



Title	The potential of glycerol in freezing preservation of turbine oil-degrading bacterial consortium and the ability of the revised consortium to degrade petroleum wastes
Author(s)	Kurachi, Kumiko; Hosokawa, Reia; Takahashi, Marina; Okuyama, Hidetoshi
Citation	International Biodeterioration & Biodegradation, 88, 77-82 https://doi.org/10.1016/j.ibiod.2013.12.005
Issue Date	2014-03
Doc URL	http://hdl.handle.net/2115/55385
Type	article (author version)
File Information	Okuyama_IBB.pdf



[Instructions for use](#)

1 The potential of glycerol in freezing preservation of turbine oil-degrading bacterial
2 consortium and the ability of the revised consortium to degrade petroleum wastes

3

4 Kumiko Kurachi, Reia Hosokaewa, Marina Takahashi, Hidetoshi Okuyama*

5 *Graduate School Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan*

6

7 **Abstract: The turbine oil (TuO)-degrading bacterial consortium Tank-2 (original**
8 **Tank-2) was preserved as a glycerol stock at -80°C from 2009 to 2012. Storage methods**
9 **have been unavailable so far for any TuO-degrading bacterial consortia or isolates. To**
10 **evaluate the usefulness of glycerol stock, the original Tank-2 consortium frozen in**
11 **glycerol at -80°C was thawed and then revived by repeated culture in mineral salts**
12 **medium (MSM) containing 0.5% (w/w) TuO (revived Tank-2). The revived Tank-2**
13 **consortium exhibited a high activity to degrade TuO, which was equivalent to that of**
14 **original Tank-2. It also degraded car engine oil, used car engine oil, Arabian light and**
15 **Vityaz crude oils and TuO in wastewater. These results indicated that a glycerol stock at**
16 **-80°C was useful for storing Tank-2. PCR-denaturing gradient gel electrophoresis**
17 **(DGGE) that targeted the V3 regions of 16 S rRNA gene sequences showed that the**
18 **DGGE band profiles of principal bacteria were significantly different between the**
19 **original and revived Tank-2 consortia and between the revived Tank-2 culture grown in**
20 **MSM containing TuO and that grown in MSM containing other types of petroleum**
21 **products. This suggested that bacterial strains inherently residing in Tank-2 could**
22 **adjust their compositions based on the storage and culture conditions.**

23

24

25

26 *Keywords:*

27 Bacterial consortia

28 Biodegradation

29 Crude oil

30 Culture revival

31 Glycerol stock

32 Turbine oil

33 Wastewater

34 _____

35 * Corresponding author.

36 E-mail address: hoku@ees.hokudai.ac.jp (H. Okuyama)

37 Graduate School Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

38 Phone, +81-11-706-4523; Fax, +81-11-703-2347

39

40 Abbreviations:

41 DGGE, denaturing gradient gel electrophoresis; EO, engine oil; ICP-AES, inductively

42 coupled plasma-atomic emission spectrometry; MSM, minimal salts medium; TLC-FID,

43 thin-layer chromatography-flame-ionization detection; TPH, total petroleum hydrocarbon;

44 TuO, turbine oil

45

46 **1. Introduction**

47

48 Turbine oil (TuO) is used for lubricating and controlling gas and steam turbine systems. TuO
49 typically comprises 95%–99.5% (w/w) of highly refined base oil (a mixture of branched
50 alkanes and cyclic alkanes) and 0.5%–5% (w/w) of additives (Hosokawa et al. 2010). The
51 biodegradability of branched and cyclic alkanes is lower than that of aliphatic hydrocarbons
52 (Gough and Rowland 1990). According to Perry (1984), the susceptibility of hydrocarbons to
53 microbial attack is in the following order: normal alkanes (*n*-alkanes) > isoalkanes > low
54 molecular weight aromatics > cyclic alkanes. Thus, TuO, particularly cyclic alkanes, is
55 assumed to be relatively recalcitrant to microbial degradation.

56 There have been numerous reports on bacteria and bacterial consortia that can
57 degrade petroleum products, including gasoline (Wongsa et al. 2004; Lu et al. 2006), diesel
58 oil (Ciric et al. 2010; Wongsa et al. 2004; Jung et al. 2010), car engine oil (Wongsa et al.
59 2004; Abioye et al. 2012), heavy oil (Wongsa et al. 2004; Aoshima et al. 2006; Hao and Lu
60 2009) and crude oil (Razak et al. 1999; Rahman et al. 2002; Sathishkumar et al. 2008).
61 However, there are only a limited number of bacteria that can degrade TuO.

62 Zvyagintseva et al. (2001) reported that *Rhodococcus erythropolis* and *Dietzia maris*
63 are TuO degraders. Two types of TuO-degrading consortia, designated Atsuta (Ito et al.
64 2008) and Tank-2 (Hosokawa et al. 2010), which had been formulated from soil samples
65 contaminated with crude oil and TuO-containing wastewater sampled at an electric power
66 plant, respectively, efficiently degraded TuO. Their capacities to degrade TuO were
67 consistently maintained as long as these consortia were continuously cultured in media that
68 contained TuO.

69 Freezing and freeze drying are common means used for long-term storage of
70 microbial cells (see: <http://www.atcc.org/CulturesandProducts/tabid/167/Default.aspx>).

71 However, regardless of the isolated bacterial strains or consortia, appropriate reservations
72 must be considered before their practical use, such as in bioaugmentation. To date, there have
73 been no reports on whether a glycerol stock, the most commonly used storage method for
74 bacterial cells, is useful for TuO-degrading bacteria or bacterial consortia.

75 In this study, the availability and usefulness of a glycerol stock of the TuO-degrading
76 consortium Tank-2, which had been stored at -80°C for 3.5 years were evaluated. This
77 revived consortium was tested for its ability to degrade TuO and types of petroleum products,
78 including lubricating oils, crude oils and TuO in the wastewater from an electric power plant.

79

80 **2. Materials and Methods**

81

82 *2.1. Microbial consortia and culture media*

83

84 To culture microbial consortia, we used minimal salts medium (MSM) that included 0.4%
85 NH_4NO_3 , 0.47% KH_2PO_4 , 0.0119% Na_2HPO_4 , 0.001% $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 0.1% $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$,
86 0.001% $\text{MnSO}_4\cdot 7\text{H}_2\text{O}$ and 0.0015% $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, pH 7.0. MSM was supplemented with
87 TuO or another type of petroleum product (Ueno et al. 2006 a, b)

88 TuO-degrading consortia Tank-2 was used throughout this study. The original Tank-2
89 was formulated in 2009 (Hosokawa et al. 2010) and had been preserved at -80°C as a
90 glycerol stock. In this study, frozen original Tank-2 was revived from storage, subjected to
91 re-testing for its capacities to degrade various types of petroleum products and these results
92 were compared with those for the original Tank-2 consortium.

93 Glycerol-stocked original Tank-2 was first thawed at room temperature. The thawed
94 Tank-2 culture (1 ml) was then transferred to a 50-ml flask containing 10 ml of MSM
95 supplemented with 0.5% (w/w) of TuO (TuO-containing MSM) and cultured for two weeks

96 at 30°C with shaking at 160 rpm. After the first reviving culture (enrichment culture), a part
97 (200 µl) of the culture was inoculated into freshly prepared TuO-containing MSM and
98 cultured (second enrichment culture) as described above. This enrichment procedure was
99 repeated more than four times. These prepared consortia were designated as revived Tank-2.

100 TuO (type FBK turbine SH), a product of Nippon Oil Corporation (Tokyo, Japan),
101 was obtained from Hokkaido Electric Power Co., Inc. Car engine oil (Toyota Castrol motor
102 oil SN 5W-30; abbreviated EO) was purchased from a local market. Used car engine oil
103 (original Toyota Castrol motor oil SN 5W-30) was obtained from a local body shop. Arabian
104 light crude oil and Vityaz crude oil (Maki et al. 2008) were provided by Idemitsu Kosan Co.,
105 Ltd. and Vityaz crude oil (Maki et al. 2008) from the Geological Survey of Hokkaido,
106 respectively. All oil types were autoclaved at 121°C for 20 min before use.

107

108 2.2. *Formulating a new microbial consortium to degrade TuO*

109

110 To formulate a new TuO-degrading consortium, wastewater samples that contained TuO were
111 collected from the oil-water separating tank at the Moiwa hydraulic plant, Hokkaido Electric
112 Power Co., Inc. located at Minami-ku, Sapporo in 2012. This was the same sampling site
113 where a wastewater sample was collected to prepare the original Tank-2 consortium in 2009.
114 Wastewater samples were processed as described previously (Hosokawa et al. 2010). Samples
115 were centrifuged at 7,000 rpm for 15 min to precipitate insoluble matters, after which the
116 pellets were suspended in 10 ml of the supernatant. This suspension was added as an
117 inoculum to 10 ml of MSM that contained TuO in a 50-ml flask. This was then incubated at
118 30°C on a rotary shaker (160 rpm) for two weeks. An aliquot of this culture (200 µl) was
119 transferred to another 50-ml flask containing 10 ml of the same medium and incubated as
120 described above. Because bacterial cultures, including cultures of the original Tank-2, formed

121 aggregates during culture, 200 μ l of each liquid culture that contained small cell aggregates
122 were inoculated directly into 10 ml of fresh TuO-containing MSM. These cultures were
123 incubated at 30°C with shaking (160 rpm) for two weeks. The formulated TuO-degrading
124 consortium was designated Moiwa-KK.

125

126 *2.3. Degradation test*

127

128 To estimate the degradation of TuO and other types of petroleum products, 200 μ l of the
129 pre-culture for revived TuO was transferred to a 50-ml flask that contained 10 ml of MSM
130 supplemented with either TuO, EO, Arabian light crude oil or Vityaz crude oil at 0.5% (w/v).
131 Culture was carried out for one or two weeks at 30°C with shaking at 160 rpm. When the
132 thawed Tank-2 consortium was used as the inoculum, the total volume (1 ml) of the original
133 Tank-2 culture in a microfuge tube that had been frozen for 3.5 years at -80°C was thawed at
134 room temperature and then transferred into the same TuO-containing medium as described
135 above.

136 TuO in wastewater was also used as the carbon source for revived Tank-2. Culture
137 media were prepared as shown in Table 1. For this test, the concentration of TuO in the
138 medium was adjusted to 1.5%, which was three times higher than that of normal
139 TuO-containing MSM. A 200- μ l aliquot of the culture grown in TuO-containing MSM was
140 inoculated directly into 10 ml of MSM supplemented with 0.5% (w/v) of the various
141 petroleum products. Culture was performed as described above.

142

143 *2.4. Extraction and analysis of hydrocarbons*

144

145 Petroleum product extraction was performed with chloroform using the modified Bligh-Dyer

146 method (Bligh and Dyer 1959), as described previously (Hosokawa et al. 2010). Total
147 petroleum hydrocarbons (TPHs) were separated into saturated, aromatic, resin and
148 asphaltene fractions and quantified by the thin-layer chromatography-flame ionization
149 detection method (TLC-FID) using an Iatroscan (Model MK-6), as described previously
150 (Goto et al. 1994; Ito et al. 2008; Hosokawa et al. 2010). When crude oils were analysed by
151 TLC-FID, the resin and asphaltene fractions were omitted from the calculations because of
152 their recalcitrant characteristics and the inclusion of cell-derived polar lipids in the
153 asphaltene fraction (Ito et al. 2008).

154

155 *2.5. Analysis of metals*

156

157 The concentrations of Al, Cr, Cu, Fe, Pb, Zn and other ions at >0.1 mg/L (ppm) in
158 TuO-containing wastewater were determined by an inductively coupled plasma-atomic
159 emission spectrometer (ICP-AES) (Model ICPE-9000). For preparations for hydride
160 generation analysis, 5 g of sample was mixed with 8 ml of nitric acid. This mixture was
161 subjected to microwave digestion first at 230°C for 40 min and then at 120°C for 15 min.
162 After cooling, the sample mixture volume was adjusted to 50 ml with distilled water. This
163 original sample solution was diluted with 2 M nitric acid at appropriate ratios and then
164 subjected to ICP-AES. Multi-element standard solution I (MERCK, Darmstadt, Germany)
165 was used as a standard.

166

167 *2.6. DNA procedures*

168

169 *2.6.1. Extraction of bacterial genomic DNA*

170 Bacterial cells were harvested by centrifugation at 7,000 rpm for 10 min at 4°C, after which

171 the pellets were freeze-dried overnight. Freeze-dried samples were suspended in 1 ml of 20
172 mM Tris–HCl buffer (pH 8.0) containing 250 mM EDTA. The suspensions were
173 homogenized in a sterilized mortar. A 200- μ l aliquot was transferred to a 2-ml sterilized
174 screw-capped tube with a O-ring (Assist Co., Ltd., Tokyo) that contained 500 mg of sterilized
175 glass beads (105–150 μ m in diameter; Polysciences, Inc., Warrington, PA), and the remainder
176 of the suspended cells was stored at -30°C .

177 A mixture of 400 μ l of an organic solvent containing phenol, chloroform, and isoamyl
178 alcohol (25:24:1, by volume) and 800 μ l of a stabilizing reagent [100 mM Tris–HCl (pH 8.0),
179 20 mM EDTA, 1.4 M NaCl and 2% (w/v) cetyltrimethyl ammonium bromide] were added to
180 the screw-capped tubes. This mixture was disrupted with a Mini-BeadbeaterTM at 5,000 rpm
181 for 1 min, and then heated at 65°C for 15 min. After cooling on ice, the sample was
182 bead-beated at 5,000 rpm for 1 min, and centrifuged at 13,000 rpm for 10 min at 4°C .
183 Supernatants were transferred to 1.5 ml sterilized tubes and precipitated with ethanol.
184 Genomic DNA was finally dissolved in 50 μ l of EDTA-containing 20 mM Tris-HCl buffer
185 mentioned above and stored at -30°C .

186

187 2.6.2. *PCR amplification of 16S rRNA gene sequences*

188 For denaturing gradient gel electrophoresis (DGGE), PCR reactions were carried out using
189 methods described by Hosokawa et al. (2010). A set of primers with a GC clamp, 338F-GC
190 (5'-CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC
191 TAC GGG AGG CAG CAG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3'), which
192 corresponded to the V3 regions of the 16S rRNA gene sequence was used. PCR was run
193 using GC-338F and 518R primers. The PCR reaction mixture (final volume of 50 μ l)
194 included 1 unit of Ex Taq DNA polymerase (TAKARA BIO, Kyoto, Japan), 200 nM dNTP
195 mixture, 25 pM of each primer, EX Taq buffer and genomic DNA as template. PCR was

196 carried out on a Mastercycler ep thermal cycler (Eppendorf AG) using the following
197 program: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 1 min at
198 94°C, 1 min at 55°C and 1 min at 72°C and finally a 5 min extension at 72°C to amplify 16S
199 rRNA gene fragments for DGGE. To amplify the whole 16S rRNA gene, the following
200 program was used: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 1
201 min at 94°C, 1 min at 55°C, 2 min at 72°C and finally a 5-min extension at 72°C.

202

203 *2.6.3. DGGE analysis*

204 DGGE used the D-code™ Universal Mutation Detection system, as described in Hosokawa
205 et al. (2010). PCR products (10 µl each) were mixed with 1 µl of alkaline gel-loading buffer
206 containing 300 mM NaOH, 6 mM EDTA, 18% (w/v) Ficoll and 0.25% (w/v) xylene cyanol
207 and then loaded on acrylamide gel containing a denaturing urea–formamide gradient ranging
208 from 20% to 60%. A 100% concentration of denaturant was defined as 7 M urea and 40%
209 (v/v) formamide (Muyzer et al. 1993). Electrophoresis was run at a constant voltage of 200 V
210 for 3 h at a constant temperature of 60°C. After DGGE, the gel was stained for 30 min in 0.5×
211 TAE containing 1 SYBR Green (BioWhittaker, Walkersville, MD, USA) and photographed
212 under UV illumination.

213

214 3. Results and Discussion

215

216 3.1. Reviving the glycerol-stocked Tank-2 consortium

217

218 The TuO-degrading consortium Tank-2 was formulated in 2009. This bacterial consortium
219 exhibited a significantly and consistently high activity to degrade TuO (>90% of total TuO
220 for two weeks at 30°C; Hosokawa et al. 2010). Since that time it has been preserved as a
221 glycerol stock at -80°C.

222 As shown in Figure 1a, TuO degradation by revived Tank-2 gradually increased up to
223 approximately 90% of the total for the third enrichment culture and this activity was
224 consistently maintained after the fourth enrichment culture (data not shown). Considering that
225 TuO degradation by the original Tank-2 consortium was also consistently approximately 90%
226 (Hosokawa et al. 2010), it was concluded that Tank-2 could be completely revived after
227 storage at -80°C for more than three years. The revived Tank-2 consortium that was enriched
228 more than three times was used for further experiments.

229 Figure 1a also shows the time-dependent increase in TuO degradation by a newly
230 prepared TuO-degrading consortium (Moiwa-KK). After the third enrichment culture, TuO
231 degradation by the revived Tank-2 and Moiwa-KK consortia was consistently approximately
232 90% and approximately 80%, respectively, of total TuO (Fig. 1b).

233

234 3.2. Degradation of different petroleum products by revived Tank-2

235

236 The original Tank-2 consortium could degrade not only TuO, but also different types of
237 petroleum products, such as diesel oil, car engine oil and Arabian light crude oil (Fig. 2a and
238 Hosokawa et al. 2010). In this study, in addition to these petroleum products, used car engine

239 oil and Vityaz crude oil were also used as the sole carbon and energy sources for the revived
240 Tank-2 consortium. Revived Tank-2 degraded TuO (63% of total), car engine oil (64% of
241 total), used car engine oil (81% of total), Arabian light crude oil (72%) and Vityaz crude oil
242 (28% of total) after one week (Fig. 2b). Except for Vityaz crude oil, > 80% of each petroleum
243 product was degraded by revived Tank-2 after two weeks (Fig. 2b). The relatively high
244 degradation activity for engine oil by the revived Tank-2 consortium was probably because
245 the main components of car engine oil, as well as TuO, are n-alkanes (Koma et al. 2003).
246 Thus, the bacterial community structure of the Tank-2 consortium may be readily adaptable
247 to a medium that contains car engine oil.

248 Arabian light crude oil was completely degraded by revived Tank-2 after two weeks.
249 However, Vityaz crude oil degradation was less than that for Arabian light crude oil (Fig. 2b).
250 This longer time-dependent degradation of Vityaz crude oil by revived Tank-2 implies that a
251 much longer culture time in MSM supplemented with Vityaz crude oil would be needed for
252 Tank-2 to adjust its microbial structure to be more adaptable to this crude oil, which contains
253 more toxic, volatile and aromatic hydrocarbons than Arabian light crude oil (Maki et al.
254 2008).

255 The original Tank-2 consortium was known to form cell aggregates during culture in
256 TuO-containing MSM and TuO degradation was considerably reduced when these bacterial
257 aggregates were not inoculated along with a liquid aliquot that contained free-living bacteria
258 (Hosokawa et al., 2010). These results suggested that free-living bacteria and bacteria
259 comprising cell aggregates were both involved in TuO degradation in the original Tank-2
260 culture. Cell aggregates were also formed in the revised Tank-2 culture with TuO-containing
261 MSM, which indicated that bacteria in cell aggregates and liquid culture were also both
262 involved in TuO degradation by revised Tank-2. In contrast, during the degradation of fresh
263 and used car engine oils, Arabian light crude oil and Vityaz crude oil by revived Tank-2, cell

264 aggregates did not form and thus, the bacterial strains involved in degrading these oil
265 products may be different from those involved in degrading TuO (see below).

266

267 *3.3. Degradation of TuO in wastewater by revived Tank-2*

268

269 The capacity to degrade TuO by revived Tank-2 was evaluated using TuO-containing
270 wastewater collected at a hydraulic plant where TuO was routinely used. The concentration of
271 TuO in this wastewater was estimated to be 3% (w/v) by TCL-FID. For the degradation test,
272 the concentration of TuO was adjusted to 1.5% (see Table 1).

273 Approximately 70% of the total TuO in wastewater was degraded by revived Tank-2
274 after one week (Table 1). This was much higher than the 37% of the total in the degradation
275 test when fresh (unused) TuO was used as a substrate. Interestingly, TuO degradation in the
276 wastewater inoculated with no revived Tank-2 was approximately 64%. These results
277 demonstrated that revived Tank-2 could utilize TuO components in wastewater as carbon
278 sources and that these components could be preferentially utilized by bacterial strains native
279 to the wastewater.

280 The relatively low TuO degradation rate by revived Tank-2 in freshly prepared MSM
281 could be explained by: (1) revived Tank-2 may inherently consist of bacteria that can degrade
282 TuO at its relatively low concentration (i.e. 1.5% TuO inhibits the growth of TuO degraders
283 in the revived Tank-2 consortium); (2) bacteria native to TuO-containing wastewater used in
284 this study can degrade TuO at concentrations as high as 1.5%; and (3) TuO wastewater may
285 contain a compound(s) that stimulates the growth of TuO degraders.

286 Because this wastewater might contain some metals derived from turbine facilities
287 and these metal contaminants might affect bacterial growth, the major metal elements in the
288 TuO-containing wastewater were analysed by ICP. Al (11 ppm), Cr (0.06 ppm), Cu (0.3 ppm),

289 Fe (17 ppm), Pb (0.16 ppm) and Zn (0.30 ppm) were the principal components detected in the
290 TuO-containing wastewater, and all of these metals were at lower than detectable levels in
291 fresh (unused) TuO. Although it has not been formulated whether elements contained in the
292 wastewater affected TuO degradation by revived Tank-2, one or more elements in this
293 wastewater may enhance the growth of TuO-degrading bacteria in this consortium. This
294 speculation is supported in that revived Tank-2 preferentially degraded used car engine oil as
295 compared to fresh car engine oil (Fig. 2b), although the metal components in the used car
296 engine oil were not analysed.

297 All of these findings suggest that bacterial growth in revived Tank-2 would be
298 enhanced rather than inhibited by elements and/or unidentified substances in the
299 TuO-containing wastewater and in used car engine oil. TuO degradation in a culture with no
300 inoculums was higher than that in the culture inoculated with the revised Tank-2 consortium
301 (Table 1). This suggested that bacteria native to the TuO-containing wastewater used in this
302 study could have adapted to this TuO-containing wastewater environment and that these
303 bacteria exhibited a higher degradation activity towards TuO than the bacteria in revived
304 Tank-2.

305

306 *3.4. Microbial community structure of Tank-2 consortia*

307

308 Based on DGGE analysis that targeted 16S rRNA gene sequences, the original Tank-2
309 consortium consisted of at least 14 principal bacterial strains (Fig. 3, bands 1–14) and its
310 DGGE band profile was consistently maintained when it was continuously cultured in MSM
311 containing TuO (Hosokawa et al. 2010). The number of DGGE bands for the freeze–thawed
312 sample of Tank-2 decreased to 10 (bands B, C, D, F, H, J, K, N, O, P and S) and this band
313 profile was significantly different from that of original Tank-2 (Fig. 3a). The intensity of each

314 band for freeze–thawed Tank-2 was low, even though a whole DNA sample extracted from 1
315 ml of frozen Tank-2 was used as a template for PCR when amplifying the 16S rRNA gene.
316 This suggested that some of the bacteria cells in the frozen Tank-2 culture might have died
317 and that their genomes had decayed during thawing.

318 Only three DGGE bands (bands 5 and C, bands 6 and D, bands 8 and F) were
319 common to the original and freeze–thawed Tank-2 consortia. The number of bands for
320 revived Tank-2 was 13 (bands A, B, D, E, G, I, L, M, N, P, Q, R and S), but its DGGE band
321 profile differed significantly from that of original Tank-2. Only bands 10 and I appeared to be
322 common to these two consortia. Interestingly, relatively dense bands, such as bands E, G, I, L
323 and M, appeared only for revived Tank-2, which suggested that bacteria corresponding to
324 some of these DGGE bands could be simply freeze–thaw-resistant, but not TuO-degrading.
325 Although TuO-containing-wastewater sampling to formulate Tank-2 and Moiwa-KK was
326 performed at the same hydraulic plant of the electric power company and the enrichment
327 processes used were also the same, the microbial community structures of these two consortia
328 were entirely different.

329 The DGGE band profiles for the cultures grown in MSM supplemented with TuO,
330 engine oil, and Arabian light and Vityaz crude oils were compared (Fig. 3b). Some bands
331 (bands B, E, G, M, N, P, Q, and R) were common to all four cultures. However, band A was
332 common and band G was dominant for the consortia containing lubricating oils (TuO and car
333 engine oil) and bands M, N, and P were commonly dominant for the consortia containing
334 crude oils (Arabian light and Vityaz). Because cycloalkanes are the major components only in
335 lubricating oils, bacteria corresponding to bands A and G may be cycloalkane degraders.
336 Similarly, bacteria corresponding to bands M, N and P in the cultures of crude oil may be
337 degraders of polycyclic aromatic hydrocarbons (PAHs), which are the major components
338 only in crude oils.

339 Figure 3c shows the DGGE band profiles for the microbial consortia cultured in
340 TuO-containing wastewater. Ten bands (bands B, C, D, F, G, M, N, P, R, and S) were detected
341 for revived Tank-2-inoculated wastewater culture that contained 1.5% TuO (lane 3 in Fig. 3c).
342 Relatively dense bands (B, D, M, and P) were detected for Tank-2 culture supplemented with
343 fresh TuO at 1.5% (lanes 2 and 3 in Fig. 3c), which suggested that bacteria corresponding to
344 these DGGE bands were native in revived Tank-2 and that these bacteria would be mainly
345 responsible for degrading a high concentration (1.5%) of TuO. Bands b, g, h, and s were
346 detected only for the 1.5% TuO-containing wastewater culture inoculated with no Tank-2
347 (lane 4 in Fig. 3c), which indicated that bacteria corresponding to these bands originated from
348 the wastewater.

349 Nine DGGE bands (bands a, c, e, f, j, l, n, p, and r in lane 5, Fig. 3c) were detected for
350 the Moiwa-KK consortium, which had been formulated in TuO (0.5%)-containing MSM.
351 Although the same TuO-containing wastewater was used, no common bands were detected
352 between the Moiwa-KK consortium and the culture containing 1.5% TuO-containing
353 wastewater. These results again suggested that the bacterial species capable of degrading
354 0.5% TuO and 1.5% TuO were different.

355

356 **4. Conclusions**

357

358 A TuO-degrading bacterial consortium, Tank-2, maintained its high capacity to degrade TuO
359 after storage for 3.5 years at -80°C and could utilize various types of petroleum products as
360 substrates without enrichment culture. TuO in wastewater and used EO were degraded well
361 by revived Tank-2. However, most of the bacterial strains in the original and the revived
362 TuO-degrading microbial consortia were different from one another. Bacterial strains
363 inherently residing in Tank-2 had the potential to flexibly adjust their composition in

364 accordance with storage and culture conditions. Tank-2, and probably the new consortium
365 Moiwa-KK, even after freeze-storage can be used as inocula for bioaugmentation to
366 remediate waters that are polluted with various types of lubricating and crude oils.

367

368

369 **Acknowledgements**

370 We thank Mr. Y. Uchiai of Hokkaido Electric Power Company Inc. for providing his assistance in the
371 sampling of TuO-containing wastewater and turbine oils. Elements in the TuO-wastewater were
372 analyzed by Ms. N. Takeda of the Open Facility, Hokkaido University.

373

374

375 **References**

376

377 Abioye, O.P., Agamuthu, P., Abdul Aziz, A.R., 2012. Biodegradation of used motor oil in soil using
378 organic waste amendments. *Biotechnology Research International* Article ID 587041, 8 pages
379 doi:10.1155/2012.

380 Aoshima, H., Hirase, T., Tada, T., Ichimura, N., Yamaguchi, H., Taguchi, M., Myoenzono, T., 2006.

381 Improvement of heavy oil degradation by *Rhodococcus erythropolis* C2. *Journal of*

382 *Environmental Biotechnology* 5, 107–109.

383 Ciric, L., Philp, J.C., Whiteley, A.S., 2010. Hydrocarbon utilization within a diesel-degrading bacterial
384 consortium. *FEMS Microbiology Letters* 303, 116–122.

385 Goto, M., Kato, M., Asumi, M., Shirai, K., Venkateswaran, K., 1994. TLC/FID method for evaluation
386 of the crude-oil-degrading capability of marine microorganisms. *Journal of Marine Biotechnology*
387 2, 45–50.

388 Gough M.A. and Rowland S.J., 1990. Characterization of unresolved complex mixtures of

389 hydrocarbons in petroleum. *Nature* 344, 648-650.

390 Hao, R., Lu, A., 2009. Biodegradation of heavy oils by halophilic bacterium. *Progress in Natural*
391 *Science* 19. 997–1001.

392 Hosokawa, R., Sakaguchi, H., Okuyama, H., 2010. Establishment and characterization of turbine
393 oil-degrading bacterial consortia. *International Biodeterioration & Biodegradation* 64, 519–524.

394 Ito, H., Hosokawa, R., Morikawa, M., and Okuyama, H., 2008. A turbine oil-degrading bacterial
395 consortium from soils of oil fields and its characteristics. *International Biodeterioration and*
396 *Biodegradation* 61, 223–232.

397 Koma D., Sakashita Y., Kubota K., Fujii Y., Hasumi F., Chung S.Y., and Kubo M., 2003. Degradation
398 of car engine base oil by *Rhodococcus* sp. NDKK48 and *Gordonia* sp. NDKY76A. *Bioscience,*
399 *Biotechnology, and Biochemistry* 67, 1590–1593.

400 Lu, S., Wang, H., Yao, Z., 2006. Isolation and characterization of gasoline-degrading bacteria from
401 gas station leaking-contaminated soils. *Journal of Environmental Sciences* 18, 969–972.

402 Maki, H., Maekawa, K., Okamoto, Y., 2008. A field experiment of Sakhalin Vityaz crude oil
403 degradation at a shore of Saroma Lake, Hokkaido (in Japanese with English summary). *Journal*
404 *of Water and Waste* 50, 1005–1011.

405 Perry, J.J., 1984. Microbial metabolism of cyclic alkanes. In: Atlas, R.M. (Ed.), *Petroleum*
406 *Microbiology*. MacMillan Publishing Company, New York, pp. 61–98.

407 Rahman, P. K. S. M., Rahman, T. J., Lakshmanaperumalsamy, P., Banat, I. M., 2002. Occurrence of
408 crude oil degrading bacteria in gasoline and diesel station soils. *Journal of Basic Microbiology* 42,
409 284–291.

410 Razak, C.N.A., Wang, W.F., Rahman, S.H.S.A., Basri, M., Salleh, A.B., 1999. Isolation of the crude
411 oil degrading marine *Acinetobacter* sp. E11. *Acta Biotechnologica* 19, 213–223.

412 Sathishkumar, M., Binupriya, A.R., Baik, S.-H., Yun, S.-E., 2008. Biodegradation of crude oil by
413 individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon
414 contaminated areas. *Clean* 36, 92 – 96.

415 Ueno, A., Hasanuzzaman, M., Yumoto, I., Okuyama, H., 2006a. Verification of degradation of diesel
416 oil by *Pseudomonas aeruginosa* strain WatG in soil microcosms. *Current Microbiology* 52,
417 182–185.

418 Ueno, A., Ito, Y., Yamamoto, Y., Yumoto, I., Okuyama, H., 2006b. Bacterial community change
419 analysis of diesel oil-contaminated soil microcosms biostimulated with Luria-Bertani medium
420 and bioaugmented with a petroleum-degrading bacterium, *Pseudomonas aeruginosa* strain WatG.
421 *Journal of Basic Microbiology* 46, 310–317.

422 Wonga, P., Tanaka, M. , Ueno, A. , Hasanuzzaman, M. , Yumoto, I., Okuyama, H., 2004. Isolation
423 and characterization of novel strains of *Pseudomonas aeruginosa* and *Serratia marcescens*
424 possessing high efficiency to degrade gasoline, kerosene, diesel oil, and lubricating oil. *Current*
425 *Microbiology* 49, 415–422.

426 Zvyagintseva I.S., Surovtseva E.G., Poglazova M.N., Ivoliiov V.S., and Belyaev S.S., 2001.
427 Degradation of machine oil by nocardioform bacteria. *Microbiology* 70, 270-276.

428
429
430

431 **Figure legends**

432

433 **Figure 1.** Degradation of turbine oil (TuO) in TuO-containing mineral salts medium by the
434 revived Tank-2 consortium and a newly prepared TuO-degrading consortium, Moiwa-KK. (a)
435 Time-dependent increases in TuO degrading activity of revived Tank-2 and Moiwa-KK. (b)
436 TuO degradation by the original and revived Tank-2 consortia and Moiwa-KK. Culture was
437 carried out for two weeks at 30°C with shaking at 160 rpm. TuO was extracted and analysed
438 as described in the text.

439

440 **Figure 2.** Degradation of different types of petroleum hydrocarbons by original and revived
441 Tank-2. (a) Original and (b) revived-Tank 2 consortia were cultured in MSM containing 0.5%
442 (w/v) of different petroleum products. The results for original Tank-2 were obtained using the
443 consortium maintained by continuous culture in MSM containing 0.5% TuO (see Hosokawa
444 et al. 2010). Culture was for two weeks at 30°C, after which any remaining petroleum
445 products were extracted and analysed as described in the text.

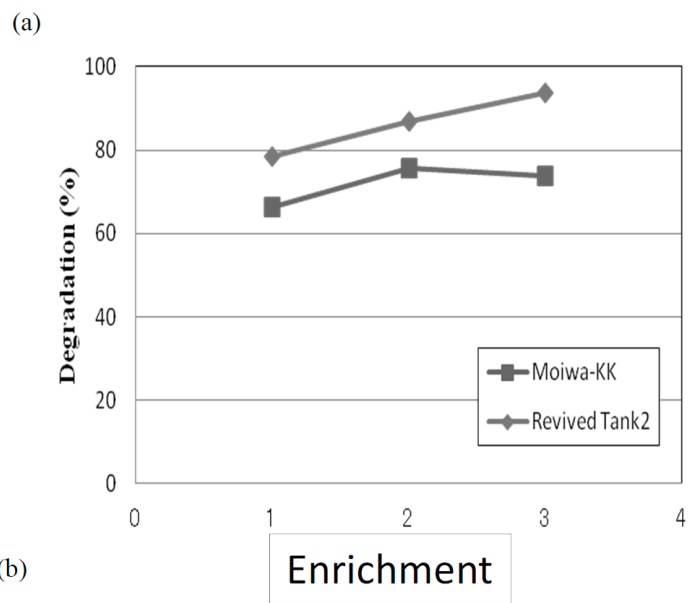
446

447 **Figure 3.** PCR-denaturing gradient gel electrophoresis (DGGE) analysis targeting the 16S
448 rRNA gene sequences in different culture types. (a) DNA for templates was extracted from
449 freeze-thawed Tank-2 (Freeze-thawed) and revived (Revived) Tank-2. Capital letters indicate
450 DGGE bands for freeze-thawed and revived Tank-2 cultures. Bands D, N, P and S were
451 commonly detected for freeze-thawed and revived Tank-2. Lane for Original indicates the
452 DGGE profile for the original Tank-2 culture by Hosokawa et al. (2010). (b) Template DNA
453 was extracted from revived Tank-2-inoculated cultures in MSM that contained either TuO
454 (TuO), unused car engine oil (Engine oil), Arabian light crude oil (Arabian light) or Vityaz
455 crude oil (Vityaz). Capital letters indicate DGGE bands corresponding to the DGGE bands

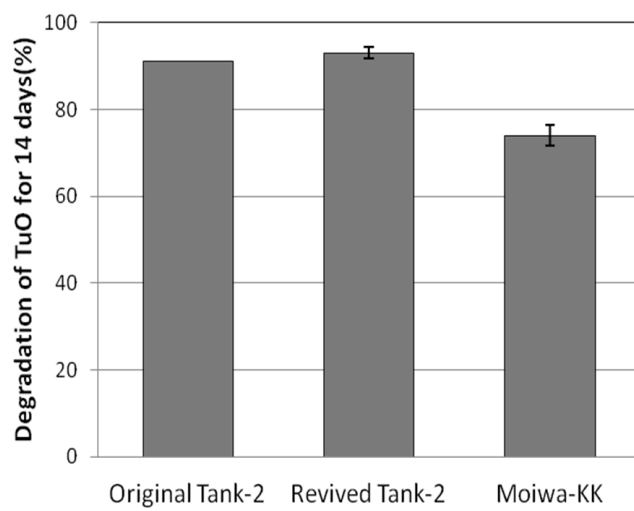
456 for freeze-thawed and revived Tank-2 cultures shown in Fig. 3a. Bands indicated by arrows
457 at the same distance from the top of gel have the same denotation. The band indicated by *
458 was detected only in the Arabian light crude oil culture. (c) Revived Tank-2 grown in MSM
459 containing 0.5% TuO was inoculated in MSM that contained either 1.5% unused TuO
460 (culture 2) or 1.5% TuO-containing wastewater (culture 3). For culture 4, no revived Tank-2
461 was inoculated into 1.5% TuO-containing wastewater. Culture 5 was a newly formulated
462 TuO-degrading Moiwa-KK consortium that was cultured in MSM containing 0.5% TuO.
463 Culture was for one week at 30°C with shaking at 160 rpm. Lane shown is the same as the
464 lane for Revived in Fig. 3a. Capital letters for lanes 1, 2 and 3 indicate DGGE bands for
465 freeze-thawed and revived Tank-2 cultures. Small letters for lanes for cultures 4 and 5
466 indicate DGGE bands arising from the TuO-containing waste water collected for this study.
467
468

1 Fig. 1

2



(b)



3

4

5

6

7

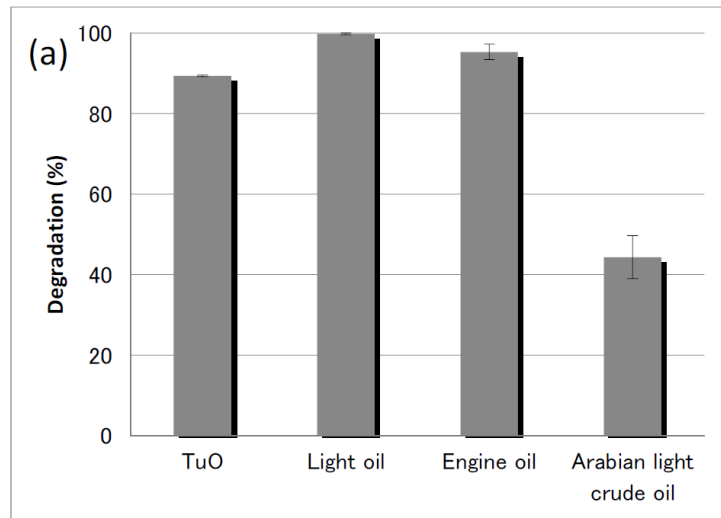
8

9

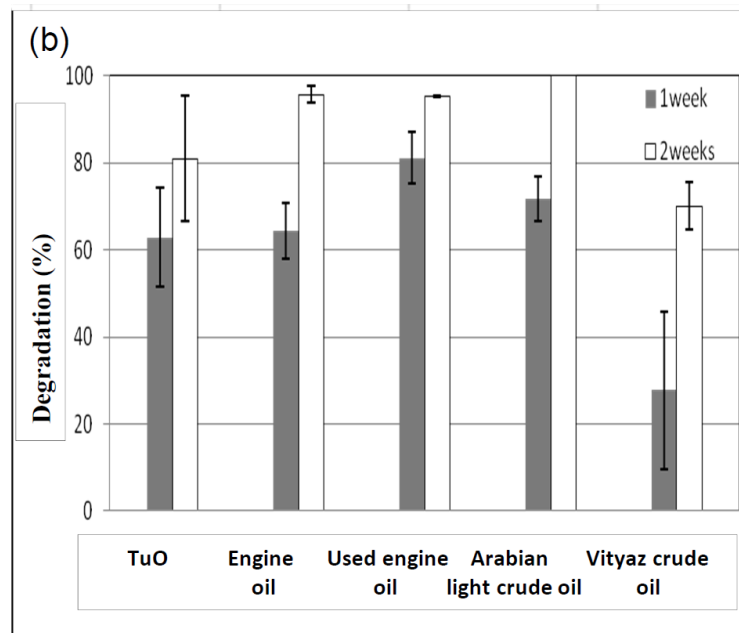
10

1

2 Fig. 2.



3



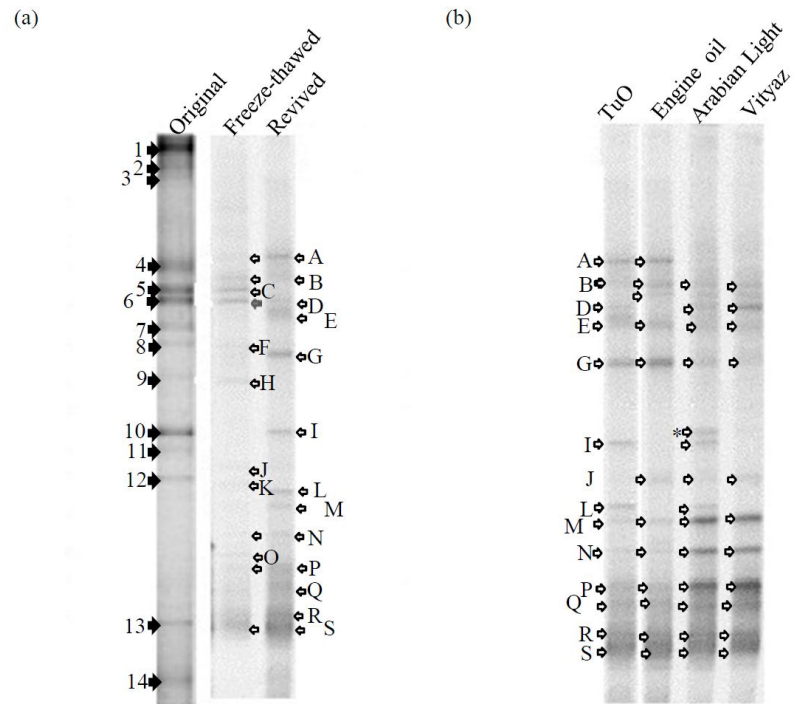
4

5

6

7

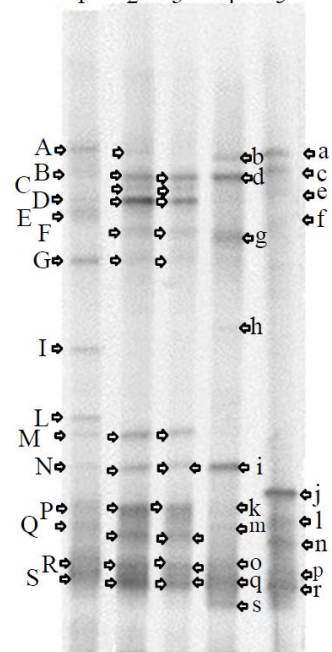
1 Fig. 3



2

(c)

TuO (%)	0.5	1.5	1.5	1.5	0.5
Wastewater	-	-	+	+	-
Revived Tank-2	+	+	+	-	-
Moiwa-KK	-	-	-	-	+
Culture	1	2	3	4	5



3

4

1 Table 1. Degradation of TuO in wastewater by revived Tank-2

Culture [*]	Media component ^{**}		Unused TuO	Degradation of TuO
	MSM and wastewater (WW)	Revived Tank-2		
Culture 2	10 ml MSM (×1) plus no WW	200 µl	1.5% added	36.7% ± 3.0%
Culture 3	5 ml MSM (×2) plus 5 ml WW	200 µl	Not added	70.7% ± 2.0%
Culture 4	5 ml MSM (×2) plus 5 ml WW	Not added	Not added	63.7% ± 3.2%
Culture 5	10 ml MSM (×1) plus no WW	Not added ^{***}	Not added	53.5% ± 6.9%

2 ^{*}, All cultures contained 1.5% (w/w) TuO.3 ^{**}, MSM (×1), mineral salts medium (MSM) at its normal concentration; MSM (×2), MSM at
4 two-times concentration; WW, original wastewater containing TuO (3%).5 ^{***}, 200 µl of Moiwa-KK was added instead of Revived Tank-2.

6

7

8

9