal over the same 6-day period (42–72%, Table 5). These removal trends were also evident in the removal profiles of the component NAs: while there was some abiotic removal in sterile biochar (Fig. 4ai), OSPW microbes contributed to near-complete degradation of HA, DA, and CHBA (Fig. 4aaii). Biotic and sterile media pH measurements were similar (7.3 \pm 0.1) after 6-day incubations, suggesting that differences in NA removal were due to biological degradation rather than microbially influenced changes in media chemistry. In experiments with N3, there was an increase in abundance with regards to CHAA, suggesting a buildup of this NA, consistent with previous OSPW microbial-8 χ NA work by Demeter et al. (2015). The researchers attributed this buildup to an incomplete biodegradation of CHBA to produce acetate and

Table 5

Removal (%) of total NAs in growth media after 6-day incubations in 8 χ NA Media and Model OSPW, calculated by Eq. (1). Sterile and OSPW cultures were incubated for 6-days with 20 g L⁻¹ biochar SB or N3. Values are the average (\pm SEM) of duplicate samples.

	8 χ NA media		Model OSPW	
	SB (%)	N3 (%)	SB (%)	N3 (%)
Sterile	22 \pm 2.3	25 \pm 8.6	28 \pm 1.3	19 \pm 2.4
OSPW cultures	72 \pm 8.1	42 \pm 3.9	87 \pm 2.7	34 \pm 0.2

CHAA via β -oxidation, a reaction described in other CHBA degradation studies (Quesnel et al., 2011); this incomplete catabolism of CHBA leads to a production of CHAA that builds up in the media, resulting in a the negative removal ratios seen on Fig. 4aiii. ACA proved to be the most recalcitrant NA, with limited removal, mostly due to biochar sorption as evidenced by low biological removal efficiencies ($\leq 10\%$, Fig. 4aiii). This observed recalcitrance

may be of little consequence *in situ* (Demeter et al., 2015), as NA toxicity studies have found that tricyclic, adamantane diamond acids (including ACA) are amongst the least toxic NAs found in OSPW (Rowland et al., 2011).

In order to elucidate if microbes could utilize biochar-attached NAs, BH_{8χ₀} assays were performed with biochar inoculated with the 8χ_{NA} pre-established community, and incubated with BH

media without supplemental carbon sources (i.e. glucose or NAs). BH_{8χ₀} N3 total protein concentrations had a significant positive correlation with time, albeit weaker than most BH_{8χ} cultures ($r^2 = 0.30$, P-value = 0.001, Table 3). Additionally, cultures inoculated with the 8χ_{NA} community and amended with 0.1 g L⁻¹ glucose did not demonstrate any significant changes in protein over the same 30-day period. Considering the lack of

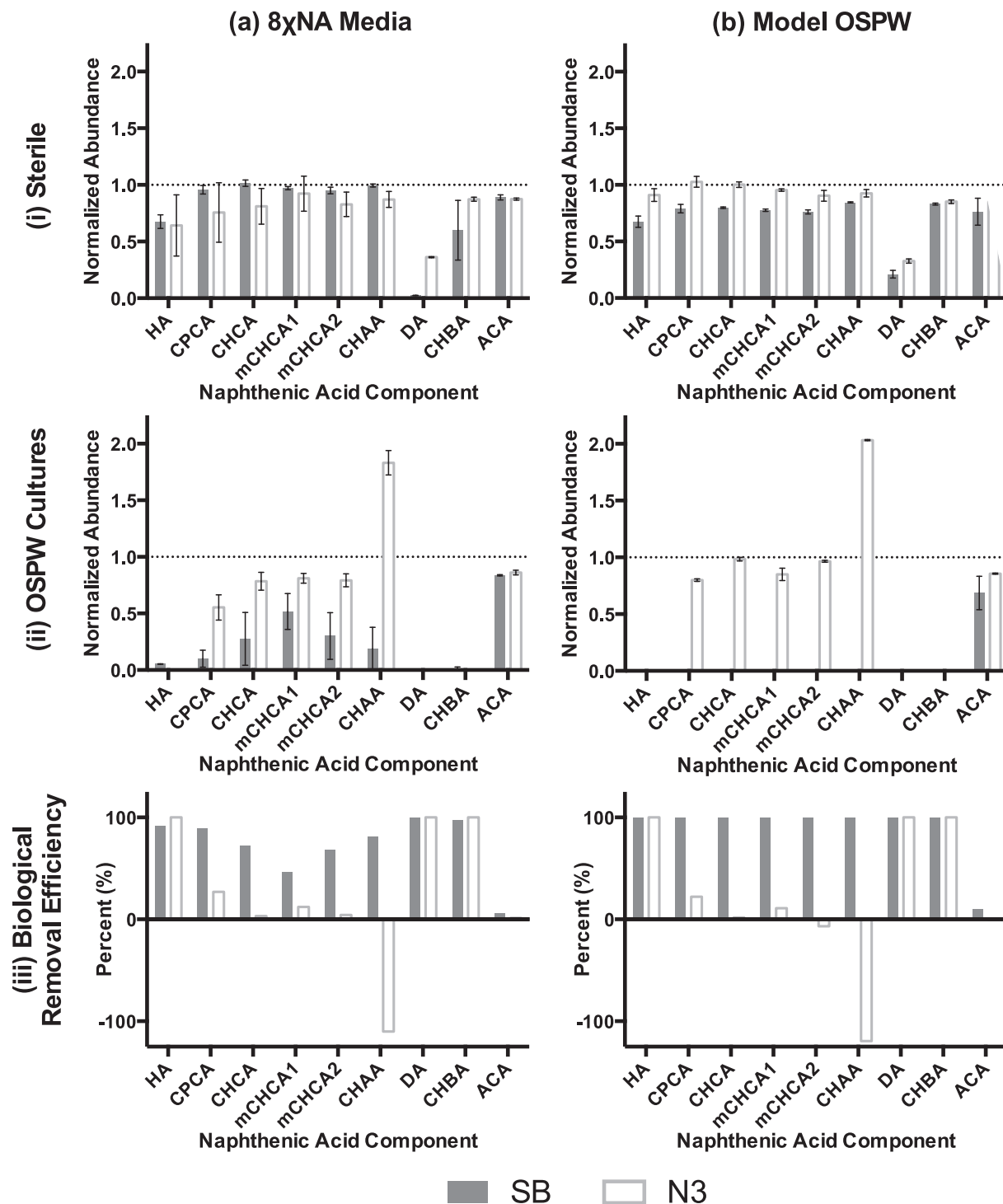


Fig. 4. Removal of naphthenic acids (NAs) in (i) sterile and (ii) OSPW cultures with biochar SB (gray bars) and N3 (white bars), from (A) 8χ_{NA} media and (B) Model OSPW. GC-FID was used to determine relative abundances of each NA component compared to an internal standard. A normalized value of '1.0' represents NA levels of untreated media; each bar represents the mean (\pm SEM) of duplicate samples. The (iii) Biological Removal Efficiency (BRE) was used as a proxy for biodegradation, and calculated as the percentage (%) of component NAs removed by the combined biochar-biofilm approach vs. sterile biochar cultures (see Eq. (2)). Positive efficiency values indicate greater removal by the combined approach than solely biochar incubations; SEM was not calculated so as to avoid statistical biases in imposing error propagation when determining removal efficiencies, as described in He et al. (2014). Cultures were incubated for 6-days with 20 g biochar L⁻¹.

growth in 8χ NA pre-established community amended with glucose, the increase in total protein accumulation seen in $BH_{8\chi}$ N3 cultures may suggest the utilization of biochar-bound NAs.

Microbial metabolism of biochar-bound organics suggests a potentially synergistic relationship when microbial degradation is paired with biochar adsorption. This synergy has been described in microbial-AC work, referred to as bioregeneration (Aktas and Çeçen, 2007). Bioregeneration is the renewal of a materials adsorptive capacity by microorganisms. In this, a material (e.g. biochar) adsorbs organics, which are metabolized by attached microbes, thus freeing binding functional groups for further adsorption. Bioregeneration has also been empirically supported with OSPW microbial biofilms metabolizing NAs while attached to AC (Islam et al., 2015).

3.3. NA removal in the presence of metals

Co-contamination of organics (i.e. NAs) and inorganics is common in industrial waste sites such as the tailings pond from which the OSPW bacterial community was sourced (Allen, 2008). Metal co-contamination has been shown to reduce the biodegradation of organic pollutants (Sandrin and Maier, 2003), thus this study also aimed to evaluate the effect of metals on OSPW community interactions with NAs.

Biochar N3 demonstrated less removal (abiotic and biotic) of NAs in the presence of metals (vs. 8χ NA Media, Table 5). Similar biological removal efficiencies were demonstrated in OSPW cultures as 8χ NA incubations, including the removal of HA, DA, CHBA, and the accumulation of CHAA and associated negative removal ratio discussed in NA removal (Fig. 4iii). Other than the biodegradation of these select NAs, little to no removal beyond that of sterile biochar N3 was evident in 6-day incubations (Fig. 4bi vs. bii). Conversely, while there was less growth in OSPW community cultures in the presence of metals (as described in Section 3.1), biochar SB had the highest NA removal (87%) in biotic cultures grown in Model OSPW (vs. 8χ NA Media, Table 5), with complete removal of all component NAs (except for ACA, Fig. 4bii). As with 8χ NA experiments, there was little variation in pH (6.9 ± 0.2) due to microbial presence, or biochar type, in Model OSPW incubations.

Biological removal efficiency values (Fig. 4biii) suggest that the enhanced NA attenuation can be attributed to the presence of the attached microbial community. Differences demonstrated in NA biodegradation associated with each support material (N3 or SB) speaks to the heterogeneity reported in the literature regarding biochar characteristics (Ahmad et al., 2014); this suggests that different types of biochar may select for different subpopulations of an environmental community, which in turn would have different metabolic potentials (Lehmann et al., 2011). The ability of the N3-associated microbes to degrade NAs appeared to be inhibited by the presence of metals, where 42% removal was evident in 8χ NA cultures vs. 34% in Model OSPW (Table 5). While some might point to the reduced microbial proliferation in Model OSPW cultures for the differences in model NA removal, biochar SB-microbes, which demonstrated comparable biofilm thickness to N3-microbes (Fig. 3), had significantly greater NA attenuation in Model OSPW than 8χ NA cultures (87 vs. 72%, Table 5). Additionally, this biodegradation and removal by SB-biofilms in Model OSPW (87%) suggests a synergism in the presence of metals, as there was greater NA removal by the combined biofilm-biochar approach than the added effects of sterile SB (22–28%) and the OSPW microbes independent of biochar (31–43%, Lemire et al., 2015).

3.4. Metal sorption

Independently, biochar and microbial biofilms each have properties conducive to the adsorption of metals: biochar has porosity to physically interact with metals, and surface functional groups

that can interact chemically (Ahmad et al., 2014), while microbes have metabolic dependent and non-dependent mechanisms of adsorption (Blanco et al., 1999; Veglio' and Beolchini, 1997), as well as the sorption potential of biofilm ECM biochemicals (Pal and Paul, 2008).

Elemental analysis via EDX detected few elements from the Model OSPW on biochar and associated biofilms; refer to Table 2 for metals and Media (Section 2.2) for Model OSPW composition. Even so, elemental analysis shows that while there is accumulation on biochar surfaces, the presence of biofilms had a concentrating effect (Fig. 5A). Of all the elements detected, the greatest accumulation was evident in biofilm-adsorbed metals, with as much as a seven-fold increase in Al (2.2 vs. 15.6 mg g^{-1}) and As (19.2 vs. 149 mg g^{-1}) on biochar SB-associated biomass compared to surrounding biochar (Fig. 5A). This was also evident when comparing areas (0.24 – 0.48 mm^2) of sterile- to biotic-biochar, where the greatest differences were seen in As (4.4 vs. 20.6) on biochar N3, as well as Al (19.2 vs. 149 mg g^{-1}) and Fe (2.6 vs. 11.6 mg g^{-1}) on biochar SB, or over four times greater accumulation on biotic-biochar (Fig. 5B).

Microbial work done with granulated AC has similarly demonstrated that biofilm attachment to AC can increase metal uptake (vs. virgin AC), both in magnitude and rate of adsorption (Scott et al., 1995). Of the elements detected, none were exclusively present on biochar or biofilms, and the increased levels on biofilms suggests the biomass acts as a sink for biochar-adsorbed elements, namely metals. Biofilm-associated functional groups make ECM anionic in nature (Pal and Paul, 2008) and thus bacterial attachment has been suggested to reduce the surface charge of

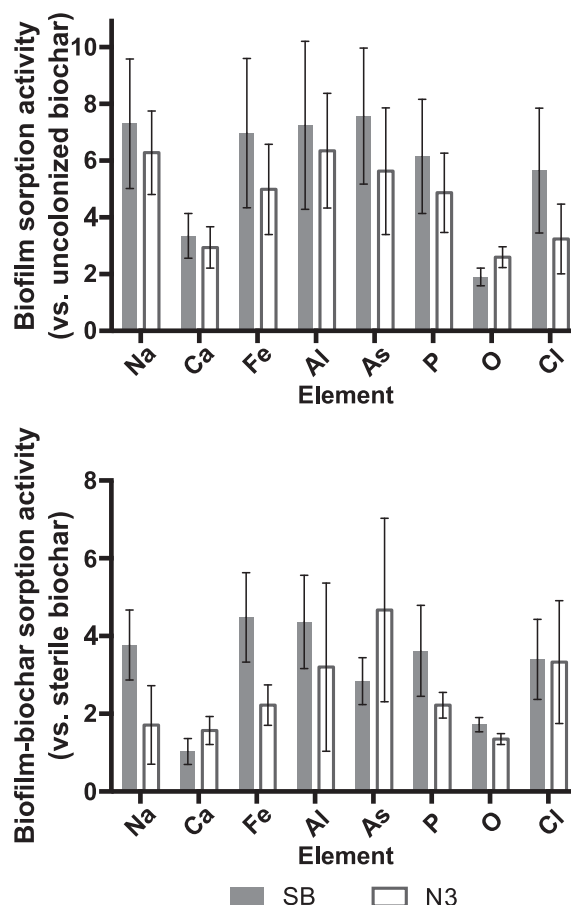


Fig. 5. Mean fold differences (\pm SEM) in elements present on (A) biofilms vs. surrounding biochar (e.g. Fig. 4C, boxes 2 vs. 1, respectively), and (B) 0.24 – 0.48 mm^2 areas of biotic vs. sterile biochar (e.g. Fig. 4B vs. A) after 30-day incubations in Model OSPW, as measured by EDX (excluding carbon).

the carbon (Rivera-Utrilla et al., 2001), which even if only locally effected (i.e. by microcolonies) will enhance the capacity to adsorb positively charged contaminants (e.g. cationic metals). While this might help elucidate the ability of biochar-associated biofilms to concentrate adsorbed metals, it was still a surprise to find so few metals from the Model OSPW in the EDX analysis.

4. Conclusions

Several carbonaceous materials were screened and two biochar samples – from softwood bark (SB) and Aspen wood – were shown to grow biofilms most effectively. Biodegradation by attached microbes was observed, and with biochar SB, a synergistic behavior between the adsorbent and biofilm was demonstrated for NA removal from water. With an initial NA concentration of 200 mg L⁻¹, biotic-SB demonstrated greater NA removal than sterile biochar (22–28%), in the absence (72%) and presence of metals (87%), outperforming 6-day NA removal demonstrated by the microbial community alone. Additionally, there was greater metal sorption by the biofilm-biochar complex than sterile biochar.

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Appendix A. Supplementary data

Supplementary data (i.e. biochar production parameters, and data from initial *P. fluorescens* screens and OSPW community assays to evaluate 6-day growth in BH_{8χ}. Appendix A also includes protein data from the 8xNA and Model OSPW pre-conditioned microbial communities, incubated for 30-days in their corresponding amended media (BH_{8χ}, BH_{M+}), unamended media (BH_{8χ0}, BH_{M+0}), BH with 0.1 g L⁻¹ glucose, as well as sterile Model OSPW incubations with each biochar. Additionally, correlations of physical characteristics of biochar samples vs. 6-day BH_C protein accumulation, rationale for Model OSPW metal working concentrations, scanning electron micrograph images, EDX spectra, summarized EDX elemental data, and pH from media before and after 6-day 8χNA and Model OSPW experiments) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2016.05.084>.

References

- Ahmad, M., Rajapaksha, A.U., Lim, J.E., Zhang, M., Bolan, N., Mohan, D., Vithanage, M., Lee, S.S., Ok, Y.S., 2014. Biochar as a sorbent for contaminant management in soil and water: a review. *Chemosphere* 99, 19–33. <http://dx.doi.org/10.1016/j.chemosphere.2013.10.071>.
- Aktaş, Ö., Çeçen, F., 2007. Bioregeneration of activated carbon: a review. *Int. Biodeterior. Biodegrad.* 59, 257–272. <http://dx.doi.org/10.1016/j.ibiod.2007.01.003>.
- Alessi, D.S., Alam, M.S., Kohler, M.C., 2014. Designer Biochar-Coke Mixtures to Remove Naphthenic Acids from Oil Sands Process-Affected Water (OSPW) (No. OSRN Report No. TR-57). Oil Sands Research and Information Network.
- Allen, E.W., 2008. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. *J. Environ. Eng. Sci.* 7, 123–138. <http://dx.doi.org/10.1139/S07-038>.
- Blanco, A., Llama, J., Serra, J.L., 1999. Biosorption of heavy metals to immobilised *Phormidium laminosum* biomass. *J. Biotechnol.* 69, 227–240. [http://dx.doi.org/10.1016/S0168-1656\(99\)00046-2](http://dx.doi.org/10.1016/S0168-1656(99)00046-2).
- Brown, L.D., Ulrich, A.C., 2015. Oil sands naphthenic acids: a review of properties, measurement, and treatment. *Chemosphere* 127, 276–290. <http://dx.doi.org/10.1016/j.chemosphere.2015.02.003>.
- Bushnell, L.D., Haas, H.F., 1941. The utilization of hydrocarbons by microorganisms. *J. Bacteriol.* 41, 653–673.
- Demeter, M.A., Lemire, J.A., Yue, G., Ceri, H., Turner, R.J., 2015. Culturing oil sands microbes as mixed species communities enhances ex situ model naphthenic acid degradation. *Front. Microbiol.* 6, 1–13. <http://dx.doi.org/10.3389/fmicb.2015.00936>.
- Frankel, M.L., Demeter, M.A., Lemire, J.A., Turner, R.J., 2016. Evaluating the metal tolerance capacity of microbial communities isolated from the Alberta oil sands process water. *PLoS ONE* 11. <http://dx.doi.org/10.1371/journal.pone.0148682>.
- Golby, S., Ceri, H., Gieg, L.M., Chatterjee, I., Marques, L.L.R., Turner, R.J., 2012. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. *FEMS Microbiol. Ecol.* 79, 240–250. <http://dx.doi.org/10.1111/j.1574-6941.2011.01212.x>.
- Hale, L., Luth, M., Crowley, D., 2015. Biochar characteristics relate to its utility as an alternative soil inoculum carrier to peat and vermiculite. *Soil Biol. Biochem.* 81, 228–235. <http://dx.doi.org/10.1016/j.soilbio.2014.11.023>.
- Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108. <http://dx.doi.org/10.1038/nrmicro821>.
- Harrison, J.J., Ceri, H., Yerly, J., Stremick, C.A., Hu, Y., Martinuzzi, R., Turner, R.J., 2006. The use of microscopy and three-dimensional visualization to evaluate the structure of microbial biofilms cultivated in the Calgary Biofilm Device. *Biol. Proced. Online* 8, 194–215. <http://dx.doi.org/10.1251/bpo127>.
- He, C., Rochfort, Q., Mcfadyen, R., 2014. Potential errors and error propagation in methods used to determine particle removal efficiency. *J. Environ. Eng.* 140, 1–5. [http://dx.doi.org/10.1061/\(ASCE\)EE.1943-7870.0000839](http://dx.doi.org/10.1061/(ASCE)EE.1943-7870.0000839).
- Herman, D.C., Fedorak, P.M., MacKinnon, M.D., Costerton, J.W., 1994. Biodegradation of naphthenic acids by microbial populations indigenous to oil sands tailings. *Can. J. Microbiol.* 40, 467–477.
- Islam, M.S., Zhang, Y., McPhedran, K.N., Liu, Y., Gamal El-Din, M., 2015. Granular activated carbon for simultaneous adsorption and biodegradation of toxic oil sands process-affected water organic compounds. *J. Environ. Manage.* 152, 49–57. <http://dx.doi.org/10.1016/j.jenvman.2015.01.020>.
- Lehmann, J., Rillig, M.C., Thies, J., Masiello, C.A., Hockaday, W.C., Crowley, D., 2011. Biochar effects on soil biota – a review. *Soil Biol. Biochem.* 43, 1812–1836. <http://dx.doi.org/10.1016/j.soilbio.2011.04.022>.
- Lemire, J.A., Demeter, M.A., George, I., Ceri, H., Turner, R.J., 2015. A novel approach for harnessing biofilm communities in moving bed biofilm reactors for industrial wastewater treatment. *AIMS Bioeng.* 2, 387–403. <http://dx.doi.org/10.3934/bioeng.2015.4.387>.
- Lo, C.C., Brownlee, B.G., Bunce, N.J., 2006. Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids. *Water Res.* 40, 655–664. <http://dx.doi.org/10.1016/j.watres.2005.12.008>.
- McKenzie, N., Yue, S., Liu, X., Ramsay, B.A., Ramsay, J.A., 2014. Biodegradation of naphthenic acids in oil sands process waters in an immobilized soil/sediment bioreactor. *Chemosphere* 109, 164–172. <http://dx.doi.org/10.1016/j.chemosphere.2014.02.001>.
- Nicolella, C., van Loosdrecht, M.C.M., Heijnen, J.J., 2000. Wastewater treatment with particulate biofilm reactors. *J. Biotechnol.* 80, 1–33.
- Nzila, A., 2013. Update on the cometabolism of organic pollutants by bacteria. *Environ. Pollut.* 178, 474–482. <http://dx.doi.org/10.1016/j.envpol.2013.03.042>.
- Pal, A., Paul, A.K., 2008. Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. *Indian J. Microbiol.* 48, 49–64. <http://dx.doi.org/10.1007/s12088-008-0006-5>.
- Quesnel, D.M., Bhaskar, I.M., Gieg, L.M., Chua, G., 2011. Naphthenic acid biodegradation by the unicellular alga *Dunaliella tertiolecta*. *Chemosphere* 84, 504–511. <http://dx.doi.org/10.1016/j.chemosphere.2011.03.012>.
- Rivera-Utrilla, J., Bautista-Toledo, I., Ferro-García, M.A., Moreno-Castilla, C., 2001. Activated carbon surface modifications by adsorption of bacteria and their effect on aqueous lead adsorption. *J. Chem. Technol. Biotechnol.* 76, 1209–1215. <http://dx.doi.org/10.1002/jctb.506>.
- Rowland, S.J., Scarlett, A.G., Jones, D., West, C.E., Frank, R.A., 2011. Diamonds in the rough: identification of individual naphthenic acids in oil sands process water. *Environ. Sci. Technol.* 45, 3154–3159. <http://dx.doi.org/10.1021/es103721b>.
- Sandrin, T.R., Maier, R.M., 2003. Impact of metals on the biodegradation of organic pollutants. *Environ. Health Perspect.* 111, 1093–1101. <http://dx.doi.org/10.1289/ehp.5840>.
- Scott, J.A., Karanjkar, A.M., Rowe, D.L., 1995. Biofilm covered granular activated carbon for decontamination of streams containing heavy metals and organic chemicals. *Miner. Eng.* 8, 221–230. [http://dx.doi.org/10.1016/0892-6875\(94\)00115-S](http://dx.doi.org/10.1016/0892-6875(94)00115-S).
- Singh, R., Paul, D., Jain, R.K., 2006. Biofilms: implications in bioremediation. *Trends Microbiol.* 14, 389–397. <http://dx.doi.org/10.1016/j.tim.2006.07.001>.
- Spokas, K.A., 2010. Review of the stability of biochar in soils: predictability of O:C molar ratios. *Carbon Manage.* 1, 289–303. <http://dx.doi.org/10.4155/cmt.10.32>.
- Suncor, 2014. SUNCOR: Rep. on sustain. 2014.
- Veglio, F., Beolchin, F., 1997. Removal of metals by biosorption: a review. *Hydrometallurgy* 44, 301–316. [http://dx.doi.org/10.1016/S0304-386X\(96\)00059-X](http://dx.doi.org/10.1016/S0304-386X(96)00059-X).

- Veksha, A., McLaughlin, H., Layzell, D.B., Hill, J.M., 2014. Pyrolysis of wood to biochar: increasing yield while maintaining microporosity. *Bioresour. Technol.* 153, 173–179. <http://dx.doi.org/10.1016/j.biortech.2013.11.082>.
- Wyndham, R.C., Costerton, J.W., 1981. Heterotrophic potentials and hydrocarbon biodegradation potentials of sediment microorganisms within the Athabasca oil sands deposit. *Appl. Environ. Microbiol.* 41, 783–790.
- Xie, T., Reddy, K.R., Wang, C., Yargicoglu, E., Spokas, K., 2015. Characteristics and applications of biochar for environmental remediation: a review. *Crit. Rev. Environ. Sci. Technol.* 45, 939–969. <http://dx.doi.org/10.1080/10643389.2014.924180>.