1 **Influence of the electron acceptor on nitrite reductase gene (*nir*) diversity in**
2 **an activated sludge community**

3
4 **Running title: Influence of the electron acceptor on *nir* diversity**

5
6 Tadashi Nittami ^{1*}, Takayuki Magura ¹, Yuko Imai ¹ and Kanji Matsumoto ¹

7 ¹ Department of Chemical Engineering, Graduate School of Engineering,
8 Yokohama National University, Yokohama, Japan

9
10 ***Corresponding Author**

11 Tadashi Nittami

12 phone: +81 (45) 3394006; fax: +81 (45) 3394006; E-mail: nittami@ynu.ac.jp

13 Postal address: Department of Chemical Engineering, Graduate School of

14 Engineering, Yokohama National University, 79-5 Tokiwadai, Hodogaya-ku,

15 Yokohama 240-8501, Japan

16 **Abstract**

17 Analyses of the nitrite reductase gene diversities (*nirK* and *nirS*) in an activated
18 sludge community fed with both nitrite and glucose were conducted. Results
19 suggest that the topology of *nirK* and *nirS* gene fragment-based phylogenetic
20 trees is influenced more by the available electron acceptor than by the carbon
21 source. A denitrification reactor was operated for 53 days and a clone library
22 constructed when the denitrifying communities in the SBR were supplied with
23 both nitrite and glucose. Half of the *nirK* and nearly all the *nirS* gene fragments
24 formed a cluster that was separate from a cluster containing *nirK* and *nirS*
25 sequences derived from other communities in nitrate-fed reactors. On the other
26 hand, *nirK* and *nirS* fragments obtained with glucose as the carbon source were
27 similar to those detected in communities fed other carbon sources.

28

29 **Key words:** activated sludge, denitrification, electron acceptor, nitrite reductase
30 genes (*nirK*, *nirS*), sequencing batch reactor (SBR), wastewater treatment

31

32 **Introduction**

33 Ammonia nitrogen in wastewater is commonly removed by sequential
34 nitrification and denitrification processes (1). Nitrification is an aerobic
35 biological process in which ammonia (NH_3) is oxidized to nitrite (NO_2^-) or
36 nitrate (NO_3^-) (2). Denitrification is an anoxic process in which NO_3^- and NO_2^-
37 are reduced to N_2 gas via nitric oxide (NO) and nitrous oxide (N_2O) in a process
38 requiring an organic carbon source (3). Inhibition of nitrite oxidation and the
39 development of a nitrogen removal process via NO_2^- reduction
40 ($\text{NH}_3 \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$) are attractive (4-14). Theoretically,
41 the oxygen requirements during the aerobic phase would be 25% lower than
42 those of conventional nitrogen removal processes via NO_3^- reduction and the
43 carbon requirements for denitrification would be 40% lower (4,5,7,14).

44 Community analysis of ammonia oxidizing bacteria (AOB) and nitrite
45 oxidizing bacteria (NOB) biodiversity in this process has also been reported.

46 Logemann *et al.* investigated the dominant AOB in a single reactor system for
47 high activity ammonia removal over nitrite (SHARON), using 16S rRNA genes
48 as phylogenetic markers (15). Egli *et al.* also analyzed AOB and NOB
49 communities in nitrification reactors by monitoring 16S rRNA and ammonium
50 monooxygenase (*amoA*) gene diversity (16). However, the denitrifying bacterial
51 populations in the process have not, as far as the authors know, been analyzed
52 because these are thought to be unimportant in the inhibition of nitrite oxidation.
53 However, the available electron acceptor (whether it is NO_3^- or NO_2^-) may
54 change the denitrifying bacterial population, and thereby affect the
55 denitrification performance of the process. Therefore, analysis of the dominant
56 denitrifying bacteria in nitrogen removal via the NO_2^- process is important in
57 any attempt to optimize conditions.

58 Because of the taxonomic diversity of denitrifying bacteria, 16S
59 rRNA-based approaches are of limited value (17). Instead, several studies have
60 targeted nitrite reductase (Nir) genes (*nirK* and *nirS*) (18, 19). The presence of

61 the *nirK* and *nirS* genes appears to be mutually exclusive in each denitrifying
62 organism (20). The objective of this study was to investigate the community
63 composition of denitrifying bacteria in activated sludge fed with NO_2^- based on
64 the population of *nirK* and *nirS* genes. The *nirK* and *nirS* populations were
65 compared to those in previous studies. Glucose was also used as a carbon source
66 because little research has been conducted to analyze the composition of
67 denitrifying communities fed carbon sources other than acetate (19), methanol
68 (18, 19) or ethanol (18).

69

70 **Materials and Methods**

71 **Denitrifying SBR**

72 A laboratory-scale reactor (Fig. 1) was operated as a sequencing batch reactor
73 (SBR) with a working volume of 6 liters. The inoculum was obtained from an
74 anaerobic-anoxic-aerobic (A_2O) biological nutrient removal plant (Yokohama,
75 Japan) that treats municipal wastewater. Influent contained 18 mg-N l^{-1} of

76 ammonium (NH_4^+) and 0.5 mg-N l^{-1} of NO_3^- . Treated effluent contained 0.7
77 mg-N l^{-1} of NH_4^+ and 6.2 mg-N l^{-1} of NO_3^- . No NO_2^- was detected in either the
78 influent or effluent. Sludge retention time (SRT) and hydraulic retention time
79 (HRT) were controlled at 9.5 d and 7.6 h respectively. The SBR was operated
80 under anoxic conditions for 53 d in a water bath at 20°C on a 6 h cycle
81 consisting of feed (20 min), anoxic reaction (4.5 h), settling (30 min), and
82 discharge of supernatant (40 min). To maintain anoxic conditions, nitrogen gas
83 was supplied for 10 min at the beginning of the anoxic reaction period. Aeration
84 was performed for 5 min at the end of the anoxic reaction period to prevent
85 activated sludge from floating during the settlement period. SRT and HRT were
86 controlled at 6 d and 12 h, respectively. Levels were maintained by discarding
87 250 ml of mixed liquor in the anoxic reaction period each cycle before aeration,
88 and 3 l of supernatant was removed at the discharge period of each cycle. The
89 mixed liquor was not discarded from days 6 to 14 in order to allow build-up of
90 the mixed-liquor suspended solids (MLSS) levels. The pH in the reactor was

91 maintained below 7.2 using H_2SO_4 .

92

93 **Synthetic wastewater (SWW)**

94 The synthetic wastewater (SWW) contained the following ingredients (g l^{-1}):

95 $\text{C}_6\text{H}_{12}\text{O}_6$, 22.5; NaNO_2 , 44.3; NH_4Cl , 0.86; KH_2PO_4 , 3.74; K_2HPO_4 , 0.28;

96 Na_2EDTA , 10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.14; and a trace element solution (40 ml)

97 prepared as previously reported (21). During the first 21 days, 30.9 g of NaNO_3

98 (instead of NaNO_2) was added into the SWW to keep the electron acceptor the

99 same as those in present in the inoculum. The concentrated SWW was diluted

100 with milli-Q water and sterilized by autoclaving. At the beginning of the feed

101 period, 60 ml of SWW was diluted 1:50 with tap water.

102

103 **Reactor analysis**

104 Activated sludge samples were collected from the SBR 7-8 times over the cycle.

105 Both NO_3^- and NO_2^- were measured using a LC-10A ion chromatograph with a

106 Shim-pack IC-A3 column (Shimadzu, Kyoto, Japan). NO_2^- denitrification rates
107 were determined by changes from 10 min to 100 min in relevant parameters
108 during one cycle after day 26. The MLSS was determined according to standard
109 methods (22).

110

111 **Activated sludge sampling and DNA extraction**

112 Activated sludge samples were taken from the SBR on days 26, 32, 35, 47, and
113 53. The collected samples were washed with Tris-EDTA buffer (10 mM
114 Tris-HCl, 1mM EDTA, pH 8) and then stored at -20°C . DNA was extracted
115 using the FastDNA SPIN Kit for Soil (Qbiogene, Irvine, CA, USA), following
116 the manufacturer's instructions, and stored at -20°C .

117

118 **PCR amplification**

119 Partial *nirK* and *nirS* gene fragments were amplified using the primer sets
120 nirK1F-nirK5R and nirS1F-nirS6R, respectively, as described by Braker *et al.*

121 (20). Touchdown PCR was carried out using the TaKaRa PCR Thermal Cycler
122 Dice Gradient (Takara Bio, Shiga, Japan) using conditions described elsewhere
123 (23), with the exception that the forward and reverse primer concentrations were
124 reduced to 0.5 $\mu\text{mol l}^{-1}$, and the concentration of MgCl_2 was reduced to 1.5
125 mmol l^{-1} . The amplified products were examined on 2% agarose gels by
126 electrophoresis.

127

128 **RFLP**

129 PCR products (5 μl) of *nirK* and *nirS* fragments were digested with 5 U of
130 *Hae*III and *Msp*I restriction endonucleases (Takara Bio), respectively, for 2 h at
131 37°C. Restriction endonucleases were deactivated by heating the reaction
132 mixture at 65°C for 10 min following digestion. Digested samples were
133 separated using electrophoresis on 8% (wt vol^{-1}) polyacrylamide gels (Bio-Rad
134 Laboratories Japan, Tokyo, Japan) for 130 min at 200 V. The gels were stained
135 with SYBR Green I (Invitrogen Japan K.K., Tokyo, Japan) for 15 min and then

136 examined on a UV transilluminator.

137

138 **Cloning and sequencing of *nirK* and *nirS***

139 Both *nirK* and *nirS* PCR products were purified using the Wizard DNA

140 Clean-Up System (Promega, Madison, USA) and then ligated with the Qiagen

141 PCR Cloning Plus Kit (QIAGEN K.K., Tokyo, Japan) following the

142 manufacturer's instructions. Subsequent transformation and determination of the

143 the *nirK* and *nirS* sequences was conducted by Takara Bio

144 (<http://www.takara-bio.com/research.htm>).

145

146 **Phylogenetic analysis**

147 The *nirK* and *nirS* nucleotide sequences were translated to amino acid sequences

148 using the Molecular Evolutionary Genetics Analysis (MEGA v. 3.1) software

149 (24) and then aligned with other *nirK* and *nirS* sequences from the DNA Data

150 Bank of Japan (DDBJ) using CLUSTAL W. Phylogenetic trees were constructed

151 using the neighbor-joining method using the Tree View program (v. 1.6.6), and
152 bootstrap values calculated from 1,000 trees.

153

154 **Nucleotide sequence accession numbers**

155 The *nirK* and *nirS* sequences data reported in this paper have been deposited in
156 the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession
157 numbers AB440209 – AB440224.

158

159 **Real-time PCR assay**

160 Quantitative PCR was performed on the Thermal Cycler Dice Real Time System
161 (Takara Bio). Amplification reactions were carried out with the SYBR Premix
162 Ex Taq (Takara Bio) in a total volume of 20 µl. Each reaction mixture contained
163 1 µl (50 ng) of template DNA, 10 µl of SYBR Premix Ex Taq, and 0.05 µl (5
164 pmol) of each primer. The real-time PCR protocols for *nirK* and *nirS* primer sets
165 were as follows: 95°C for 10 s, and 50 cycles at 95°C for 8 s, 60°C for 40 s; and

166 95°C for 15 s, 60°C for 30 s, 95°C for 15 s; respectively. All measurements
167 were carried out in triplicate.

168 To quantify the copy numbers of the target DNA, standard curves were
169 obtained by plotting the threshold cycle values as a function of the log of the
170 copy number of the target DNA. Ten-fold serial dilutions of purified PCR
171 products that were prepared for cloning were used as standards. DNA
172 concentrations in the purified PCR products were measured by spectroscopy
173 using a U-2000A spectrophotometer (Hitachi, Tokyo, Japan). The copy numbers
174 of the PCR products were then calculated using the measured DNA
175 concentration as previously reported (25).

176

177 **Results**

178 **Reactor performance**

179 The denitrification performance of the SBR was assessed by measuring NO_3^-
180 and NO_2^- levels on days 1, 6, 15, 21, 26, 32, 35, and 47. Fig. 2 shows the NO_3^-

181 and NO_2^- levels at timepoints of 10 min and 260 min into the anoxic reaction
182 period in a single cycle on each sampled day. The SBR community showed NO_3^-
183 and NO_2^- reduction on all days except days 6, 26, and 32. Fig. 2 shows MLSS
184 levels in samples taken on the same days. MLSS was kept at approximately 1 g
185 l^{-1} after day 6 except for day 32 when the SBR community did not show NO_2^-
186 reduction. NO_2^- denitrification rates were assessed after changing the electron
187 acceptor to NO_2^- as shown in Fig. 3 by measuring NO_2^- levels after 10 min and
188 100 min when NO_2^- levels decreased linearly. On days 26 and 32, denitrification
189 performance was poor (approximately $0 \text{ mg-N g-MLSS}^{-1} \text{ h}^{-1}$), although rates
190 increased to $5.64 \text{ mg-N g-MLSS}^{-1} \text{ h}^{-1}$ and $4.55 \text{ mg-N g-MLSS}^{-1} \text{ h}^{-1}$ by days 35
191 and 47, respectively.

192

193 **Quantification of *nirK* and *nirS* genes**

194 Changes in population levels of denitrifying bacteria in the SBR were assessed
195 by quantifying *nirK* and *nirS* gene copies. Real-time PCR assays were

196 conducted with samples taken on days 26, 32, 35, 47, and 53. On day 26
197 (immediately before changing the electron acceptor to NO_2^-), copy numbers of
198 both *nirK* and *nirS* were very low (*nirK*: $4.18 \times 10^2 \pm 1.60 \times 10^1$ copies per
199 ng-DNA, *nirS*: $6.28 \times 10^4 \pm 2.90 \times 10^3$ copies per ng-DNA). These numbers then
200 increased and reached $10^4 - 10^5$ copies per ng-DNA and $10^5 - 10^6$ copies per
201 ng-DNA, respectively, after day 32 (Fig. 3).

202

203 **RFLP analysis**

204 To assess if the denitrifying bacterial community composition had changed,
205 RFLP analyses of PCR *nirK* and *nirS* amplicons were conducted on days 26, 35,
206 47, and 53 (Fig. 4). Band patterns for *nirK* on days 26 and 35 appeared to be
207 different from those obtained from samples taken on days 47 and 53 due to the
208 presence of an extra band of approximately 300 bp. For *nirS*, only the bands in
209 the day 26 sample appeared different from those samples taken on the other
210 three days. On day 26, a band >500 bp appeared while a band around 200 bp

211 disappeared. These RFLP results implied that the composition of the
212 denitrifying community had changed between days 26 and 47 of operation.
213
214 **Cloning analysis**
215 PCR products of *nirK* and *nirS* in samples collected on day 47 produced 35 and
216 45 clones respectively. Partial DNA sequences of all 80 clones were determined
217 and then translated into amino acid sequences. Their distribution is shown in Fig.
218 5. With the *nirK* library, three sequences (MGR-47KH11, MGR-47KB12, and
219 MGR-47KF9) were shared by about 77% of the total clones and the remainder
220 contained eight sequences. The *nirK* sequences of MGR-47KH11,
221 MGR-47KB12, and MGR-47KF9 were closely related to those of *Rhizobium* sp.
222 R-24658 (accession no. AM230834), *Alcaligenes* sp. 'TJOPY2 (A-I)' (accession
223 no. EF202173), and *Alc.* sp. 'CJANPY1 (A-II)' (accession no. EF202175) with
224 the following similarities of identity: 123/133, 126/133 and 118/133,
225 respectively.

226 With the *nirS* library, three sequences (MGR-47SA8, MGR-47SA7, and
227 MGR-47SH10) were shared by about 96% of the library, and the rest was
228 comprised of two sequences. Moreover, MGR-47SA8 alone occurred in 87% of
229 the sequenced clones. Sequences of MGR-47SA8, MGR-47SA7, and
230 MGR-47SH10 were closely related to *nirS* of *Pseudomonas* sp. R-25061
231 (accession no. AM230904), *Dechloromonas* sp. R-28400 (accession no.
232 AM230913), and *Ps.* sp. R-25061 (accession no. AM230904) with the following
233 shared similarities: 162/171, 156/175 and 161/171, respectively.

234

235 **Phylogenetic analysis**

236 A phylogenetic analysis was performed on the amino acid sequences derived
237 from the *nirK* and *nirS* sequences (Fig. 6). The trees shown in Fig. 6 were
238 composed mainly of the *nirK* and *nirS* sequences reported previously by Heylen
239 *et al.* (26), and their naming system has been adopted here (26). This tree also
240 includes *nirK* and *nirS* sequences detected in other studies from denitrifying

241 reactors fed with acetate and methanol. The sequences “K-A# and S-A#” and
242 “K-M# and S-M#” were reported in NO_3^- denitrifying reactors fed acetate and
243 methanol, respectively (19). In addition, the sequences “IMR37K# and
244 IMR37S#” and “IMR64K# and IMR64S#” were detected mainly in communities
245 from methanol-fed reactors with NO_3^- and NO_2^- as electron acceptors,
246 respectively (27). The methanol-fed reactor of Nittami *et al.* (27) was operated
247 under the same conditions as the SBR used in this study with the exception of
248 the feed substrates.

249 The tree topology of *nirK* products showed three distinct clusters (Fig. 6). The
250 largest cluster (cluster I clones) contained 20 sequences (MGR-47KH11 and six
251 other sequences) out of the 35 sequences obtained in this study, while cluster II
252 contained 15 (MGR-47KB12, MGR-47KF9 and two other sequences). The tree
253 topology of *nirS* products showed four clusters. Cluster Ia contained 3
254 sequences (i.e., MGR-47SA7 and one additional sequence) out of the 45
255 sequences obtained in this study, while cluster Ib contained 42 sequences (i.e.,

256 MGR-47SA8, MGR-47SH10 and one additional sequence).

257

258 **Discussion**

259 This study aimed to investigate the community composition of denitrifying

260 bacteria in activated sludge fed with NO_2^- . Therefore, a clone library was

261 constructed from a sample believed to represent the denitrifying communities in

262 the SBR fed with NO_2^- . To determine if NO_2^- was utilized, the water indices

263 including NO_2^- levels were monitored (Fig. 2). As mentioned above, NO_3^- was

264 dosed as the electron acceptor until day 21 to ensure the electron acceptor was

265 the same as that used in the inoculum. However, Fig. 2 shows that denitrification

266 was barely detectable and that the MLSS had decreased by 21% during the first 6

267 days. Although NO_3^- was utilized later, NO_2^- accumulated on day 15 and 21. In

268 particular, on day 15, 43% of the utilized NO_3^- -N had accumulated as NO_2^- -N.

269 Therefore, not only denitrifying bacteria but also many nitrate-respiring bacteria

270 appeared to use NO_3^- on day 15. The electron acceptor was changed to NO_2^- on

day 21, and denitrification was not detected until day 32. However, rates had recovered by day 35 and 47. This recovery may be explained by a change in the denitrifying bacterial community as the community *nirK* and *nirS* gene fragments were clearly different on day 47 and 35, respectively (Fig. 4). As previously reported (27), some denitrifying bacteria may not carry out a high rate of denitrification with NO_2^- . Fig. 3 shows NO_2^- denitrification rates and copy numbers of *nirK* and *nirS* genes after day 26. Although NO_2^- denitrification rates were barely detectable until day 32, the copy numbers of *nirK* and *nirS* genes increased on day 32 (Fig. 3). The increase in NO_2^- denitrification rate did not correspond to changes in the copy numbers of *nirK* and *nirS* genes. Possible reasons for this are (i) compatibility of the PCR primer sets or (ii) changes in the denitrifying bacterial community (27). As shown in Fig. 2, total NO_x^- -N levels at 260 min were reduced to half of those present at 10 min on day 21. Therefore, it is likely that some denitrifying bacteria had already accumulated before day 21. However, the copy numbers of each *nir* gene on day 26 were <5% compared to

286 those on day 47. This low copy number seems to depend on the compatibility of
287 the primer sets (20) as previously reported (19, 26). On the other hand,
288 denitrifying activity was not present on day 32 irrespective of the increase of
289 copy numbers of both *nir* genes, particularly the *nirK* gene (Fig. 3). The authors
290 propose that this discrepancy depends on changes in the denitrifying bacterial
291 community composition. In other words, the increase in the abundance of *nir*
292 genes arose from some denitrifying bacteria having very low denitrification
293 activity with NO_2^- . In support of this hypothesis, Fig. 4 shows that the
294 community composition of *nirK* gene fragments remained unchanged until day
295 35. The sample for clone library generation was collected on day 47 when the
296 denitrifying communities in the SBR were supplied with NO_2^- and glucose. As
297 mentioned above, the phylogenetic trees of *nirK* and *nirS* gene fragments (Figs.
298 6A and 6B) were constructed based on the clone library from the day 47 sample.
299 Throbäck *et al.* (28) reported that *nirK* clone distribution seems to be habitat
300 specific, differing between soil, activated sludge, and marine habitats. In their

301 tree, *nirK* amplicons from activated sludge dominated a single subcluster. Heylen
302 *et al.* also reported two features of *nirK* gene fragment diversity: (i) *nirK*
303 sequences from denitrifiers isolated from activated sludge were markedly
304 different from other publicly available *nirK* sequences, and (ii) identical *nirK*
305 sequences were retrieved from taxonomically distinct strains (26). They also
306 proposed that *nirK* sequence similarities reflect the habitats of the denitrifiers,
307 and hypothesized that horizontal gene transfer (HGT) events involving *nirK*
308 genes have occurred. In the study by Heylen *et al.*, *nirK* sequences from
309 denitrifiers isolated from activated sludge in a municipal wastewater treatment
310 plant were closely related to each other, with an average similarity of 88.2% (26).
311 These sequences were grouped into their cluster I. In this study, 57% (20/35) of
312 *nirK* sequences also grouped into cluster I (Ia) as shown in Fig. 6A. Most of the
313 *nirK* sequences obtained by Osaka *et al.* (K-A# and K-M#) are also in cluster I
314 (Ia), except for that of the K-A4 clone. Furthermore, the *nirK* sequences
315 (IMR37K#) obtained by Nittami *et al.* (27) also fall into cluster I (Ia and Ib).

316 However, 43% (15/35) of *nirK* sequences obtained in this study and some of
317 the *nirK* sequences (IMR64K#) of Nittami *et al.* (27) are found in cluster II. This
318 suggests that the *nirK* clustering pattern is not based solely on the habitat of the
319 denitrifiers. The majority of the *nirK* clones in cluster II have been detected in
320 NO₂⁻-fed denitrifying reactors. Therefore, the clustering patterns seem to reflect
321 the electron acceptor that is available to the denitrifying populations. Incidentally,
322 the tree of *nirK* clones created by Nittami *et al.* (27) was also clearly divided into
323 two clusters based on the added electron acceptor (NO₃⁻ and NO₂⁻). In this case,
324 97% of *nirK* clones obtained from a NO₃⁻-fed sludge sample (IMR37K#) grouped
325 into one cluster, and 93% of *nirK* clones obtained from a NO₂⁻-fed sludge sample
326 (IMR64K#) grouped into the other cluster.

327 All *nirS* sequences of Osaka *et al.* (S-A# and S-M#) and some *nirS*
328 sequences of Nittami *et al.* (IMR37SA7) group into cluster Ia as shown in Fig.
329 6B, and the majority of these clones were recovered from NO₃⁻ denitrifying
330 reactors. However, 93% (42/45) of the *nirS* sequences obtained in this current

study fall into cluster Ib. Moreover, IMR64SA3 and IMR64SA1 sequences from the NO_2^- denitrifying reactor (27) fall into cluster Ib and IVb, respectively. Heylen *et al.* reported that *nirS* phylogeny generally parallels 16S rRNA gene phylogeny at the family or genus level, with the exception of a few strains (26). Therefore, they also commented that *nirS* genes would be more recalcitrant to transfer than *nirK* genes if HGT was occurring. However, as noted above, the results of this study suggest that *nirS* distribution in clusters seems to be determined by which electron acceptor is available. In addition, the authors previously reported (27) that the tree of *nirS* sequences seems to be divided based on which electron acceptor (NO_3^- or NO_2^-) is available. Etchebehere and Tiedje (29) described a denitrifying strain containing two *nirS* genes. They suggested that one of these was acquired by HGT to increase its competitive ability under high nitrate loading conditions. If this occurs with populations using NO_2^- , some denitrifying strains may form a similar separate clade.

As far as can be determined from the data in Fig. 6, the added carbon source

346 had little to no effect on the topology of the *nir* trees. It is difficult to understand
347 the *nirK* and *nirS* phylogenetic diversity of denitrifying bacteria that use glucose
348 as their carbon source. It appears that *nirK* and *nirS* tree topology changes much
349 more in response to changes in the available electron acceptor than to those in
350 the carbon source.

351

352 **Acknowledgements**

353 The authors gratefully acknowledge Prof. R. Seviour for his suggestions and
354 manuscript proofreading. The authors thank the Environmental Planning Bureau
355 of the Yokohama City Government for providing the activated sludge. This
356 research was supported by the Japan Society for the Promotion of Science
357 (JSPS), Grant-in-Aid for Young Scientists (B) 17710059, and Grant-in-Aid for
358 Scientific Research (B) 18310054.

359

360 **References**

- 361 **1. Ip, S.Y., Bridger, J.S., and Mills, N.F.:** Effect of alternating aerobic and
362 anaerobic conditions on the economics of the activated sludge system. *Water*
363 *Sci. Technol.* **19(5-6)**, 911-918 (1987).
- 364 **2. Bock, E., Koops, H.P., and Harns, H.:** Cell biology of nitrifying bacteria, p.
365 17-38. *In* Prosser, J.I. (ed.), *Nitrification*, IRL Press, Oxford (1986).
- 366 **3. Tiedje, J.M.:** Ecology of denitrification and dissimilatory nitrate reduction to
367 ammonium, p.179-244. *In* Zehnder, A.J.B. (ed.), *Biology of anaerobic*
368 *microorganisms*, John Wiley & Sons, New York (1988).
- 369 **4. Turk, O., and Mavinic, D.S.:** Preliminary assessment of a shortcut in
370 nitrogen removal from wastewater. *Can. J. Civ. Eng.* **13**, 600-605 (1986).
- 371 **5. Turk, O., and Mavinic, D.S.:** Benefits of using selective inhibition to remove
372 nitrogen from highly nitrogenous wastes. *Environ. Technol. Lett.* **8**, 419-426
373 (1987).
- 374 **6. Turk, O., and Mavinic, D.S.:** Maintaining nitrite build-up in a system
375 acclimated to free ammonia. *Water Res.* **23**, 1383-1388 (1989).

- 376 **7. Abeling, U., and Seyfried, C.F.:** Anaerobic-aerobic treatment of high
377 strength ammonium wastewater nitrogen removal via nitrite. *Water Sci.*
378 *Technol.* **26**, 1007-1015 (1992).
- 379 **8. Garrido, J.M., van Benthum, W.A.J., van Loosdrecht, M.C.M., and**
380 **Heijnen, J.J.:** Influence of dissolved oxygen concentration on nitrite
381 accumulation in a biofilm airlift suspension reactor. *Biotechnol. Bioeng.* **53**,
382 168-178 (1997).
- 383 **9. Bae, W., Baek, S., Chung, J., and Lee, Y.:** Optimal operational factors for
384 nitrite accumulation in batch reactors. *Biodegradation.* **12**, 359-366 (2002).
- 385 **10. Ruiz, G. Jeison, D., and Chamy, R.:** Nitrification with high nitrite
386 accumulation for the treatment of wastewater with high ammonia
387 concentration. *Water Res.* **37**, 1371-1377 (2003).
- 388 **11. Ruiz, G., Jeison, D., Rubilar, O., Ciudad, G., and Chamy, R.:**
389 Nitrification-denitrification via nitrite accumulation for nitrogen removal
390 from wastewaters. *Bioresour. Technol.* **97**, 330-335 (2006).

- 391 **12. Chung, J., Shim, H., Lee, Y.W., and Bae, W.:** Comparison of influence of
392 free ammonia and dissolved oxygen on nitrate accumulation between
393 suspended and attached cells. *Environ. Technol.* **26**, 21-33 (2005).
- 394 **13. Yang, Q., Peng, Y., Liu, X., Zeng, W., Mino, T., and Satoh, H.:** Nitrogen
395 removal via nitrite from municipal wastewater at low temperatures using
396 real-time control to optimize nitrifying communities. *Environ. Sci. Technol.*
397 **41**, 8159-8164 (2007).
- 398 **14. Blackburne, R., Yuan, Z., and Keller, J.:** Demonstration of nitrogen
399 removal via nitrite in a sequencing batch reactor treating domestic
400 wastewater. *Water Res.* **42**, 2166-2176 (2008).
- 401 **15. Logemann, S., Schantl, J., Bijvank, S., van Loosdrecht, M., Kuenen, J.G.,**
402 **and Jetten, M.:** Molecular microbial diversity in a nitrifying reactor system
403 without sludge retention. *FEMS Microbiol. Ecol.* **27**, 239-249 (1998).
- 404 **16. Egli, K., Langer, C., Siegrist, H.-R., Zehnder, A.J.B., Wagner, M., and**
405 **van der Meer, J.R.:** Community analysis of ammonia and nitrite oxidizers

during start-up of nitrification reactors. *Appl. Environ. Microbiol.* **69**,
 3213–3222 (2003).

**17. Geets, J., de Cooman, M., Wittebolle, L., Heylen, K., Vanparys, B., De
 Vos, P., Verstraete, W., and Boon, N.:** Real-time PCR assay for the
 simultaneous quantification of nitrifying and denitrifying bacteria in
 activated sludge. *Appl. Microbiol. Biotechnol.* **75**, 211–221 (2007).

18. Hallin, S., Throbäck, I.N., Dicksved, J., and Pell, M.: Metabolic profiles
 and genetic diversity of denitrifying communities in activated sludge after
 addition of methanol or ethanol. *Appl. Environ. Microbiol.* **72**, 5445–5452
 (2006).

**19. Osaka, T., Yoshie, S., Tsuneda, S., Hirata, A., Iwami, N., and Inamori,
 Y.:** Identification of acetate- or methanol-assimilating bacteria under
 nitrate-reducing conditions by stable-isotope probing. *Microb. Ecol.* **52**,
 253–266 (2006).

20. Braker, G., Fesefeldt, A., and Witzel, K.-P.: Development of PCR primer

421 systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect
 422 denitrifying bacteria in environmental samples. Appl. Environ. Microbiol.
 423 **64**, 3769–3775 (1998).

424 **21. Pai, S.-L., Chong, N.-M., and Chen, C.-H.:** Potential applications of
 425 aerobic denitrifying bacteria as bioagents in wastewater treatment. Bioresour.
 426 Technol. **68**, 179–185 (1999).

427 **22. Wilder, B.H.:** Solids, p. 2-54-2-60. In Clesceri, L.S., Greenberg, A.E., and
 428 Eaton, A.D. (ed.), Standard Methods for the Examination of Water and
 429 Wastewater, 20th edn. American Public Health Association, Washington DC
 430 (1998).

431 **23. Braker, G., Zhou, J., Wu, L., Devol, A.H., and Tiedje, J.M.:** Nitrite
 432 reductase genes (*nirK* and *nirS*) as functional markers to investigate
 433 diversity of denitrifying bacteria in Pacific northwest marine sediment
 434 communities. Appl. Environ. Microbiol. **66**, 2096–2104 (2000).

435 **24. Kumar, S., Tamura, K., and Nei, M.:** MEGA3: Integrated software for

436 molecular evolutionary genetics analysis and sequence alignment. Brief
 437 Bioinform., **5**, 150-163 (2004).

438 **25. Whelan, J.A., Russell, N.B., and Whelan, M.A.:** A method for the absolute
 439 quantification of cDNA using real-time PCR. J. Immunol. Methods. **278**,
 440 261-269 (2003).

441 **26. Heylen, K., Gevers, D., Vanparys, B., Wittebolle, L., Geets, J., Boon, N.,**
 442 **and de Vos, P.:** The incidence of *nirS* and *nirK* and their genetic
 443 heterogeneity in cultivated denitrifiers. Environ. Microbiol. **8**, 2012–2021
 444 (2006).

445 **27. Nittami, T. Imai, Y., Nakamura, K., and Matsumoto, K.:** Operation of a
 446 methanol denitrification reactor fed with NO_3^- and NO_2^- as electron acceptors
 447 and analysis of denitrifying bacterial genes. Journal of Japan Society on
 448 Water Environment. **31**, 269-274 (2008). (in Japanese)

449 **28. Throbäck, I.N., Enwall, K., Jarvis, A., and Hallin, S.:** Reassessing PCR
 450 primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of

451 denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. **49**, 401–417
452 (2004).

453 **29. Etchebehere, C., and Tiedje, J.M.:** Presence of two different active *nirS*
454 nitrite reductases genes in a denitrifying *Thauera* sp. from a high
455 nitrate-removal-rate reactor. Appl. Environ. Microbiol. **71**, 5642–5645
456 (2005).

457

458 **Figure legends**

459 I

460 Fig. 1. Schematic diagram of the SBR used in the study.

461

462

463 II

464 Fig. 2. Changes in water quality indices in the SBR. Levels of NO_3^- -N (circles),
465 and NO_2^- -N (squares) at 10 min (closed) and 260 min (open) in the anoxic reactor
466 cycles in samples taken on days 1, 6, 15, 21, 26, 32, 35, and 47 are indicated.
467 MLSS levels of biomass samples (grey diamonds) taken on the same days are
468 also indicated. NO_3^- was added as the electron acceptor until day 21 (indicated as
469 “ NO_3^- ”), and then changed to NO_2^- (indicated as “ NO_2^- ”).

470

471

472 III

473 Fig. 3. Community analysis of SBR sludge based on *nirK* (grey bars) and *nirS*
474 (open bars) as determined by real-time PCR. Samples were analyzed in triplicate,
475 and mean values $\pm$ S.D. are shown. Denitrification rates (closed circles) on days
476 26, 32, 35, and 47 are also indicated.

477

478

479 IV

480 Fig. 4. Changes in restriction fragment length polymorphism (RFLP) patterns of
481 the *nirK* and *nirS* PCR products obtained from the denitrifying sludge on days 26,
482 35, 47, and 53. PCR products of *nirK* and *nirS* were digested with *Hae*III and
483 *Msp*I, respectively. A DNA size standard was loaded into the middle lane.

484

485

486 V

487 Fig. 5. Distribution of amino acid sequences represented by *nirK* and *nirS* clones
488 in samples taken on day 47. Each bar shows the percentage occupied by each
489 clone. The closed bars show MGR-47KH11 (*nirK*) and MGR-47SA8 (*nirS*), the
490 dark grey bars show MGR-47KB12 (*nirK*) and MGR-47SA7 (*nirS*), the light grey
491 bars show MGR-47KF9 (*nirK*) and MGR-47SH10 (*nirS*), and the open bars show
492 other clones.

493

494

495 VI

496 Fig. 6. Phylogenetic analysis of *nirK* and *nirS* by the neighbor-joining method.

497 Sequences obtained in this study are shown in black and the numbers are

498 included in parentheses. The scale bar represents a difference of 10 amino acids

499 per 100 residues. Bootstrap values >750 and in the range of 500-750 are indicated

500 at the nodes with closed circles and open circles, respectively. (A) The figure is

501 composed of partial *nirK* sequences based on 133 amino acids. (B) The figure is

502 composed of partial *nirS* sequences based on 171-175 amino acids.

503

5. Figure 1

5. Figure 2

5. Figure 3

5. Figure 4
[Click here to download high resolution image](#)

5. Figure 5

5. Figure 6A
[Click here to download high resolution image](#)

5. Figure 6B
[Click here to download high resolution image](#)