1 **Differential protein expression during growth on model and commercial**

2 **mixtures of naphthenic acids in *Pseudomonas fluorescens* Pf-5.**

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4 Running Title: Protein expression during growth on NAs

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25

26 **Summary**

27   Naphthenic acids (NAs) are carboxylic acids with the formula ($C_nH_{2n+z}O_2$), and

28 are the toxic, persistent constituents of oil sands process-affected waters (OSPW),

29 produced during oil sands extraction. Currently, the proteins and mechanisms involved

30 in NA biodegradation are unknown. Using LC-MS/MS shotgun proteomics, we

31 identified proteins overexpressed during growth of *Pseudomonas fluorescens* Pf-5 on

32 a model NA (4'-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) and commercial NA mixture

33 (Acros). By day 11, >95% of *n*-BPBA was degraded. With Acros, a 17% reduction in

34 intensity occurred with 10-18 carbon compounds of the *Z* family -2 to -14 (major NA

35 species in this mixture). A total of 554 proteins (*n*-BPBA) and 631 proteins (Acros)

36 were overexpressed during growth on NAs; including several transporters (e.g. ABC

37 transporters), suggesting a cellular protective response from NA toxicity. Several

38 proteins associated with fatty acid, lipid and amino acid metabolism were also

39 overexpressed; including acyl-CoA dehydrogenase and acyl-CoA thioesterase II,

40 which catalyse part of the fatty acid beta-oxidation pathway. Indeed, multiple enzymes

41 involved in the fatty acid oxidation pathway were upregulated. Given the presumed

42 structural similarity between alkyl-carboxylic acid side chains and fatty acids, we

43 postulate that *P. fluorescens* Pf-5 was using existing fatty acid catabolic pathways

44 (among others) during NA degradation.

45

46 **Keywords**: *Pseudomonas fluorescens*; toxicity; naphthenic acids; tailing ponds; oil

47 sands process-affected water (OSPW); proteomics.

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Introduction

In the Athabasca region of Northern Alberta, Canada, surface mining and caustic hot water extraction of bitumen has resulted in the accumulation of vast quantities of wastewaters known as oil-sands process-affected water (OSPW) (Quagraine et al., 2005; Siddique et al., 2011). There are still no approved strategies to treat and safely release OSPW, thus it is contained in large tailings ponds which cause considerable environmental concern (Giesy et al., 2010). One of the major challenges for reclaiming these ponds is the presence of naphthenic acids (NAs) which are the main toxic components of OSPW and demonstrate both acute and chronic toxicity to a variety of aquatic organisms (Headley and McMartin, 2004; Frank et al., 2009; Morandi et al., 2015; Beddow et al., 2016). NAs are also highly persistent under field conditions and a major long-term strategy for remediation is to age the water in end-pit lakes, but this strategy is highly uncertain and may take decades (Gosselin et al., 2010). For effective OSPW remediation and site reclamation, effective modes of NA degradation or removal will be crucial.

Despite their recalcitrance and toxicity, very little is known about the mechanisms of NA biodegradation and the enzymes involved (Whitby, 2010). A few studies using model NAs have shown that several microorganisms (e.g. *Pseudomonas putida*) can metabolise single-ringed NAs by the beta-oxidation pathway (Smith et al., 2008; Johnson et al., 2013; Clothier and Gieg, 2016). In addition, a *Mycobacterium* sp. was found to degrade aromatic NAs by both the beta- and omega-oxidation pathways (Johnson et al., 2012). Although such studies have made some headway, the proteins involved in NA biodegradation (e.g. transport and metabolic pathways) are still unknown. Their identification has in part been hampered

75   by the chemical complexity of environmental NAs and the identification of the

76   individual components.

77   It is known that NAs are a complex class of aliphatic, cycloaliphatic and

78   aromatic mono-carboxylic acids ($C_nH_{2n+z}O_2$), where n is the number of carbon atoms

79   and Z is either zero or a negative even integer representing hydrogen deficiency due

80   to double-bonds or rings (Brient et al., 1995; Clemente and Fedorak, 2005; Whitby,

81   2010). However, only a few NA structures have been identified in OSPW, including tri-

82   , tetra-, or pentacyclic monocarboxylic acids and monoaromatic species (Rowland et

83   al., 2011a, b, c; 2012; Wang et al., 2013; West et al., 2013). Although aromatic

84   alkanoic acids make up a comparatively small proportion of NA mixtures (<10% in

85   crude oils; Hsu et al., 2000), they are significant contributors to the overall toxicity and

86   recalcitrance of NAs in OSPW (Headley and McMartin, 2004); including notably acting

87   as environmental androgen receptor antagonists (Thomas et al., 2009).

88   Previous studies have shown that microbial biodegradation decreases OSPW

89   toxicity over time (Quagraine et al., 2005; Frank et al., 2009; Johnson et al., 2011) and

90   the use of microorganisms is a potential remediation strategy for oil sands operators.

91   However, NA biodegradation is affected by chemical structure and the more

92   recalcitrant NAs contain multiple branched alkyl chains and methyl substitution of the

93   cycloalkane rings (Smith et al., 2008; Johnson et al., 2012) as well as the highly

94   branched and multi-ringed diamondoid NAs found in tailings ponds (Demeter et al.,

95   2015; Ahad et al., 2018; Paulssen and Gieg, 2019; Folwell et al., 2020). Despite their

96   recalcitrance, several NA-degrading communities and species have been identified

97   including *Pseudomonas putida* and *Pseudomonas fluorescens* (Del Rio et al., 2006;

98   Johnson et al., 2013). Using *P. fluorescens* Pf-5 as a model organism, this study aimed

99   to identify proteins differentially expressed during growth on a model NA and

100 commercial NA mixture. Identification of such overexpressed proteins could then be

101 targeted as a novel approach for improving OSPW reclamation in the future.

102

103 **Experimental Procedures**

104 *Naphthenic acids used in this study*

105   The (4′-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) used in this study (**Fig 1A**)

106 was synthesized using a modified Haworth synthesis (Smith et al., 2008). The

107 commercial mixture of NAs was obtained from Acros Organics, UK (EINECS 215-662-

108 8).

109

110 *Pseudomonas fluorescens Pf-5 culture*

111   *P. fluorescens* Pf-5 was obtained from Dr Andrew Spiers (University of Abertay,

112 Aberdeen, UK) and maintained on Luria Bertani (LB) agar (Sambrook et al., 1989).

113 Cultures of *P. fluorescens* Pf-5 were grown overnight at 30°C and 110 rpm in LB broth

114 to an optical density OD_{600} between 0.6 and 0.7. The inoculum was centrifuged at

115 3435 × *g* for 15 min (Hereaus Multifuge 3-SR) and cell pellets washed three times with

116 sterile minimal salts medium (MSM) to remove any trace carbon carried over from the

117 LB medium (Johnson et al., 2011). Cells were inoculated (1% (v/v)) into triplicate 100

118 mL MSM in 120 mL sterile serum bottles containing either *n*-BPBA (final concentration

119 of 10 mg L^{-1}), commercial NA mixture (Acros Organics, UK) (final concentration 100

120 mg L^{-1}) or 1% (w/v) sodium pyruvate. All serum bottles were capped with a PTFE-lined

121 crimp sealed septum. Killed controls were also prepared by addition of $HgCl_2$ (2% (w/v)

122 final concentration) to the inoculum prior to inoculation. Viability was checked by

123 overnight growth on LB agar at 30°C. Abiotic controls were also prepared as well as

124 procedural blanks containing *P. fluorescens* Pf-5 (1% v/v) but no NAs. All bottles were

125 incubated at 110 rpm in the dark at 30°C and OD_{600} was measured

126 spectrophotometrically to monitor growth. Destructive sampling of triplicate bottles

127 was carried out whereby cultures were centrifuged at 9,466 x g for 10 min (Hereaus

128 Multifuge 3-SR). This was performed during exponential growth phase at days 0 and

129 11 (for cultures grown on n-BPBA and the commercial NA mixture) and 0 and 14 h (for

130 cultures grown on pyruvate) based on comparable OD_{600} measurements (between

131 0.026 and 0.056). Proteins from cell pellets were extracted immediately and

132 supernatants were frozen at -20°C prior to ethyl acetate extraction of NAs.

133

134 *NA extraction and analysis*

135 　　　　NAs were extracted from the supernatants using ethyl acetate as previously

136 described (Smith et al., 2008; Johnson et al., 2011). n-BPBA extracts were analysed

137 by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890 GC as

138 previously described (Johnson et al., 2011). For experiments with the commercial

139 NAs, extracts were reduced to dryness under a gentle stream of nitrogen, spiked with

140 400 ng of ^{13}C-myristic acid (as an internal standard), and reconstituted in 1 mL of 10

141 mM ammonium acetate in 75:25 H_2O/acetonitrile (HPLC grade, Fisher Scientific).

142 Samples were analyzed on a Shimadzu LC 20XR LC system using a Waters BET

143 Phenyl (15 cm × 1.0 mm × 1.6μm d.p., Waters). 10 mM ammonium acetate (MS grade,

144 Sigma-Aldrich) in Milli-Q water (Solvent A) and 10 mM ammonium acetate dissolved

145 in 60:40 methanol/acetonitrile (Solvent B) were used as eluents. The mobile phase

146 composition was held at 5% B for 2 min, followed by a linear gradient to 99% B in 16

147 min, followed by an isocratic hold at 99% B for 2 min. The solvent composition was

148 returned to 5% B and remained at this composition for 10 min prior to the next injection.

149 The flow rate was 100 μL min^{-1}. An API 5600 (AB Sciex, Framingham, MA) time of

150 flight high-resolution mass spectrometer with an electrospray source operating in

151 negative ionization mode was used for detection. Acquisition was performed in scan

152 mode from m/z 100 to m/z 650. Data were acquired using Analyst TF and

153 chromatographic peaks were integrated with Multiquant 2.0. software (AB Sciex,

154 Framingham, MA) as previously described (Ross et al., 2012). All peak areas were

155 normalized to the peak area of the internal standard. The Z=0 family was removed

156 from the data set, as these species have been identified as saturated fatty acids (Ross

157 et al., 2012). The species C10 Z=-8 was also removed from the LC data set as this

158 corresponded to the 4-phenylbutanoic acid added as an internal standard.

159

160 *Protein extraction and quantification*

161   Cell pellets were resuspended in four volumes of sodium dodecyl sulphate

162 (SDS) sample buffer [comprising 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2%

163 (w/v) SDS, 12 mM dithiothreitol and one Pierce Protease Inhibitor Tablet per 50 mL]

164 and boiled for 10 min. The cell debris was removed by centrifugation at 11357 × g for

165 5 min and the supernatant was transferred to a sterile microcentrifuge tube and stored

166 at -80°C.

167

168 *In-gel digestion with trypsin*

169   A procedure that is optimized for digestion of whole cell lysates was used as

170 previously described (Metodiev, 2011). The protein samples containing 20 mg total

171 protein in SDS PAGE buffer were loaded onto standard Laemmli-type polyacrylamide

172 gels and allowed to stack and enter the resolving gel but not to separate. The protein

173 bands were excised and digested with trypsin as described in Alldridge et al. (2008).

174 The peptides were extracted and dried in a vacuum concentrator and reconstituted in

175   20 µL of LC/MS-grade water containing 0.1% (v/v) formic acid. Peptide concentration

176   was measured by spectrophotometry using a NanoDrop spectrophotometer and 1 mL

177   aliquot of the reconstituted peptide sample.

178

179   *LC-MS/MS analysis of peptides*

180   Peptide analysis was performed as described by Greenwood et al. (2012).

181   Briefly, 2 mg total peptides per sample were injected automatically from the microplate,

182   desalted online, separated on a 15 cm long pulled-tip nanocolumn, and analyzed by

183   electrospray-ionization tandem mass spectrometry on a hybrid high-resolution

184   LTQ/Orbitrap Velos instrument (Thermo Scientific). The raw data files were converted

185   to mzXML format using the ReAdW program and uploaded onto the LabKey server for

186   analysis. The open-source search engine X! Tandem was used to identify the proteins

187   and acquire spectral count data (Craig and Beavis, 2004). The primary statistical

188   evaluation and filtering of the protein identification and spectral count data was

189   performed as described in Alldridge et al. (2008) using the peptide and protein prophet

190   programs (Nesvizhskii et al., 2003) integrated into the LabKey CPAS (Computational

191   Proteomics Analysis System version 2.2). Peptides and proteins were filtered at 0.3%

192   false discovery rate (FDR) to obtain the final dataset. Proteins were quantified by

193   counting the number of MS/MS spectra matched to corresponding proteins.

194   Sequences from Uniprot for *P. fluorescens* (strain ATCC BAA-477 / NRRL B-23932 /

195   Pf-5) were used to perform protein identification. Spectral counts were normalized to

196   the run yielding the highest number of spectral counts (17660) by the TSpC Total

197   Spectral Counts method (Dong et al., 2007) to account for small observed differences

198   between runs (spectral counts ranged from 14580 to 17660 per run).

199

Bioinformatics Analysis

Differential expression analysis was performed on 1261 proteins by analysis of variance (ANOVA) and Tukey's HSD test with Benjamini–Hochberg post hoc corrections (Benjamini and Hochberg, 1995) within the XLSTAT-Premium Version 2016.1 (Addinsoft) 'OMICs' package. Proteins with mean spectral counts less than 3 in any one treatment were excluded from this statistical analysis. Further analysis of individual proteins that demonstrated a significant increase in abundance compared with controls was carried out using NCBI's blastp programme (Atschul et al., 1990; McGinnis and Madden, 2004) and Conserved Domains Database (CDD) (Marchler-Bauer et al., 2015) . Pathway analysis was carried out by examination of Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg) pathways for *P. fluorescens* Pf-5. Protein enzyme commission numbers were obtained from the identification described above and relevant pathways examined for the presence of upregulated proteins.

Results and Discussion

Growth of *P. fluorescens* Pf-5 and NA degradation

P. fluorescens Pf-5 grew on all NAs tested with no growth observed in the abiotic or killed controls. After a short lag phase, growth was observed after 6 h on pyruvate and after 4 days on *n*-BPBA and the commercial NA mixture (Acros) (**Supplementary Fig S1**), which is similar to previous NA biodegradation studies (Johnson et al., 2011; 2013). Degradation of *n*-BPBA occurred by day 11 as shown by a reduced signal in *n*-BPBA (retention time 16.59 mins) compared with the abiotic control (**Fig 2**). During *n*-BPBA degradation, a metabolite (4′-*n*-butylphenyl)ethanoic acid (*n*-BPEA) was produced (**Fig 1B**) which was absent in the abiotic control. This *n*-

BPEA metabolite has been found previously with mixed enrichment cultures (Johnson et al., 2011); and pure cultures of a *Mycobacterium* sp. (Johnson et al., 2012); and *Pseudomonas putida* KT2440 (Johnson et al., 2013). Additional unidentified peaks (with m/z between 311-341) were also observed that had longer retention times, suggesting they are less polar or less water soluble compounds. These findings confirmed that *P. fluorescens* Pf-5 degraded *n*-BPBA and that the initial degradation steps involved the removal of two carbons from the carboxyl side chain indicative of beta-oxidation as previously shown (Johnson et al., 2011; 2012; 2013). In total <5% of the *n*-BPBA was remaining after 11 days of incubation. Previous NA biodegradation studies have also shown that *P. fluorescens* and *P. putida* can degrade model NAs such as cyclohexane carboxylic acids (Blakeley and Papish, 1982; Del Rio et al., 2006; Johnson et al., 2013). Pseudomonads also increased in abundance during the degradation of the highly branched aromatic NA (*sec*-BPBA) by mixed enrichment cultures (Johnson et al., 2011).

With the Acros commercial NA mixture, we found compositional changes in C/Z number compared with killed controls (**Fig 3, Supplementary Fig S2**). Overall, there was a 17% reduction in the intensity of 10-18 carbon compounds of the *Z* family -2 to -14, which are the major NA species in this commercial mixture. These findings showed that *P. fluorescens* Pf-5 degraded specific carbon compounds in the commercial mixture of NAs. However, we also observed an increased production of compounds with larger C/Z numbers, which may be an artefact of the metabolism of endogenous substrates released from dead bacterial cells. Quesnel et al. (2011) also showed a loss of 11–17 carbon compounds of the *Z* family -2 with the unicellular alga *Dunaliella tertiolecta* with tailings associated NAs. Although *P. fluorescens* Pf-5 was

249   able to degrade *n*-BPBA and the Acros commercial NA mixture, it should be noted that

250   these NAs are not the same as those found in OSPW.

251

252   *Overview of LC-MS/MS shotgun proteomic analysis*

253   The proteomes of *P. fluorescens* Pf-5 were analysed and a total of 139,921

254   spectral counts were obtained that were assigned to 2,239 proteins. Following

255   normalisation, all very low abundance proteins (i.e. < mean of three normalised

256   spectral counts in any treatment) were removed prior to statistical analysis, leaving

257   1,261 proteins which represented 88% of the total detected normalised spectral

258   counts. Over 72% (1,042) of these proteins were detected during growth on all three

259   growth substrates, with an additional 43 proteins that were only detected during growth

260   on both *n*-BPBA and Acros commercial NAs. A further one or eight proteins were only

261   detected during growth on *n*-BPBA or Acros commercial NAs respectively (**Fig 4A**).

262   However, ordination analysis revealed that the proteomes were highly different

263   between all three treatments (**Fig 4B**), also due to large changes in the relative

264   abundance of many of the proteins detected during growth under all conditions.

265   Proteomes from replicates were however highly similar within each treatment (**Fig 4B**).

266   A total of 696 proteins were significantly differentially expressed between cells

267   grown on *n*-BPBA or Acros commercial NAs compared with controls. Specifically, 554

268   proteins were significantly differentially expressed between cells grown on *n*-BPBA

269   versus pyruvate (**Fig 4C**), of which 274 were significantly higher in relative abundance

270   when grown on *n*-BPBA. Similarly, 631 proteins were significantly differentially

271   expressed with cells grown on commercial NAs (**Fig 4D**) of which, 314 were

272   significantly higher in relative abundance in the cells grown on Acros commercial NAs

273   (**Supplementary Table 1**). Of the significantly differentially expressed proteins

274 identified when *P. fluorescens* Pf-5 was exposed to NAs, several were related to

275 transport and metabolism (e.g. lipid and amino acid metabolism) as well as energy

276 production and conversion (based on COG ontology) (**Supplementary Table 1, Fig**

277 **5**).

278

279 *Transporter proteins and outer membrane porins*

280 Several putative membrane transporter proteins significantly increased in

281 relative abundance with both NA treatments, including a long chain fatty acid transport

282 protein (UNIPROT ID Q4K8C7, p=0.000); an arginine-ornithine antiporter (Q4K7R5

283 p=0.001); a transporter substrate-binding protein (Q4K8Z9 p=0.000); a sodium/

284 proline symporter PutP (Q4KJE3, p=0.015) and a major facilitator family transporter

285 (Q4K562, p=0.048). Of particular note was a significant upregulation in putative

286 glucose ATP (energy-dependent efflux pumps)-binding cassette (ABC) transporters

287 (e.g. Q4K7T5, Q4K7T2, Q4KI80, Q4K401, all p<0.03; Q4KJN6, p=0.002). For

288 example, Q4K401 increased >15-fold, whilst a putative polyamine ABC transporter

289 substrate-binding protein (Q4KDC0, p=0.00) increased 8-fold following exposure to

290 both NAs compared with controls. Other notable differentially expressed ABC

291 transporter substrate-binding proteins included Q4KFZ7 (p=0.002); Q4KHV4, Q4K828

292 (both p=0.000) that increased between 3- and 5-fold and a putative ATP-binding

293 cassette domain containing protein Q4K832, (p=0.013) which increased >2-fold with

294 both NA treatments.

295 ABC transporters are used for the uptake of many substances and it may be

296 the observed upregulation was due to NA transport across the cell membrane. In

297 addition, some ABC transporter proteins are important for the detoxification of

298 xenobiotics, as they actively transport chemicals and their metabolites out of cells,

299 protecting the cell from any potential toxic effects (Hessel et al., 2013; Klaassen and

300 Lauren, 2010). ABC transporters have also been shown to be actively involved in the

301 excretion of polycyclic aromatic hydrocarbons from a range of organisms (Alharbi et

302 al., 2016; Bard, 2000). However, a variety of chemicals and metabolites can inhibit

303 members of the ABC superfamily of transporter proteins (Kurth et al., 2015; Smital and

304 Kurelec, 1997) including OSPW (and the potential NAs therein) (Alharbi et al., 2016).

305 We postulate that ABC transporter inhibition occurs as a stress response following NA

306 exposure, thus protecting the cell from NA toxic effects.

307   Several putative outer membrane proteins also had significantly higher spectral

308 counts with both NA treatments compared with controls. For example, an OmpA family

309 protein (Q4KFI8, p=0.000), had >540 and >700 mean spectral counts with n-BPBA

310 and Acros commercial NAs respectively, compared to 330 spectral counts in the

311 controls. OmpA is a proposed porin in the outer membrane of Gram-negative bacteria

312 such as *Pseudomonas* spp. that allows slow membrane penetration by small

313 compounds, has been implicated in cellular responses to environmental stress (Van

314 der Heijden et al., 2016) and plays a role in bacterial pathogenesis (Confer and

315 Ayalew, 2013). Another outer membrane protein OprG (Q4K583, p=0.000), also had

316 significantly higher spectral counts with both NA treatments compared with controls.

317 OprG is part of the OmpW family with proposed roles in Fe transport (McPhee et al.,

318 2009). Regulation of Fe transport is one strategy bacteria utilise in the repair of redox

319 stress-induced damage (Andrews et al., 2003) and we postulate that cells were

320 overexpressing both OmpA and OprG as a stress response to NA toxicity. Given that

321 NAs are highly toxic to a range of organisms including bacteria (Frank et al., 2009;

322 Whitby, 2010; Morandi et al., 2015), it was not surprising that there was a significantly

323 higher relative abundance of transporter proteins following NA exposure

324 (**Supplementary Table 1**).

325

326 *Lipid/ fatty acid metabolism*

327 Several proteins associated with lipid metabolism had significantly higher

328 relative abundance of spectral counts following NA exposure (**Fig 5A**). Given that alkyl

329 side chains NAs have been found previously in Acros commercial NAs (Hao et al.,

330 2005) and the structural similarity between alkyl-carboxylic acid side chains and fatty

331 acids, it is not surprising that proteins involved in lipid metabolism were over

332 expressed. We postulate that *P. fluorescens* Pf-5 was utilising existing fatty acid

333 metabolism metabolic proteins (among others) during NA degradation. For example,

334 an acyl-CoA dehydrogenase MmgC (Q4KC62, p=0.011) increased 8-fold (with *n*-

335 BPBA) and 7-fold (with Acros commercial NAs) compared with controls. This protein,

336 along with an acyl-CoA thioesterase II (ACOT2) (Q4K8Q2, p=0.004) which itself

337 showed >3-fold higher expression with Acros commercial NAs, acts in the alpha- and

338 beta-oxidation of various lipids (Hunt et al., 2012) and so their overexpression is likely

339 to be a response to the upregulation of the pathways degrading acetyl/acylcoAs. In

340 mammalian systems, when ACOT2 is upregulated, beta-oxidation capacity increases

341 (Fujita et al., 2011; Momose et al., 2011). It is not surprising therefore that proteins

342 associated with alpha- and beta-oxidation of lipids were overexpressed given that the

343 aerobic transformation of NAs occurs via alpha-/ beta-oxidation (Blakeley and Papish,

344 1982; Rontani and Bonin, 1992; Johnson et al., 2011).

345 In addition to ACOT, an enoyl-CoA hydratase (Q4KC63, p=0.001) was also

346 detected in significantly more abundance, increasing by 3-fold for both NA treatments

347 compared to controls (**Fig 5A**). Enoyl-CoA hydratase is essential for metabolizing fatty

acids in the beta-oxidation pathway to produce acetyl CoA and ATP. A putative enoyl-CoA hydratase was recently shown to contribute to biofilm formation in an antibiotic tolerant denitrifying bacterium and pathogen *Achromobacter xylosoxidans* (Cameron et al., 2019). When the gene (*echA*) encoding a putative enoyl-CoA hydratase was disrupted, a decrease in biofilm accumulation occurred, increasing the organism's susceptibility to antibiotics (Cameron et al., 2019). In our study, a poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase protein (Q4KKC4, $p=0.000$) which is involved in biofilm formation (Wang et al., 2004), was also significantly more abundant when cells were exposed to both NA treatments, remaining undetected in the control. Although biofilm formation was not measured herein, it is well known that biofilms facilitate substrate degradation (Nicolella et al., 2000; Singh et al. 2006; Chakraborty et al., 2012) including NAs (Golby et al., 2012; Choi et al., 2014; Demeter et al., 2015; Folwell et al., 2016) and overexpression of these proteins involved in biofilm formation may facilitate NA removal in OSPW.

Several putative dehydrogenases had significantly higher relative abundance with both NA treatments (**Fig 5A**) including: an acyl-CoA dehydrogenase (Q4K5J0, $p=0.005$); 3-hydroxyisobutyrate dehydrogenase (Q4KIP8, $p=0.016$); a dihydrolipoyl dehydrogenase (Q4KDP5, $p=0.039$) a Glu/Leu/Phe/Val dehydrogenase (Q4KI29, $p=0.003$); and an isovaleryl-CoA dehydrogenase (Q4K9P7, $p=0.026$) which participates in valine, leucine, and isoleucine degradation. In addition, several putative oxidoreductases, carboxylases and transferases increased significantly in relative abundance with both NA treatments including: FAD-dependent oxidoreductase (Q4KED0, $p=0.026$); NADH-quinone oxidoreductase (Q4K9S5, $p=0.012$); NAD(P)-dependent oxidoreductases (Q4KBD2, Q4KC60, $p<0.014$); FAD-binding oxidoreductase (Q4KEZ1, $p=0.003$); flavin oxidoreductase/NADH oxidase (Q4KHN1,

373   $p=0.001$); NADH quinone oxidoreductases (Q4K9T0, Q4K9S5, $p<0.012$); the

374   acetyl/propionyl/methylcrotonyl-CoA carboxylases (Q4K9P4, Q4K9P6, $p<0.024$);

375   acetyl-CoA C-acyltransferase (Q4KC61, $p=0.003$), and acetyl-CoA C-acetyl

376   transferases (Q4KIT5, Q4KEA5, $p<0.003$). Additionally, a flavoprotein (Q4KFP5,

377   $p=0.001$) and long-chain fatty acid--CoA ligase (Q4K7V1, $p=0.029$) increased

378   significantly in relative abundance with both NA treatments compared to controls.

379   It was notable that five proteins were significantly differentially expressed with

380   the Acros commercial NAs but not detected with either n-BPBA or the controls. These

381   were three putative dehydrogenases (acyl-CoA dehydrogenases (Q4K8Z4, Q4KFF6,

382   $p<0.005$); 3-hydroxyacyl-CoA dehydrogenase (Q4KFM7, $p=0.000$); and two

383   carboxylases, namely acetyl/ propionyl/ methylcrotonyl-CoA carboxylase alpha

384   subunit (Q4K8Z2, $p=0.000$) and acyl-CoA carboxylase beta subunit (Q4K8Z5,

385   $p=0.000$) (**Supplementary Table 1**). Acetyl-CoA carboxylase catalyses the

386   carboxylation of acetyl-CoA to produce malonyl-CoA, which is a substrate for fatty acid

387   biosynthesis (Tong, 2005). It is possible that this suite of proteins were involved in

388   degrading certain carboxylic acids within the Acros commercial mixture, possibly the

389   10-18 carbon compounds of the Z family -2 to -14 that we observed a reduction in (**Fig**

390   **3**), although this is yet to be confirmed.

391   Importantly, we found multiple proteins involved in sequential reactions in fatty

392   acid degradation were upregulated with both NA treatments (**Supplementary Fig S3**),

393   and an example of such a pathway is given (**Fig 6**). We postulate that *P. fluorescens*

394   Pf-5 cells were utilising their existing fatty acid metabolism machinery to metabolise

395   the NAs tested. Since these fatty acid pathways are conserved, a wide variety of

396   species are likely to have the enzymatic potential to biodegrade NAs and a range of

397   NA-degrading microorganisms have been identified (reviewed in Skeels and Whitby,

398   2019). It is possible however, that under certain conditions Pseudomonads may have

399   a competitive advantage, not only by withstanding NA toxicity, but as likely NA-

400   degrading genera. This is supported by Johnson et al. (2011) who showed that

401   Pseudomonads increased in abundance during the degradation of certain NAs in

402   mixed communities. Thus, targeting the Pseudomonads in OSPW may be a

403   bioremediation strategy for oil sands operators for enhanced NA removal.

404

405   *Amino Acid metabolism*

406   A total of 24 proteins significantly upregulated by >2-fold were related to amino

407   acid metabolism (**Fig 5B**) and notable upregulated proteins are detailed in the amino

408   acid metabolism KEGG pathway (**Supplementary Fig S4**). Specifically, an ornithine

409   carbamoyltransferase (OTCase, Q4K7R3, p=0.000) was upregulated 3-fold (with both

410   *n*-BPBA and Acros commercial NAs) compared with controls. There are two classes

411   of OTCase, anabolic and catabolic that function in arginine metabolism (Cunin et al.,

412   1986). All anabolic OTCases, except those found in *Pseudomonas* spp. catalyze both

413   directions of the reaction (Cunin et al., 1986). In some prokaryotes including

414   Pseudomonads, OTCase is able to degrade arginine by the reverse mechanism in the

415   arginine dihydrolase pathway (Cunin et al., 1986). In *P. putida*, OTCase is inhibited by

416   relatively high concentrations of arginine (Stalon et al., 1977).

417   In addition to OTCase, another protein which had significantly higher

418   abundance with both NA treatments was a putative glutaminase-asparaginase AnsB

419   (Q4KEX6, p=0.001), which is widely distributed in microorganisms and had a wide

420   substrate-specificity (Hüser et al., 1999; Wriston and Yellin 1973). Another protein

421   which increased in relative abundance (between 11 to 12-fold) with both NA

422   treatments was a putative arginine N-succinyl transferase (Q4K836, p=0.039) which

423 uses succinyl-CoA and L-arginine as substrates to produce CoA and N_2-succinyl-L-

424 arginine. Other proteins that significantly increased >2-fold following exposure to both

425 NA treatments were: NAD glutamate dehydrogenase (Q4KD35, $p=0.000$), which is

426 found in most microbes and converts glutamate to α-ketoglutarate (Wootton, 1983);

427 D-amino acid dehydrogenase (Q4K3T7, p=0.007), 3-deoxy-7-phosphoheptulonate

428 synthase class II (Q4K8T7, $p=0.028$), glycine dehydrogenase aminomethyl-

429 transferring protein (Q4K416, $p=0.018$); urocanate hydratase (Q4KJN8, $p=0.010$)

430 involved in histidine degradation; a Fe(II)-containing non-heme oxygenase (4-

431 hydroxyphenylpyruvate dioxygenase, Q4KB91, $p=0.001$) involved in tyrosine

432 catabolism, a dipeptidase (Q4KAU0, $p=0.001$) and an amino peptidase (Q4K8F8,

433 $p=0.001$) (**Fig 5B**).

434

435 *Energy production and conversion, secondary metabolism*

436 Several proteins putatively involved in secondary metabolism also had a higher

437 relative abundance with both NA treatments (**Fig 5C**) including: a

438 glycerophosphodiester phosphodiesterase (Q4KFA5, $p=0.026$); alcohol

439 dehydrogenases (e.g. Q4K4Z2, Q4K9B8, both $p<0.019$) which catalyzes the

440 conversion of the primary alcohol to an aldehyde, and aldehyde dehydrogenases

441 which oxidise aldehydes to carboxylic acids (e.g. Q4KAB3, $p=0.025$, Q4K837,

442 $p=0.011$) (**Supplementary Table S1, Fig 5C**). It is notable that aldehyde

443 dehydrogenases also catalyze xenobiotic metabolism (Sladek 2002) and could

444 potentially be involved in NA metabolism herein, although this remains inconclusive.

445 One protein identified as a putative alpha-ketoacid dehydrogenase subunit

446 (Q4KDP3, $p=0.000$), which catalyzes the oxidative decarboxylation of branched, short-

447 chain alpha-ketoacids significantly increased 25-fold (with *n*-BPBA) and 21-fold (with

448   Acros commercial NAs). Other notable proteins were: proline dehydrogenase

449   (Q4KJE4, *p*=0.000); aconitate hydratase (Q4KFD5, *p*=0.001); and a succinate

450   dehydrogenase (Q4KFZ3, *p*=0.001) which converts succinate to fumarate as part of

451   the Krebs cycle. Several Cytochrome b, c, or d family proteins including multiple

452   subunits of cytochrome c oxidases including: Q4KFE2, Q4K6H5, Q4KFE7, Q4KKM0,

453   Q4KFE0, Q4KKJ7, Q4K6H4 and Q4K5Q1, (all *p*<0.004) and Q4K6Q0 (p=0.01), along

454   with an iron-sulfur cluster-binding protein (Q4KII3, *p*=0.002), which are all involved in

455   the electron transport chain also had significantly higher abundance with both NA

456   treatments compared with controls.

457   Several proteins relating to inorganic ion metabolism were also significantly

458   differentially expressed with both NA treatments. For example, a copper-containing

459   nitrite reductase (CuNiR) which reversibly reduces nitrite to NO (Q4K5B4, *p*=0.000)

460   increased >8 to 9-fold. Increased expression of this denitrifying enzyme could arise

461   from the ammonium in the MSM media or from the liberation of nitrite from nitrate

462   esters. In support of this, a putative pentaerythritol trinitrate reductase (Q4KHN1,

463   *p*=0.001) which reductively liberates nitrite from nitrate esters and degrades

464   xenobiotics such as 2,4,6-trinitrotoluene (TNT) (French and Bruce, 1996; French et

465   al., 1998) was significantly increased with both NA treatments. Ammonium oxidation

466   pathways have been well characterised in Pseudomonads and typically involve

467   ammonia monooxygenase producing hydroxylamine which is subsequently oxidised

468   to nitrite (Hollocher et al., 1981). As a result, CuNiR could then be upregulated to deal

469   with excess nitrite. An azurin-related protein (Q4KJ49, *p*=0.000) also had high spectral

470   counts with both NA treatments. Azurin is a periplasmic Cu-containing cupredoxin

471   protein capable of electron transfer reactions including being an electron donor to

472   CuNiR in the denitrification pathway (Zumft, 1997). It has also been implicated as a

part of cellular response to redox stress. Notably, azurin knockout strains of *P. aeruginosa* showed increased sensitivity to hydrogen peroxide or paraquat redox stress but with no impairment to growth on nitrite or nitrite (Vijgenboom et al., 1997).

Molybdenum is an essential micronutrient for microorganisms and molybdoenzymes are widespread among prokaryotes where they catalyze steps in carbon, sulfur and nitrogen metabolism (Schwarz et al., 2009, Mendel and Schwarz, 2011; Mendel, 2013), are involved in chemotaxis towards electron acceptors, environmental stress responses (Schwartz and Mendel, 2006; Baraquet et al., 2009; Leimkuhler and Lobbi-Nivol, 2016), and play a role in pollutant detoxification (e.g. chromium Cr(VI) and arsenic (III)) (Islam et al., 2004; Chovanec et al., 2012; Slyemi and Bonnefoy, 2012; Kruger et al., 2013). When competing anions are present, molybdoenzymes require specific uptake systems including high affinity ABC transporters (Hagen, 2011) and this supports the upregulation of ABC transporters found herein. In our study, a molybdenum (Moco) cofactor biosynthesis protein (Q4KAB0, *p*=0.035) was significantly differentially expressed >11-fold (with Acros commercial NAs) and 3.5-fold (with *n*-BPBA). Yet interestingly, membrane nitrate reductase (Nar) (among other proteins known to contain a Moco cofactor in *P. fluorescens* Pf5) was not found to be upregulated in our study. Molybdenum metabolism is tightly connected to Fe-S cluster synthesis (Mendel, 2013) and in our study, bacterioferritin (*Bfr2*), an iron uptake protein (Q4K560, *p*=0.000) with likely roles in iron storage, mobilization and homeostasis (Rivera, 2017), was also significantly expressed (between 5 and 7.5-fold with both NA treatments). In *P. aeruginosa*, *Bfr2* bacterioferritin provides resistance to hydrogen peroxidase (Ma et al., 1999) and we postulate that *Bfr2* (along with molybdoenzyme) upregulation was an oxidative stress

497 response to NA toxicity and both were involved in NA detoxification, although this

498 remains to be determined.

499

500 *Other cellular functions: cell wall biogenesis and chemotaxis*

501   Proteins associated with other cellular functions were also significantly more

502 abundant (by >2-fold) in both NA treatments compared with controls included a signal

503 transductor histidine kinase (Q4K8D2, *p*=0.000); a polysaccharide export protein

504 (Q4K8Y1, *p*=0.012) which is involved in cell wall biosynthesis (**Supplementary Table**

505 **1**) and several methyl-accepting chemotaxis proteins (e.g. Q4KE19, Q4KBC3,

506 <*p*=0.003) which are involved in cell motility were also in higher relative abundance in

507 both the NA treatments and are likely a chemotactic response to NA toxicity.

508

509 **Conclusions**

510   Little was previously known about the mechanisms involved in NA

511 biodegradation and the enzymes or other proteins involved in the metabolism of this

512 class of OSPW contaminants. Here we sought to elucidate proteins significantly

513 upregulated during NA biodegradation of a model NA and commercial NA mixture with

514 *P. fluorescens* Pf-5 as a model microorganism. Several differentially expressed

515 proteins were identified following NA exposure that were involved in metabolism - lipid,

516 fatty acid and amino acid degradation pathways- suggesting that *P. fluorescens* Pf-5

517 may be utilising its existing lipid, fatty acid metabolism metabolic proteins (among

518 others) during NA biodegradation. Multiple membrane porins, transporters and

519 chemotaxis proteins were also significantly upregulated and likely represent a general

520 cellular response to oxidative stress and the cell's detoxification mechanisms to

521 protect the cell from environmental stress and NA toxicity. However, additional

522 proteins which were also highly upregulated such as CuNiR and MoCo biosynthesis

523 proteins and their significance in NA degradation remains to be determined. In

524 conclusion, we provide new empirical evidence of potential proteins that could be

525 targeted in further overexpression or synthetic biology studies with more authentic

526 OSPW NAs as a novel approach for improving OSPW reclamation in the future.

527

531

532 **Author contributions**

533 CW and MH conceived the idea and wrote the original proposal to obtain the funding

534 for the PhD studentship, co-supervised by MF. RJ and CW conceived and designed

535 the proteomics experiments along with KS. MM performed the proteomics work, whilst

536 JM, MR, LC, LG contributed to the measurements of the degradation experiments. RJ,

537 KS, LC, MR, and MM conducted all the experimental work and analysed the

538 experimental data. MH and BM contributed to the bioinformatics analysis. All authors

539 prepared the manuscript.

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911 **Titles to figures**

912 **Fig 1** Structure of (4′-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) **(A)** and (4′-*n*-

913 butylphenyl)ethanoic acid (*n*-BPEA) **(B).**

914

915 **Fig 2** Degradation of *n*-BPBA and production of (4′-*n*-butylphenyl)ethanoic acid (*n*-

916 BPEA) metabolite by *Pseudomonas fluorescens* Pf-5 (**A**) compared to abiotic

917 controls (**B**) following 11 days of incubation.

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919 **Fig 3** Heat map of the percentage change for Acros commercial NA species, plotted

920 by carbon number and Z, following 11 days of incubation. Percent changes are

921 calculated relative to killed control following 11 days of incubation.

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923 **Fig 4** Overview of the proteomic analysis of *P. fluorescens* Pf-5 growing on naphthenic

924 acids (NAs) compared with pyruvate controls. Venn diagram comparing the common

925 and unique proteins detected and identified during growth on the three substrates. (**A**).

926 PCA analysis highlighting highly similar replicate proteomes that differ significantly

927 with growth substrate (**B**), Volcano plots of normalized LC-MS/MS spectral counts

928 comparing *P. fluorescens* Pf-5 during growth on NAs compared with pyruvate controls

929 (**C** and **D**). Red points: proteins where $p < 0.05$ and above two-fold difference; Green

930 point above horizontal dotted line: proteins where $p < 0.05$ but below two-fold

931 difference; Green points below horizontal dotted line: proteins above two-fold

932 difference but not statistically significant; Blue points: proteins below two-fold

933 differential expression and not statistically significant; Horizontal dashed line: $p=0.05$;

934 Vertical dashed lines: two-fold difference. Thus all red points represent proteins that

935   are above two-fold difference (vertical dashed lines) and statistically significant

936   (horizontal dashed line, $p = 0.05$).

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938   **Fig 5** Mean spectral count n-BPBA, (black bars), Acros commercial NA mixture (grey

939   bars), and control (white bars) for metabolism functional COG categories.

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941   **Fig 6** Partial enzymatic pathway diagram for fatty acid degradation in *Pseudomonas*

942   *fluorescens* Pf5