Biodegradation of alkyl branched aromatic alkanoic naphthenic acids by *Pseudomonas putida* KT2440

Running Title: Characterisation of aromatic NA degradation by *Pseudomonas putida* KT2440

Richard J. Johnson[1], Ben E. Smith†[2], Steven J. Rowland[3] and Corinne Whitby[1*]

[1]Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex, CO4 3SQ, UK

[2]Oil Plus Ltd., Dominion House, Kennet Side, Newbury, RG14 5PX, UK

†Present address: BP Exploration, Exploration & Production Technology, Sunbury Business Park, Chertsey Road, Sunbury-on-Thames, Middlesex, TW16 7LN. UK

[3]Petroleum & Environmental Geochemistry Group, Biogeochemistry Research Centre, School of Geography, Earth & Environmental Sciences, University of Plymouth, Plymouth, PL4 8AA, UK

*Corresponding author

Tel: +44 (0)1206 872062 Fax: +44 (0) 1206 872592

Email: cwhitby@essex.ac.uk

Article Type: Special Issue ISMOS-3

Abstract:

The majority of the world's crude oil reserves consist of highly biodegraded heavy and super heavy crude oils and oil sands that have not yet been fully exploited. These vast resources contain complex mixtures of carboxylic acids known as naphthenic acids (NAs). NAs cause major environmental and economic problems, as they are recalcitrant, corrosive and toxic. Although aromatic acids make up a small proportion of most NA mixtures, they have demonstrable toxicities to some organisms (e.g. some bacteria and algae) and ideally need to be removed or reduced by remediation. The present study analysed the ability of *Pseudomonas putida* KT2440 to degrade highly recalcitrant aromatic acids, as exemplified by the alkyl phenyl alkanoic acid (4′-*t*-butylphenyl)-4-butanoic acid) (*t*-BPBA) and the more degradable (4′-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA). *n*-BPBA was completely metabolized after 14 days, with the production of a persistent metabolite identified as (4′-*n*-butylphenyl)ethanoic acid (BPEA) which resulted from removal of two carbon atoms from the carboxyl side chain (beta-oxidation) as observed previously with a mixed consortium. However, when *n*-BPBA concentration was increased two-fold, degradation decreased by 56% with a concomitant six-fold decrease in cell numbers, suggesting that at greater concentrations, *n*-BPBA may be toxic to *P. putida* KT2440. In contrast, *P. putida* KT2440 was unable to degrade the highly recalcitrant *t*-BPBA even after 49 days. These findings have implications for NA bioremediation in the environment.

Keywords: *beta*-oxidation/ naphthenic acids/ bioremediation.

50 **1. INTRODUCTION**

51 The largest single crude oil accumulations in the world are found as partially
52 biodegraded oil sands deposits in Alberta (Canada, 900 billion barrels) and Eastern
53 Venezuela (Venezuela, 1,200 billion barrels) (Head et al. 2003). The extraction of
54 bitumen from the vast highly degraded oil sands deposits in Northern Alberta,
55 Canada has resulted in the accumulation of ≥ 840 million m^3 of oil sands tailings and
56 oil sands process water (OSPW), which are stored in large tailings ponds (Siddique
57 et al. 2011). Concerns have been raised about the possible environmental impacts of
58 tailings ponds due to the presence of recalcitrant, toxic organic acids, collectively
59 known as naphthenic acids (NAs) (Headley and McMartin 2004; Whitby 2010;
60 Siddique et al. 2011).

61 Naphthenic acids (NAs) are complex mixtures of cycloaliphatic and alkyl-
62 substituted acyclic carboxylic acids present in petroleum. NAs also contain smaller
63 amounts of other compounds such as aromatic, olefinic, hydroxyl and dibasic acids
64 (Headley and McMartin, 2004). Although aromatic NAs make up a small percentage
65 of some NA mixtures (e.g. Rowland et al. 2011a,b,c), they may contribute
66 disproportionately to the overall toxicity and recalcitrance of NAs (Headley and
67 McMartin 2004; Johnson et al. 2011a). One solution for the removal of these
68 recalcitrant, toxic compounds is to apply microorganisms for bioremediation.
69 However, the mechanisms by which this is achieved are poorly understood.

70 A previous study isolated both *Pseudomonas putida* and *Pseudomonas*
71 *fluorescens* from OSPW and demonstrated >95 % degradation of both commercial
72 and environmental NA mixtures by a co-culture of *P. putida* and *P. fluorescens* (Del
73 Rio et al. 2006). Johnson et al. (2011a) also identified an increase in abundance of
74 16S rRNA gene sequences relating to *Pseudomonas* spp. (among others) during the

75 degradation of alkyl branched aromatic NAs (Johnson et al. 2011a). Furthermore,
76 *Pseudomonas putida* KT2440 has been shown to metabolise a wide variety of
77 substrates including phenylacetic acid and other phenylalkanoic acids (Jimenez et al.
78 2002; Gilbert et al. 2003).
79 Although to date, there is no information regarding the ability of *P. putida*
80 KT2440 to metabolise alkyl branched aromatic NAs, the diverse metabolism of *P.*
81 *putida* KT2440 appears to make it an excellent potential candidate for NA
82 degradation. The overall aims of this study were to investigate the catabolic ability of
83 *P. putida* KT2440 to degrade different alkyl branched aromatic NAs and to identify
84 any metabolites produced during degradation. Such information would enable the
85 elucidation of the functional genes and metabolic pathways involved in aromatic NA
86 degradation, thus facilitating the bioremediation of these recalcitrant aromatic NAs in
87 the environment.
88
89 **2. MATERIALS AND METHODS**
90 *2.1 Bacterial Strain*
91 *Pseudomonas putida* KT2440 (DSMZ 6125) was obtained from the DSMZ
92 culture collection (Braunschweig, Germany). Exponential cultures were grown at
93 30°C for 24 h with shaking (120 rpm) on Luria-Bertani (LB) medium (Sambrook et al.,
94 1989) to a cell density of 1 x 10^8 cells mL^{-1}.
95
96 *2.2. Aromatic carboxylic acid synthesis*
97 The two butylphenyl butanoic acid (BPBA) isomers used in this study were: (4′-*n*-
98 butylphenyl)-4-butanoic acid (*n*-BPBA) and (4′-*t*-butylphenyl)-4-butanoic acid) (*t*-

99 BPBA). Both BPBAs were synthesized using a modified Haworth synthesis as
100 described previously (Smith, 2006; Smith et al. 2008).
101
102 *2.3. Degradation of n-BPBA and t-BPBA by P. putida KT2440*
103 Degradation experiments were set up by inoculating *P. putida* KT2440 (2%
104 v/v) into 25 mL minimal salts medium (MSM) containing either *n*- (at final
105 concentrations of either 2, 3 or 4 mg L^{-1}) or *t*-BPBA (final concentration of 4 mg L^{-1})
106 as described previously (Johnson et al. 2011a). Killed controls (to determine whether
107 any abiotic loss had occurred) were prepared by Tyndallization of the inoculum
108 before BPBA addition, and were checked by growth on LB agar and incubating
109 overnight at 30 °C prior to inoculation. Abiotic controls containing MSM with the
110 individual BPBA isomer (dissolved in 0.1 M NaOH and added to a final concentration
111 of 4 mg L^{-1}) were also prepared. Procedural blanks containing either *P. putida*
112 KT2440 inoculated (2% v/v) in MSM only or MSM supplemented with 0.1M NaOH
113 (10 µl) were also performed. Destructive sampling was carried out at days 0, 11, 31
114 and 49. Cell counts were performed on cultures grown on *n*- and *t*-BPBA as well as
115 procedural blanks by dilution plating onto LB agar, with phosphate buffered saline
116 (pH 7.4; Sambrook et al. 1989) as the diluent and incubated at 30 °C for 24 h.
117
118 *2.4. BPBA extraction and gas chromatography mass spectrometry analysis*
119 BPBA extraction was performed as described previously (Johnson et al.,
120 2011a). Briefly, all glassware was soaked overnight in Decon[90] (Decon Laboratories,
121 Pennsylvania, USA), rinsed three times with distilled water, baked until dry at 110
122 °C, rinsed three times with acetone (Fisher Scientific, Massachusetts, USA) and
123 autoclaved. The internal standard, 4-phenylbutyric acid (Acros Organics, Thermo

124 Fisher Scientific, Massachusetts, USA) (10 mg) dissolved in 1 mL methanol (HPLC
125 grade, Fisher Scientific) was added to each supernatant from above (final
126 concentration 2 mg L^{-1}). Each BPBA isomer from the supernatant was extracted by
127 acidifying to pH <2.0 (using conc. HCl) and extracted three times with 15 mL HPLC-
128 grade ethyl acetate (Fisher Scientific) as described by Smith et al. (2008). Solvent
129 extracts were pooled, dried with 5-10 g anhydrous Na_2SO_4 (Fisher Scientific) for > 90
130 min and the organic acids concentrated by rotary evaporation (Buchi/Rotavapor,
131 Flawil, Switzerland) at 40 °C. Samples were transferred to a GC vial (Chromacol,
132 Welwyn Garden City, UK), sparged with nitrogen and reacted with 50 µl *N,O*-
133 bis(trimethylsilyl)trifluoroacetamide (Supelco, Missouri, USA) at 60°C for 20 min to
134 form trimethylsilyl derivatives. Derivatized samples were resuspended in 1 mL
135 dichloromethane (HPLC grade, Acros Organics) and separated by gas
136 chromatography-mass spectrometry (GC-MS) using an Agilent 7890 GC interfaced
137 with an Agilent 5975C MS. Run conditions were a 1 µL splitless injection (injector
138 temperature of 250 ºC) onto a 30 m x 250 µm x 0.25 µm HP-5MS column. Oven
139 temperatures were increased from 40 to 300 ºC at 10 ºC min^{-1} followed by a 10 min
140 hold at 300 ºC. Helium was used as the carrier gas at a constant flow rate of 1 mL
141 min^{-1}. The transfer line and source were held at 230 ºC. The mass
142 spectrophotometer was a quadrupole operated in full-scan mode (scan range 50-550
143 Da).
144
145 *2.5. Statistical Analysis*
146 Statistical analysis was performed using SPSS PASW statistics version 18.0.0.
147

148 **3. RESULTS AND DISCUSSION**

149 Degradation of *n*- and *t*-BPBA by *P. putida* KT2440 was investigated and the
150 results are presented in Figs. 1 & 2. With both *n*- and *t*-BPBA incubations, killed
151 controls (Fig. 1 A, circled) behaved accordingly and showed no abiotic losses. *P.*
152 *putida* KT2440 was able to metabolise *n*-BPBA after 14 days incubation and this is
153 the first report of the partial degradation of butyl substituted aromatic butanoic acids
154 by *P. putida* KT2440. Although *P. putida* KT2440 is known to degrade a variety of
155 other aromatic compounds (Nelson et al. 2002), it was unable to transform *t*-BPBA
156 following 49 days incubation (Fig. 1A). The inability of *P. putida* KT2440 to degrade *t*-
157 BPBA may be due to the toxicity of *t*-BPBA. It has been shown previously that *t*-
158 BPBA is two-fold more toxic to the bacterium, *Vibrio fisheri,* compared to *n*-BPBA
159 (Johnson et al. 2011a). In addition, the recalcitrance of *t*-BPBA by *P. putida* KT2440
160 may also be due to other factors such as the more branched side chain of *t*-BPBA
161 (compared to *n*-BPBA) causing possible steric hindrance, *P. putida* KT2440 may not
162 have the enzymes required to degrade *t*-BPBA, or there could be a missing
163 enzymatic co-factor, such as iron, in the media.

164 During degradation of *n*-BPBA, a major metabolite was produced at day 14,
165 which increased in abundance following a decrease in *n*-BPBA (Fig. 1B). Gas
166 chromatograms of *n*- and *t*-BPBA degradation (Figs 2A & B) showed that the
167 metabolite produced during *n*-BPBA degradation had a retention time of 15.10 min
168 and persisted at day 49 (Fig. 2A). By contrast *P. putida* KT2440 was unable to
169 metabolise *t*-BPBA by day 49 and no metabolites were detected during the course of
170 the experiment (Fig. 2B). Interpretation of the mass spectrum of the TMS ester of the
171 major metabolite produced during *n*-BPBA degradation by *P. putida* KT2440 (Fig. 3)
172 showed that it was identical to that previously identified (Johnson et al. 2011a), as

173 (4'-*n*-butylphenyl)ethanoic acid (*n*-BPEA) by comparison with a synthetic sample.
174 The production of the *n*-BPEA metabolite, which persisted at day 49, suggests that
175 beta-oxidation was the initial route for *n*-BPBA metabolism, as has been previously
176 described by a mixed microbial consortium (Johnson et al. 2011a) and an
177 environmental isolate (Johnson et al., 2011b). Beta-oxidation is a ubiquitous pathway
178 and has been found in many organisms including *P. putida* (De Waard et al. 1993;
179 Eggink et al. 1992; Nelson et al. 2002).

180 Both aromatic and alicyclic compounds containing side chains with odd
181 numbers of carbons are much more easily biodegraded as the ring structure can be
182 cleaved through beta-oxidation (Quagraine et al. 2005; Whitby 2010). However,
183 although beta-oxidation is capable of oxidising alkanoic side chains, it stops at the
184 ethanoic group if the number of carbon atoms in the alkanoic side chain is even
185 (Beam and Perry 1974; Rontani and Bonin 1992). The two BPBAs studied here
186 contain butanoic side chains with an even number of carbon atoms and therefore,
187 they and their BPEA metabolites have the ability to accumulate in the environment
188 (Quagraine et al. 2005).

189 In contrast, other microorganisms such as *Alcaligenes* sp. PHY 12 have been
190 shown to overcome the preference for odd carbon chains during the degradation of
191 cyclohexylacetic acid, by initially alpha-oxidising the substrate to
192 cyclohexylcarboxylic acid before using beta-oxidation to open the cyclohexyl ring
193 (Rontani and Bonin 1992). In the absence of an alpha-oxidation pathway the ring
194 structure has been shown to be cleaved through phenylacetic acid in *Pseudomonas*
195 *putida* U using the phenylacetyl-CoA catabolon (phA) which contains a transport
196 system, a phenylacetic acid activating enzyme, a ring hydroxylation complex, a ring
197 opening enzyme, a beta-oxidation like system and regulatory genes (Olivera et al.

198 1998). A phA catabolon homologous to that found in *P. putida* U has been identified
199 in *P. putida* KT2440, suggesting that the enzymes are present. It is unlikely that *n*-
200 BPEA toxicity resulted in the inability of *P. putida* KT2440 to further transform this
201 metabolite, since Johnson et al. (2011a) has previously showed that *n*-BPEA is over
202 two-fold less toxic than the parent compound *n*-BPBA (by Microtox^{TM}, Azur
203 Environmental, Fairfax, USA). It is therefore possible that other factors such as
204 bioavailability or incubation time may have played a role in the recalcitrance of *n*-
205 BPEA.

206 In addition to BPEA metabolites, previous studies using a microbial
207 consortium (Johnson et al. 2011a) and *Mycobacterium aurum* IS2.3 (Johnson et al.,
208 2011b) demonstrated the presence of an additional metabolite identified as (4'-
209 carboxybutylphenyl)ethanoic acid) during BPBA degradation, which showed
210 carboxylation of the butyl side chain. Furthermore, Johnson et al. (2011b) showed
211 that (4'-carboxy-*t*-butylphenyl)ethanoic acid was produced via two different pathways
212 (i.e. either omega oxidation of *t*-BPEA to oxidise the *tert*-butyl side chain, or initial
213 omega oxidation of the *tert*-butyl side chain of *t*-BPBA to produce (4'-carboxy-*t*-
214 butylphenyl)-4-butanoic acid followed by beta-oxidation of the alkanoic acid side
215 chain. However, in the present study, these metabolites were not found when *P.*
216 *putida* KT2440 was grown in the presence of either *n*- or *t*-BPBA.

217 Several *P. putida* strains are well known for the degradation of aromatic
218 hydrocarbons and their degradation pathways have been mapped (Jimenez et al.,
219 2002). *P. putida KT2440* contains the enzymes involved in the *ortho* aromatic
220 degradation pathway but not the *meta* pathway, as it does not contain the TOL
221 plasmid (Jimenez et al. 2002) which contains the genes for *meta* cleavage pathway
222 including an alkyl monooxygenase (Franklin et al. 1981). As *P. putida* KT2440 is

223 unable to break down the aromatic ring, it would seem that the method of cleavage
224 would not be through *ortho* or intradiol cleavage. This would seem evident as the
225 less abundant *meta* pathway has a wider substrate specificity. The *meta* pathway is
226 able to utilise substituted aromatic compounds such as xylene, toluene and
227 polyaromatic hydrocarbons (PAHs), whereas the *ortho* pathway is more specific and
228 is involved in the degradation of catechol and protocatechuate (Van Der Meer et al.
229 1992; Worsey and Williams 1975). If dioxygenation of the (4'-
230 carboxybutylphenyl)ethanoic acids and subsequent *meta* cleavage were to ensue,
231 then this compound could be converted into malonate, acetate and 2-oxoglutarate,
232 which can be fed into central metabolism, leading to complete mineralisation of *n*-
233 BPBA. It would therefore be advantageous for NA bioremediation to utilise
234 microorganisms containing the TOL plasmid and therefore be capable of *meta* ring
235 cleavage. As well as monoaromatic compounds, *P. putida* strains have also been
236 shown to utilise these pathways in the degradation of polyaromatic hydrocarbons
237 such as phenanthrene, fluorene and naphthalene (Yang et al. 1994).

238 The effect of increased substrate concentration on *n*-BPBA degradation by *P.*
239 *putida* KT2440 was also investigated and the data are presented in Fig. 4.
240 Degradation of *n*-BPBA decreased by 70, 57 and 14% for *n*-BPBA concentrations of
241 2, 3 and 4 mg L^{-1} respectively at day 49 (Fig. 4). However, for *n*-BPBA at
242 concentrations of 2 and 3 mg L^{-1}, the differences in the percentage degradation were
243 not statistically significant (p=0.716). In contrast, there was a statistically significant
244 difference in the degradation for *n*-BPBA concentrations at 4 mg L^{-1} compared to
245 either 3 mg L^{-1} (p=0.021) or 2 mg L^{-1} (p=0.010).

246 In addition, viable cell counts of *P. putida KT2440* from day 31, performed by
247 dilution plating are presented in Figure 5. These showed a significant decrease in

248 cell numbers (from 7.67 x 10^6 cfu mL^{-1} to 1.27 x 10^6 cfu mL^{-1}) with increasing
249 concentration of *n*-BPBA from 0 to 4 mg L^{-1} (p= 0.022) (Fig. 5). This suggested that
250 at greater concentrations, *n*-BPBA had a greater toxic effect.
251
252 **4. CONCLUSION:**
253 In conclusion, this study investigated the catabolic ability of *P. putida* KT2440
254 to degrade different alkyl branched aromatic NAs. Whilst *n*-BPBA was readily
255 degraded within days via beta-oxidation of the alkanoic acid side chain, *P. putida*
256 KT2440 was unable to metabolise *t*-BPBA. A major metabolite was identified (*n*-
257 BPEA) that was produced during *n*-BPBA degradation. In addition, increasing
258 concentrations of *n*-BPBA resulted in a decrease in the degradative ability of *P.*
259 *putida* KT2440 and a decrease in cell numbers, suggesting that there was a toxic
260 effect. Information obtained from this study is important as it would enable the
261 elucidation of the functional genes and metabolic pathways involved in aromatic NA
262 degradation and thus facilitate the bioremediation of these recalcitrant aromatic NAs
263 in the environment.
264
265 **ACKNOWLEDGEMENTS:** This work was supported by a NERC CASE studentship
266 with Oil Plus Ltd (REF: NER/S/A/2006/14134) and the University of Essex.
267
268
269
270
271
272

273 **REFERENCES:**

274 Beam, H.W., Perry, J.J., 1974. Microbial degradation and assimilation of *n*-alkyl-
275 substituted cycloparaffins. Journal of Bacteriology 118, 394-399.
276 Del Rio, L.F., Hadwin, A.K., Pinto, L.J., MacKinnon, M.D., Moore, M.M., 2006.
277 Degradation of naphthenic acids by sediment micro-organisms. Journal of
278 Applied Microbiology 101, 1049-61.
279 Eggink, G., De Waard, P., Huijberts, G.N.M., 1992. The role of fatty acid
280 biosynthesis and degradation in the supply of substrates for poly (3-
281 hydroxyalkanoate) formation in *Pseudomonas putida*. FEMS Microbiology
282 Letters 103, 159-63.
283 Franklin, F.C., Bagdasarian, M., Bagdasarian, M.M., Timmis, K.N., 1981. Molecular
284 and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida*
285 and cloning of genes for the entire regulated aromatic ring meta cleavage
286 pathway. Proceedings of the National Academy of Sciences of the United
287 States of America 78, 7458-7462.
288 Gilbert, E.S., Walker, A.W., Keasling, J.D., 2003. A constructed microbial consortium
289 for biodegradation of the organophosphorus insecticide parathion. Applied
290 Microbiology and Biotechnology 61, 77-81.
291 Head, I.M., Jones, D.M., Larter, S.R., 2003. Biological activity in the deep subsurface
292 and the origin of heavy oil. Nature 426, 344-52.

293 Headley, J.V., McMartin, D.W., 2004, A Review of the Occurrence and Fate of
294 Naphthenic Acids in Aquatic Environments. Journal of Environmental Science
295 and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering
296 39, 1989-2010.

297 Jimenez, J.I., Minambres, B., Garcia, J.L., Diaz, E., 2002, Genomic analysis of the
298 aromatic catabolic pathways from *Pseudomonas putida* KT 2440.
299 Environmental Microbiology 4, 824-41.
300 Johnson, R.J., Smith, B.E., Sutton, P.A., McGenity, T.J., Rowland, S.J., Whitby, C.,
301 2011a. Microbial biodegradation of aromatic alkanoic naphthenic acids is
302 affected by the degree of alkyl side chain branching. The ISME Journal 5, 486-
303 96.
304 Johnson, R.J., West, C., Swaih, A.M., Folwell, B.D., Smith, B.E., Rowland, S.J. and
305 Whitby, C. 2011b. Aerobic biotransformation of alkyl branched aromatic
306 alkanoic naphthenic acids via two different pathways by a new isolate of
307 *Mycobacterium*. Environmental Microbiology, (in press).
308 Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martins dos
309 Santos, V.A.P., Fouts, D.E., Gill, S.R., Pop, M., Holmes, M., 2002. Complete
310 genome sequence and comparative analysis of the metabolically versatile
311 *Pseudomonas putida* KT 2440. Environmental Microbiology 4, 799-808.
312 Olivera, E.R., Miambres, B., GarcIa, B., Muiz, C., Moreno, M.A., Ferrndez, A., DIaz,
313 E., GarcIa, J.L., Luengo, J.M., 1998. Molecular characterization of the
314 phenylacetic acid catabolic pathway in *Pseudomonas putida* U: the
315 phenylacetyl-CoA catabolon. Proceedings of the National Academy of Sciences
316 of the United States of America 95, 6419-24.
317 Quagraine, E.K., Headley, J.V., Peterson, H.G., 2005. Is biodegradation of bitumen a
318 source of recalcitrant naphthenic acid mixtures in oil sands tailing pond waters?
319 Journal of Environmental Science and Health. Part A, Toxic/Hazardous
320 Substances & Environmental Engineering 40, 671-84.

321 Rontani, J.F., Bonin, P., 1992. Utilization of *n*-alkyl-substituted cyclohexanes by a
322 marine *Alcaligenes*. Chemosphere 24, 1441-6.
323 Rowland, S.J., West, C.E., Scarlett, A.G., Jones, D., Frank, R.A., 2011a.
324 Identification of individual tetra- and pentacyclic naphthenic acids in oil sands
325 process water by comprehensive two-dimensional gas chromatography/mass
326 spectrometry. Rapid Communications in Mass Spectrometry 25, 1198-204.
327 Rowland, S.J., West, C.E., Scarlett, A.G., Jones, D., Boberek, M., Pan, L., Ng, M.,
328 Kwong, L., Tonkin, A., 2011b. Monocyclic and monoaromatic naphthenic acids:
329 synthesis and characterisation, Environmental Chemistry Letters [doi:
330 10.1007/s10311-011-0314-6].
331 Rowland, S. J., West, C.E., Scarlett, A.G., Jones, D., Frank, R. and Hewitt L.M.
332 (2011c) .Steroidal aromatic naphthenic acids in oil sands process-affected
333 water: structural comparisons with environmental estrogens. Environmental
334 Science & Technology (in press).
335 Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory
336 manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
337 Siddique, T., Penner, T., Semple, K., Foght, J.M., 2011. Anaerobic Biodegradation of
338 Longer-Chain *n*-Alkanes Coupled to Methane Production in Oil Sands Tailings.
339 Environmental Science & Technology 45, 5892-9.
340 Smith, B.E., Lewis, C.A., Belt, S.T., Whitby, C., Rowland, S.J., 2008. Effects of alkyl
341 chain branching on the biotransformation of naphthenic acids. Environmental
342 Science and Technology 42, 9323-8.
343 Smith BE. 2006. Naphthenic Acids: Synthesis, Characterisation and Factors
344 Influencing Environmental Fate, PhD Thesis, University of Plymouth, Plymouth,
345 UK.

346 Van Der Meer, J.R., De Vos, W.M., Harayama, S., Zehnder, A.J., 1992. Molecular
347 mechanisms of genetic adaptation to xenobiotic compounds. Microbiology and
348 Molecular Biology Reviews 56, 677-94.
349 de Waard, P., van der Wal, H., Huijberts, G.N., Eggink, G., 1993. Heteronuclear
350 NMR analysis of unsaturated fatty acids in poly(3-hydroxyalkanoates). Study of
351 beta-oxidation in *Pseudomonas putida*. The Journal of Biological Chemistry
352 268, 315-9.
353 Whitby, C., 2010. Microbial naphthenic acid degradation. Advances in Applied
354 Microbiology 70, 93-125.
355 Worsey, M.J., Williams, P.A., 1975. Metabolism of toluene and xylenes by
356 *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL
357 plasmid. Journal of Bacteriology 124, 7-13.
358 Yang, Y., Chen, R.F., Shiaris, M.P., 1994. Metabolism of naphthalene, fluorene, and
359 phenanthrene: preliminary characterization of a cloned gene cluster from
360 *Pseudomonas putida* NCIB 9816. Journal of Bacteriology 176, 2158-64.
361
362
363
364
365
366
367
368
369
370

371 **Legends to Figures**

372 **Fig. 1.** Degradation of *n*- and *t*-BPBA and production of a major metabolite by
373 *Pseudomonas putida* KT2440. (A): Percentage Recovery of *n*-BPBA (■) and *t*-BPBA
374 (●) by *P. putida* KT2440 relative to the abiotic controls. Killed controls are circled.
375 (B): Production of a metabolite identified as that corresponding to the ethanoic acid
376 equivalent of *n*-BPBA, with a retention time of 15.10 min. Error bars represent
377 standard error of the mean (*n*=3).

378

379 **Fig. 2.** Example gas chromatograms showing degradation of *n*-BPBA (A) and *t*-
380 BPBA (B) over time. The internal standard was 4-phenylbutyric acid.

381

382 **Fig. 3.** Mass spectrum of a trimethylsilylated metabolite of *n*-BPBA degradation,
383 eluting at 15.10 mins. The metabolite was identified as *n*-BPEA, by comparison of
384 the mass spectrum with those of synthetic *n*-BPEA (TMS ester).

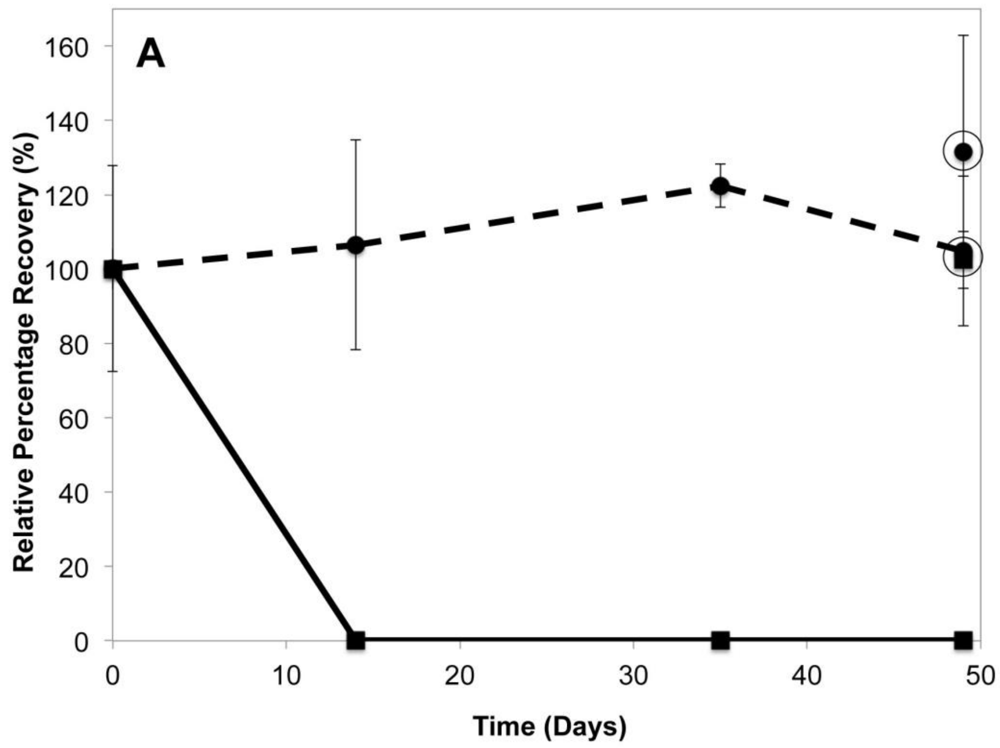
385

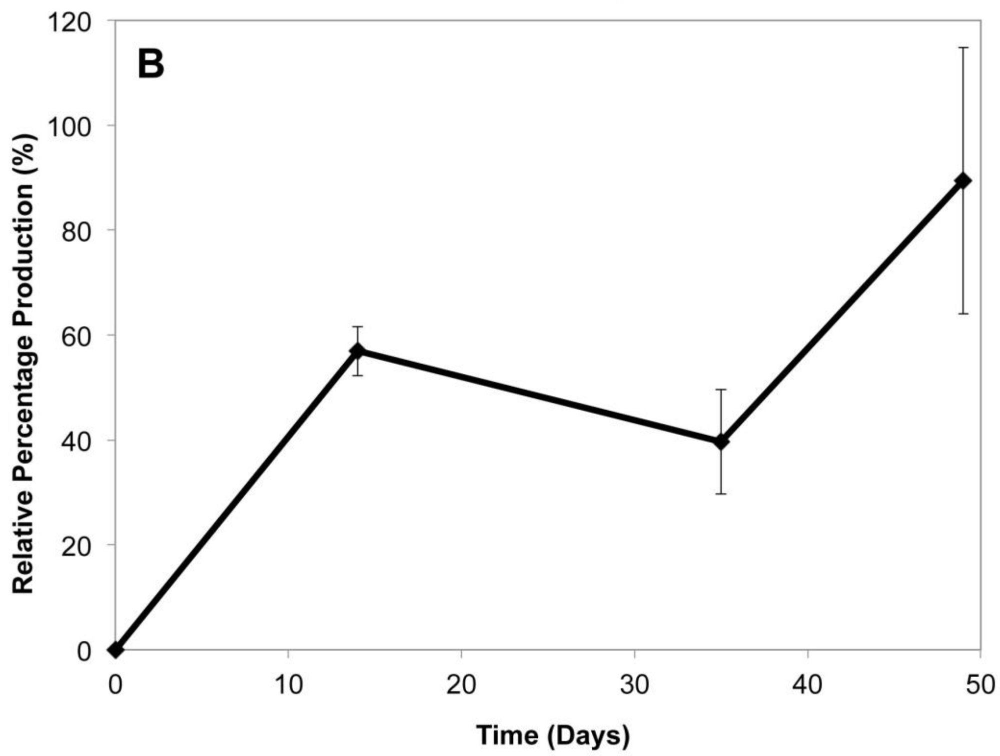
386 **Fig. 4.** Recovery of *n*-BPBA after 49 days incubation with *P. putida* KT2440. Shown
387 are the mean percentage recoveries of *n*-BPBA after 49 days incubation with either
388 2, 3 or 4 mg L^{-1} of *n*-BPBA. Bars represent the mean of triplicate cultures and error
389 bars represent standard error of the mean (*n* = 3).

390

391 **Fig. 5.** The Relationship between *n*-BPBA concentration and viable cell counts of *P.*
392 *putida* KT2440 after day 31. Error bars represent standard error of the mean (*n*=3).

393



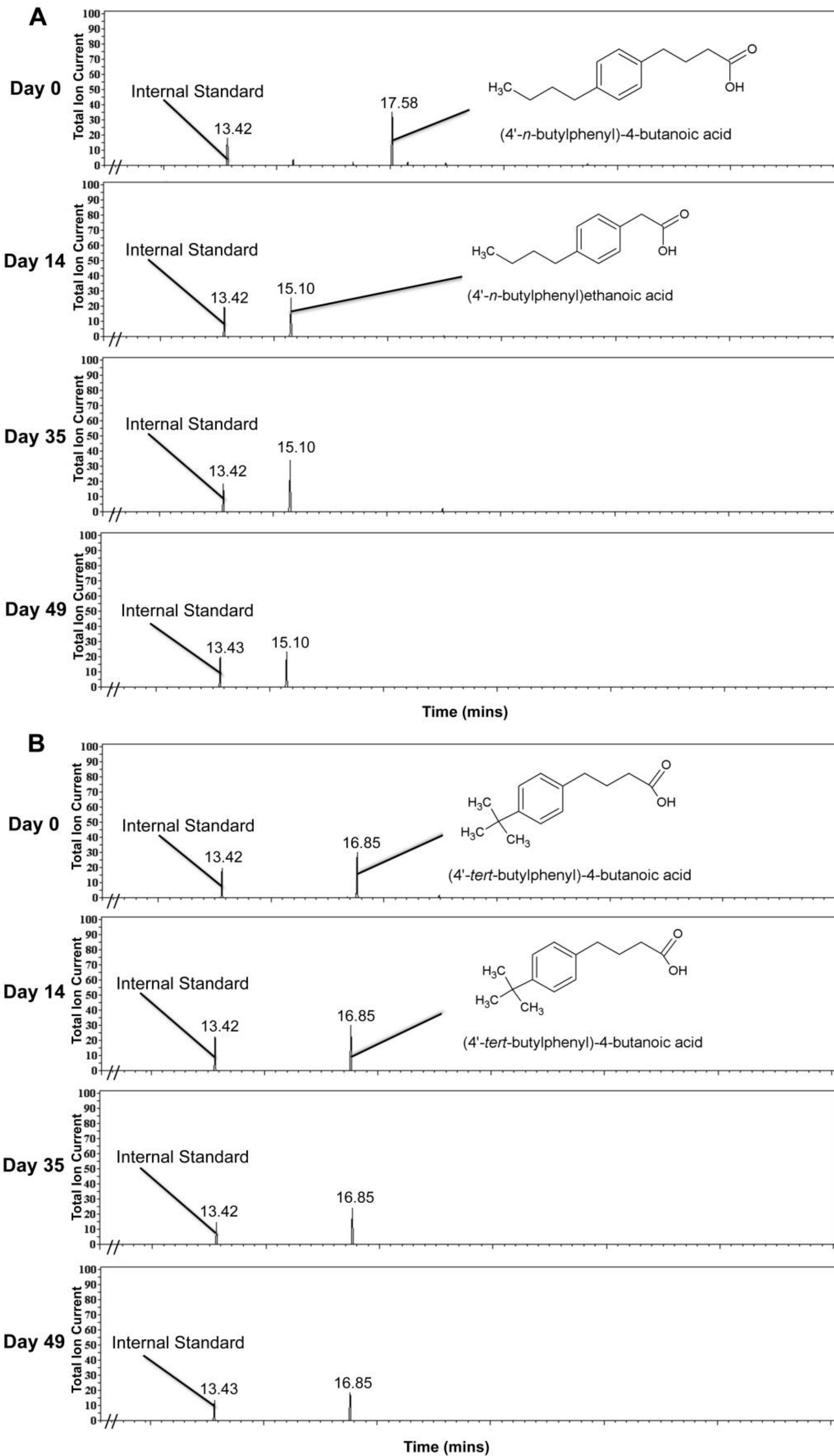


394

395 **Fig. 1.**

396

397

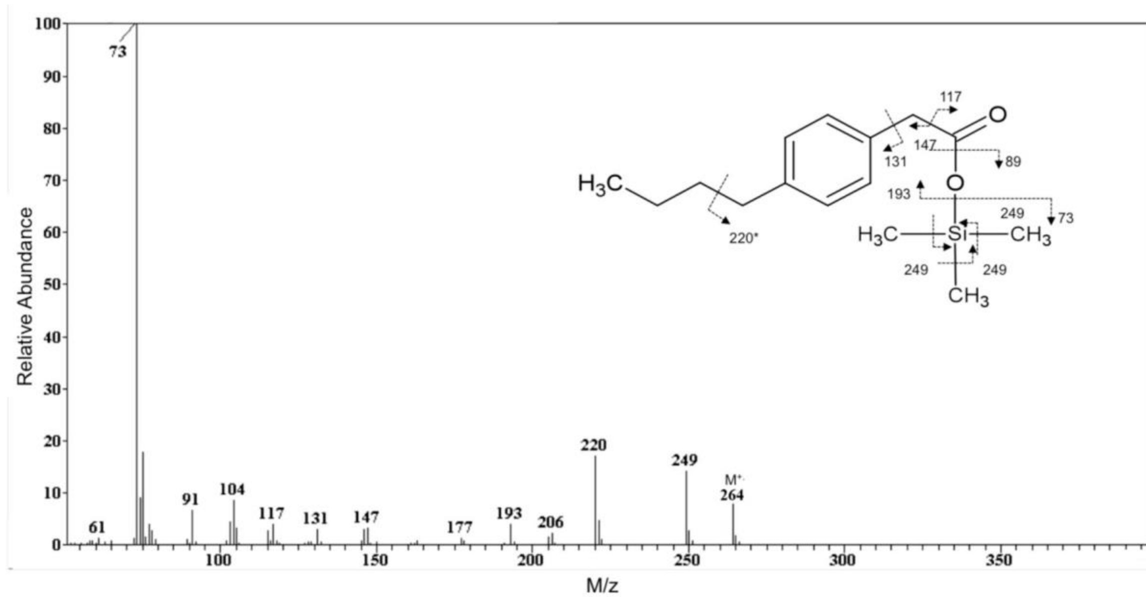


398 **Fig. 2**

399

400

401



402

403

404 **Fig. 3.**

405

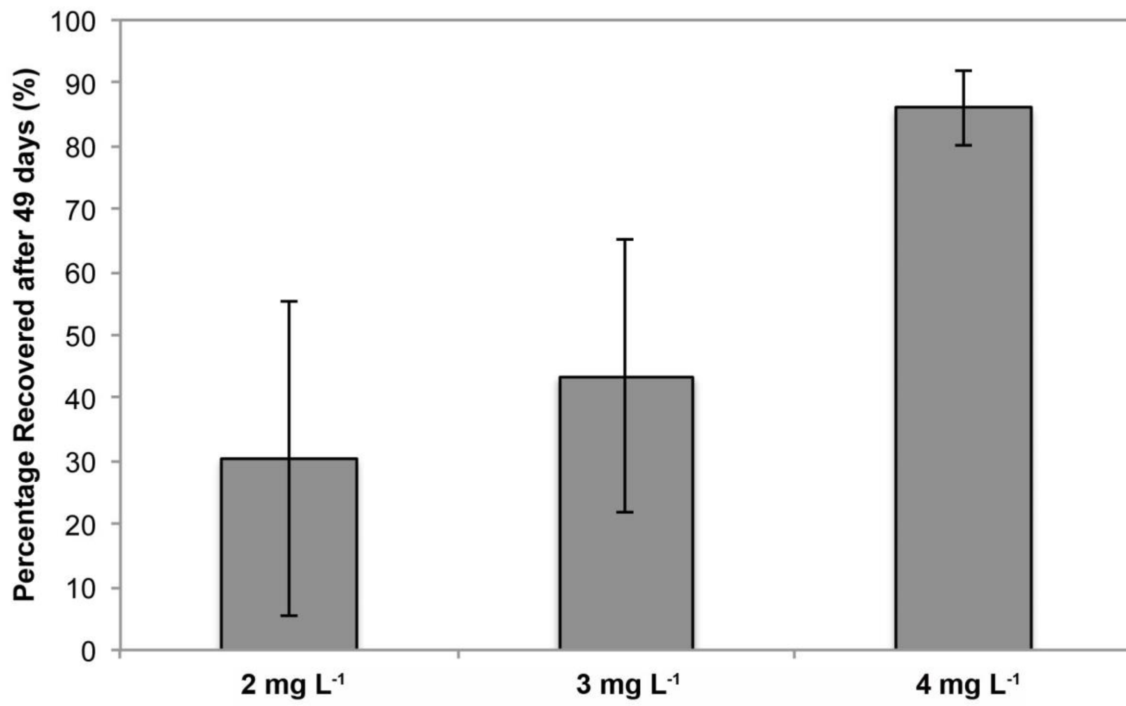
406

407

408

409

410



411

412 **Fig. 4.**

413

414

415

416

417

418

419

420

421

422

423

424

425

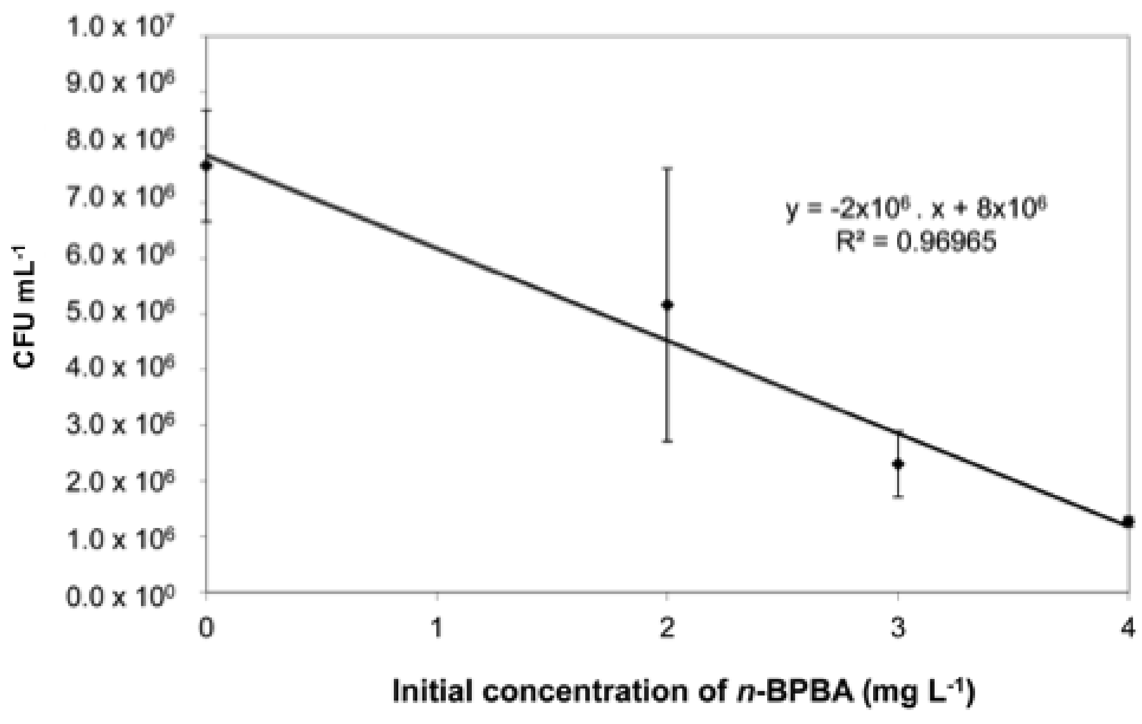
426

427

428

429

431



434

435

436

437

438

439 **Fig. 5.**

440

441

442

443

444

445

446

447

448

449

450

451

452