



Title	Growth of nitrite-oxidizing Nitrospira and ammonia-oxidizing Nitrosomonas in marine recirculating trickling biofilter reactors
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1 **Supporting information**

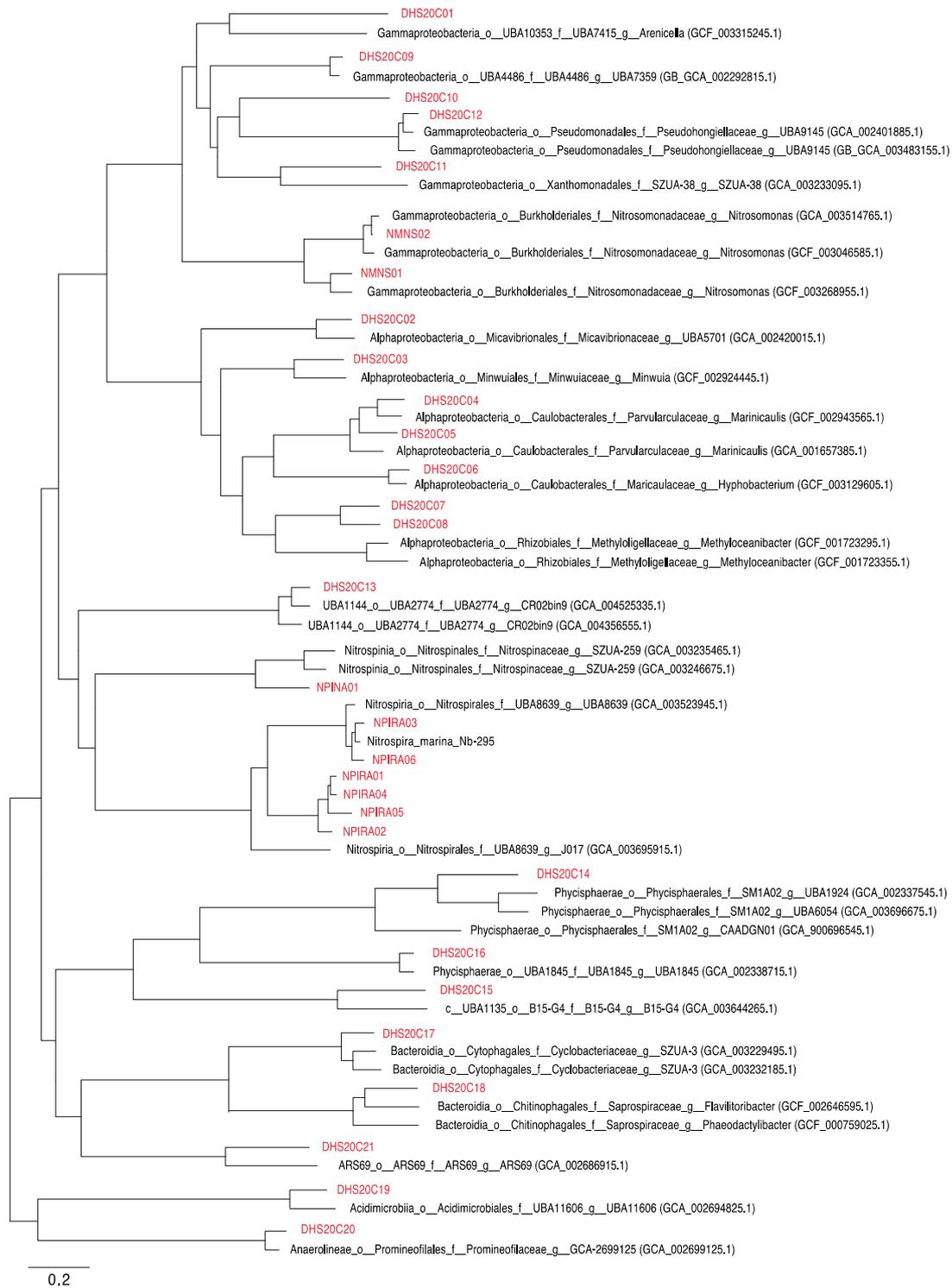
2 **Growth of nitrite-oxidizing *Nitrospira* and ammonia-oxidizing**
3 ***Nitrosomonas* in marine recirculating trickling biofilter reactors**

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5 **Naoki Fujii⁵, Tomonori Kindaichi⁵, Yoshiyuki Suzuki², Takahiro Watari³,**

6 **Masashi Hatamoto³, Takashi Yamaguchi⁶, Nobuo Araki² & Satoshi Okabe¹**

7 This manuscript contains 2, 8, and 2 supplementary figures, table and text.

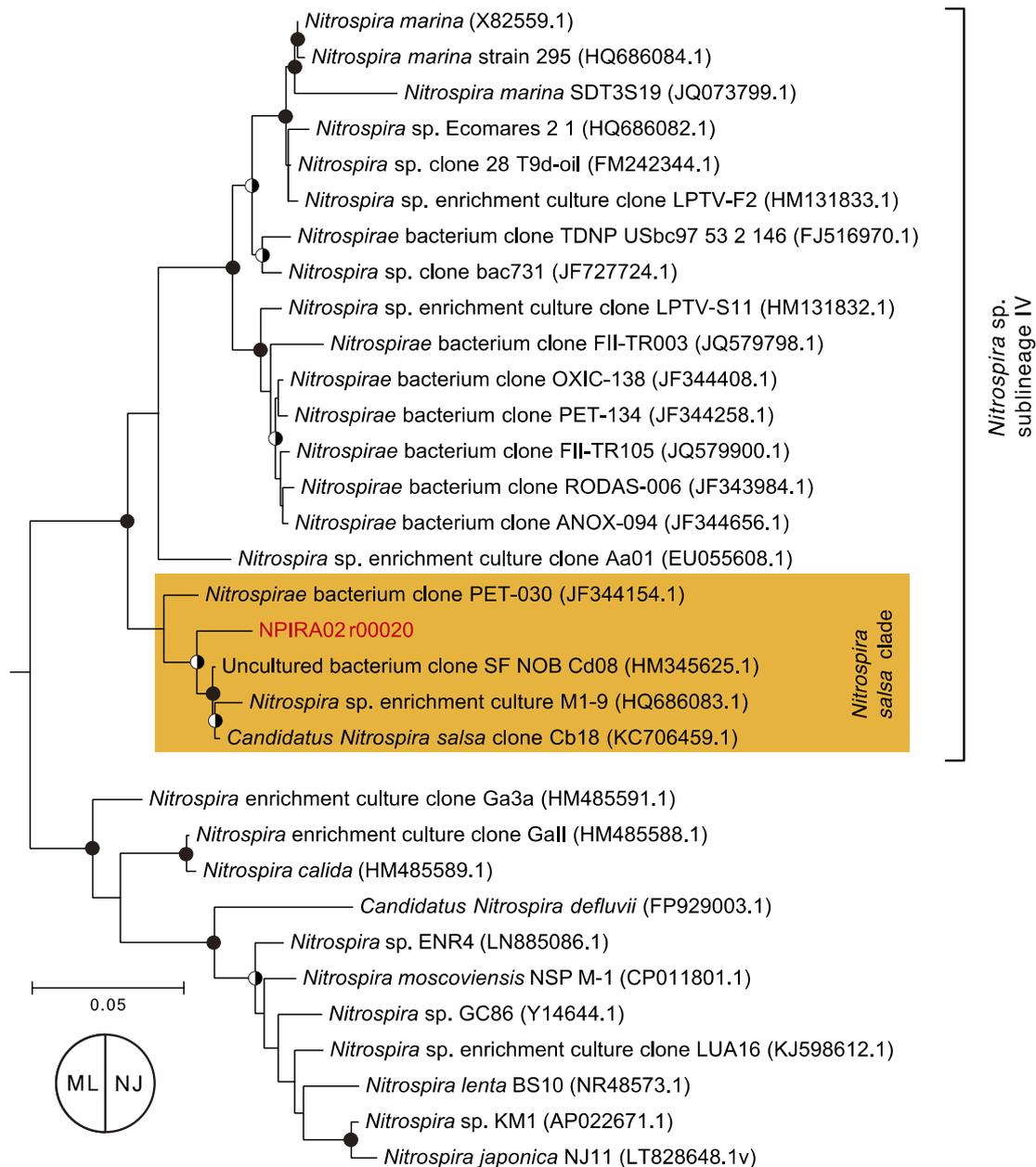


8

9 **Figure S1. Phylogeny of the 30 metagenomic bins obtained in the present study. The**

10 phylogenetic position of the obtained metagenomic bins were shown with red color. The scale

11 bar represents 20% sequence divergence.



12

13 **Figure S2. 16S-rRNA gene based maximum likelihood (ML) tree showing phylogenetic**

14 **position of the NPIRA02 bin.** Branching points that support probability >80% in the

15 bootstrap analysis (based on 500 replicates), estimated using the ML method and the neighbor

16 joining (NJ) method are shown as filled symbol. The 16S rRNA gene sequence located on the

- 17 NPIRA02 bin was affiliated into the clade containing *Nitrospira salsa* 16S rRNA gene
- 18 sequence (KC706459.1). The scale bar represents 5% sequence divergence.

19 **Table S1 Average nucleotide identity (ANI) values of the obtained *Nitrospira* bins.** 1; NPIRA01, 2; *Nitrospirae* bacterium SPGG5 (GCA_001643555.1),
 20 3; *Nitrospirae* bacterium (GCA_003228495.1), 4; MAG-cas150m-170 (PRJNA548657), 5; NPIRA02, 6; *Nitrospiraceae* bacterium (GCA_003523945.1), 7;
 21 NPIRA03, 8; *Nitrospirae* bacterium (GCA_003233615.1), 9; NPIRA06, 10; NPIRA04, 11; NPIRA05, 12; *Nitrospirae* bacterium (GCA_003696975.1),
 22 13; *Nitrospira* sp. bin75 (GCA_002238765.1), 14; *Nitrospirales*_bacterium_isolate_MH-Pat-all_autometa_1-10 (WLXC01000001.1), 15; *Nitrospirae*
 23 bacterium (GCA_003235785.1), 16; *Nitrospira marina* Nb-295 (PRJNA262287), and 17; MAG-cas50m-175 (PRJNA548657).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	100%	69.9%	71.0%	70.6%	78.8%	71.0%	71.6%	70.7%	70.8%	89.3%	85.4%	69.7%	69.9%	70.9%	70.7%	70.7%	70.6%
2		100%	71.7%	77.4%	70.2%	70.1%	70.3%	71.4%	70.2%	70.1%	70.2%	70.1%	72.0%	70.5%	70.4%	70.2%	77.4%
3			100%	70.9%	71.4%	73.4%	73.1%	79.9%	73.1%	71.7%	70.6%	69.9%	70.7%	71.2%	78.2%	73.1%	71.1%
4				100%	70.6%	70.3%	70.3%	70.9%	70.1%	70.6%	70.3%	69.9%	71.5%	71.1%	70.4%	70.7%	99.9%
5					100%	71.4%	71.5%	70.8%	70.9%	78.3%	77.6%	69.8%	70.3%	71.6%	70.9%	71.0%	70.7%
6						100%	83.3%	72.7%	84.2%	72.5%	75.3%	70.0%	70.5%	70.6%	74.0%	84.1%	70.7%
7							100%	72.5%	85.1%	71.8%	74.7%	69.5%	70.1%	70.6%	73.5%	88.2%	70.7%
8								100%	72.6%	71.4%	70.7%	69.9%	70.1%	71.1%	76.8%	72.7%	71.0%
9									100%	71.4%	77.3%	69.7%	70.3%	70.6%	73.7%	86.1%	70.6%
10										100%	84.6%	69.9%	70.4%	72.1%	71.1%	71.6%	71.1%
11											100%	69.5%	70.0%	70.6%	71.1%	74.9%	70.5%
12												100%	70.7%	70.2%	69.9%	69.9%	70.1%
13													100%	70.4%	69.9%	70.4%	71.6%
14														100%	70.6%	70.9%	71.2%
15															100%	73.8%	70.8%
16																100%	71.0%
17																	100%

25 **Table S2 Gene sets involved in central metabolism found in *Nitrospira* (i.e., NPIRA)**

26 **bins.**

27 **(Table S2 is available as the separated excel file.)**

28 **Table S3 The blastP analysis of Hao protein found in the obtained *Nitrospira* bin.** The
 29 blastP analysis was performed using the NPIRA02_01710 protein as the query sequence and
 30 *nr* database. *: canonical nitrite-oxidizing *Nitrospira*, †: complete ammonia oxidation
 31 (commamox) *Nitrospira*.

Organisms	Product	Accession No.	Identity	<i>e</i> -value
<i>Nitrospirales</i> bacterium	cytochrome <i>C</i> ₅₅₂	NKB80548.1	82.6	0
<i>Nitrospiraceae</i> bacterium	TPA: cytochrome <i>C</i> ₅₅₂ partial	HBP89707.1	85.7	0
<i>Nitrospira moscoviensis</i> *	putative octaheme cytochrome <i>c</i>	ALA57709.1	45.7	2.1E-139
<i>Nitrospira japonica</i> *	conserved protein of unknown function	SLM48584.1	42.0	9.0E-137
<i>Nitrospira nitrosa</i> †	Hydroxylamine dehydrogenase Candidatus	CUS31385.1	25.6	1.4E-17
<i>Nitrospira nitrificans</i> †	Hydroxylamine dehydrogenase Candidatus	CUS32689.1	24.9	1.6E-17
<i>Nitrospira inopinata</i> †	Hydroxylamine dehydrogenase Candidatus	CUQ65042.1	23.8	2.0E-17

33 **Table S4 Gene sets involved in central metabolism found in *Nitrosomonas* (i.e., NMNS)**

34 **bins**

35 **(Table S4 is available as the separated excel file.)**

36 **Table S5 Operational conditions of the marine recirculating bioreactors where**

37 ***Nitrospira* sublineage IV population outnumbered AOB/AOA population.**

Reactor	Salinity	Temp.	pH	DO mg/L	NH ₄ ⁺ * μM	NO ₂ ⁻ μM	NO ₃ ⁻ μM	Reference
down-hanging sponge (DHS) reactor	33‰	20°C	7.3	8	7.9	9.3	4,900	This study
Moving-bed biofilter	24–30‰	15–20°C	7	9–10	5–60	10–40	6,000	[1]
Bio ball filter	26.3 psu	<i>n.a.</i>	8.8	6.5	18	<i>n.d.</i>	1,800	[2]
Trickling filter	20‰	19–30°C	7.7	>6	35	15.7	2,000	[3], [4]

38 *n.a.*; not available, *n.d.*; not detected

39 * total ammonia concentration, TAN

40 **References**

- 41 1. Keuter S, Kruse M, Lipski A, Spieck E. Relevance of *Nitrospira* for nitrite oxidation in a
 42 marine recirculation aquaculture system and physiological features of a *Nitrospira*
 43 *marina*-like isolate. *Environ Microbiol* 2011; **13**: 2536–2547.
- 44 2. Brown MN, Briones A, Diana J, Raskin L. Ammonia-oxidizing archaea and nitrite-
 45 oxidizing nitrospiras in the biofilter of a shrimp recirculating aquaculture system. *FEMS*
 46 *Microbiol Ecol* 2013; **83**: 17–25.

- 47 3. Foesel BU, Gieseke A, Schwermer C, Stief P, Koch L, Cytryn E, et al. *Nitrosomonas*
48 Nm143-like ammonia oxidizers and *Nitrospira marina*-like nitrite oxidizers dominate the
49 nitrifier community in a marine aquaculture biofilm. *FEMS Microbiol Ecol* 2008; **63**:
50 192–204.
- 51 4. Gelfand IY, Barak Y, Even-Chen Z, Cytryn E, Rijn J van, Krom MD, et al. A novel zero
52 discharge intensive seawater recirculating system for the culture of marine fish. *J World*
53 *Aquaculture Soc* 2003; **34**: 344–358.

54 **Table S6. Oligonucleotide primers used for quantitative PCR (qPCR) assays.** Annealing
 55 temperature and extension time are 60°C and 20 s, respectively.

Target	Primers	Sequence (5'-3')	Reference
Prokaryotic 16S rRNA gene	515F 806r	GTGCCAGCMGCCGCGGTAA GGACTACHVGGGTWTCTAAT	Caporaso <i>et al.</i> (2011)
AOB <i>amoA</i>	amoA1F amoA2Rv1	GGGGTTTCTACTGGTGGT CCCCTSKGSRAAKCCTTCTTC	Rotthauwe <i>et al.</i> , 1997 This study
<i>Nitrospira</i> <i>nxB</i>	nxB169f nxB638r	TACATGTGGTGGAACA CGGTTCTGGTCRATCA	Pester <i>et al.</i> , 2014
<i>Nitrospira</i> 16S rRNA gene	Nspra675F Nspra746R	GCGGTGAAATGCGTAGAKATCG TCAGCGTCAGRWAYGTTCCAGAG	Graham <i>et al.</i> , 2007

56 References

- 57 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh,
 58 P.J., *et al.* (2011) Global patterns of 16S rRNA diversity at a depth of millions of
 59 sequences per sample. *Proc. Natl. Acad. Sci. U. S. A.* **108**: 4516–4522.
- 60 Graham, D.W., Knapp C.W., Van Vleck, E.S., Bloor, K., Lane, T.B., and Graham, C.E.
 61 (2007) Experimental demonstration of chaotic instability in biological nitrification.
 62 *ISME J* **1**: 385–393.

- 63 Pester, M., Maixner, F., Berry, D., Rattei, T., Koch, H., Lückner, S., *et al.* (2014) NxrB
64 encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic
65 marker for nitrite-oxidizing *Nitrospira*. *Environ Microbiol* **16**: 3055–3071.
- 66 Rothhauwe, J-H., Witzel, K-P., and Liesack, A.W. (1997) The ammonia monooxygenase
67 structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural
68 ammonia-oxidizing populations. *Appl Environ Microbiol* **63**: 4704–4712.

69 **Table S7 Accession numbers of the obtained bins.**

Bin	Accession No.
NPIRA01	BQIU01000001-BQIU01000203
NPIRA02	BQIV01000001-BQIV01000092
NPIRA03	BQIW01000001-BQIW01000218
NPIRA04	BQIX01000001-BQIX01000633
NPIRA05	BQIY01000001-BQIY01000595
NPIRA06	BQIZ01000001-BQIZ01000303
NMNS01	BQJA01000001-BQJA01000199
NMNS02	BQJB01000001-BQJB01000117
NPINA01	BQJC01000001-BQJC01000014
DHS20C01	BQJD01000001-BQJD01000119
DHS20C02	BQJE01000001-BQJE01000012
DHS20C03	BQJF01000001-BQJF01000234
DHS20C04	BQJG01000001-BQJG01000133
DHS20C05	BQJH01000001-BQJH01000386
DHS20C06	BQJI01000001-BQJI01000271
DHS20C07	BQJJ01000001-BQJJ01000027
DHS20C08	BQJK01000001-BQJK01000006
DHS20C09	BQJL01000001-BQJL01000420
DHS20C10	BQJM01000001-BQJM01000065
DHS20C11	BQJN01000001-BQJN01000210
DHS20C12	BQJO01000001-BQJO01000282
DHS20C13	BQJP01000001-BQJP01000173
DHS20C14	BQJQ01000001-BQJQ01000273
DHS20C15	BQJR01000001-BQJR01000011
DHS20C16	BQJS01000001-BQJS01000478
DHS20C17	BQJT01000001-BQJT01000462
DHS20C18	BQJU01000001-BQJU01000161
DHS20C19	BQJV01000001-BQJV01000589
DHS20C20	BQJW01000001-BQJW01001044

71 **Table S8 Calculation of ΔG_r .** The calculations were performed under 1) the standard
72 condition at pH7, 2) the condition found in NH_4^+ -feeding DHS reactor, 3) the initial condition
73 of batch incubation, and 4) the condition where the concentrations of reactant and products
74 are one millimolar at pH 7. Additional explanations are available as **Supplementary text 1.**

75 (Table S8 is available as the separated Excel file.)

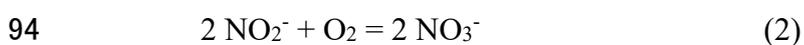
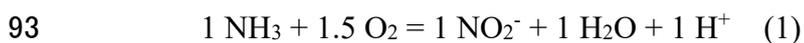
76

77 **Supplementary text 1**

78 **Gibbs free-energy change (ΔG_r) of aerobic ammonia and nitrite oxidation in the**
79 **operated DHS reactors**

80 The DHS reactors fed with NH_4^+ or NO_2^- have been operated at 20°C for > 1 y continuously
81 without the change of operational conditions and disturbance (See the section Experimental
82 procedures for details of operational conditions). Typical concentrations of NH_4^+ , NO_2^- , NO_3^-
83 and dissolved oxygen (DO) and pH values were as following [2]; 7.9 μM of NH_4^+ , 9.3 μM of
84 NO_2^- , 4.9 mM of NO_3^- , 250 μM of DO, and pH 7.3.

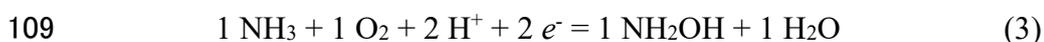
85 The ΔG_r of aerobic ammonia (Eq. 1) and nitrite oxidation reaction (Eq. 2) were
86 calculated by using the standard equation ($\Delta G_r = \Delta G_r^\circ + RT \ln k$), where, ΔG_r° ; Standard free-
87 energy changes, R ; gas constant (0.008314 kJ mol⁻¹ K⁻¹), T ; temperature (K), k ; equilibrium
88 constant calculated from Eq.1 and Eq.2. The ΔG_r° were as following; -26.57 kJ mol⁻¹ for
89 NH_3 , 16.4 kJ mol⁻¹ for dissolved oxygen (*i.e.*, $\text{O}_{2\text{aq}}$), 0 kJ mol⁻¹ for H^+ , -37.2 kJ mol⁻¹ for
90 NO_2^- , -111.34 kJ mol⁻¹ for NO_3^- , -23.4 kJ mol⁻¹ for NH_2OH [3], and -237.17 kJ mol⁻¹ for
91 H_2O . NH_3 concentration was calculated from the NH_4^+ concentration by considering pH
92 equilibrium ($pK_a = 9.25$).



95 The ΔG_r were calculated under 1) the standard condition at pH7, 2) the condition
96 found in NH_4^+ -feeding DHS reactor (described above), 3) the initial condition of the batch
97 incubations with the addition of 0.5 mM NH_4^+ or 0.5 mM NO_2^- and $^{14}\text{CO}_2$, and 4) the
98 condition where the concentrations of reactant and products are one millimolar. As for the
99 condition 3), initial concentration of oxidized products (NO_2^- or NO_3^-) was assumed to be
100 100-folds lower than the initial NH_4^+ or NO_2^- concentration.

101 The ΔG_r of aerobic ammonia oxidation in the operated DHS reactor and at the batch
102 incubation were -271.7 and -283.3 kJ mol- NH_3^{-1} , respectively (**Table S8**). The ΔG_r of
103 aerobic nitrite oxidation in the operated DHS reactor and at the batch incubation were -57.0
104 and -83.5 kJ mol- NO_2^{-1} , respectively. Therefore, aerobic ammonia oxidation yields 4.8- and
105 3.4-folds higher free energies than aerobic nitrite oxidation in the DHS reactor and at the
106 batch incubation, respectively.

107 The oxidation reaction of ammonia to NH_2OH is described as following [1], and the
108 $\Delta G_r'^{\circ}$ of the reaction is -170.5 kJ mol- NH_3^{-1} ;



110 Calculations are available as **Table S8**.

111 **Reference**

- 112 1. Lancaster KM, Caranto JD, Majer SH, Smith MA. Alternative bioenergy: updates to and
113 challenges in nitrification metalloenzymology. *Joule* 2018; **2**: 421–441.

- 114 2. Oshiki M, Aizuka T, Netsu H, Oomori S, Nagano A, Yamaguchi T, et al. Total ammonia
115 nitrogen (TAN) removal performance of a recirculating down-hanging sponge (DHS)
116 reactor operated at 10 to 20°C with activated carbon. *Aquaculture* 2020; **520**: 734963.
- 117 3. van der Star WRL, van de Graaf MJ, Kartal B, Picioreanu C, Jetten MSM, van
118 Loosdrecht MCM. Response of anaerobic ammonium-oxidizing bacteria to
119 hydroxylamine. *Appl Environ Microbiol* 2008; **74**: 4417–4426.

120 **Supplementary text 2**

121 **2-1) Metabolic potentials of the NPIRA bins**

122 Metabolic potentials of the obtained NPIRA bins were examined based on
123 presence/absence of functional genes required. In the following description, the NPIRA05 bin
124 was not included because the completeness of the NPIRA05 bin (59%) was lower than others
125 (81 to 97%) (**Table 1**). Commamox *Nitrospira* oxidizes ammonia to NH₂OH by ammonia
126 monooxygenase (Amo), and the formed NH₂OH is subsequently oxidized to NO by
127 hydroxylamine dehydrogenase (Hao) (Daims *et al.*, 2015; van Kessel *et al.*, 2015). The
128 formed NO is finally oxidized to NO₂⁻ by the as-yet unidentified enzymes and/or abiotic
129 reaction (Carantoa and Lancaster, 2017). Orthologues of commamox *Nitrospira amoCAB*
130 (threshold *e*-value of blastp search 10⁻¹⁵) were not found in the NPIRA bins and also in the
131 other known *Nitrospira* sublineage IV genomes (**Fig. 3**). As for the *hao*, the NPIRA2 and
132 NPIRA3 bins had the orthologues of *Nitrospira* octaheme cytochrome *c* (OCC) instead of
133 commamox *Nitrospira hao*, which showed high similarities with the OCC found in the
134 canonical NO₂⁻-oxidizing *Nitrospira* genomes (*Nitrospira moscoviensis* and *Nitrospira*
135 *japonica* genomes) (**Table S3**). In the *Nitrospira moscoviensis* genome, the OCC consisted of
136 a gene cluster containing a Rieske/cytochrome *b* complex and other genes involved in
137 nitrogen acquisition, whereas such a gene cluster was not located in the NPIRA bins. The

138 *Nitrospira* OCC is currently believed to contribute to assimilatory nitrite reduction and/or the
139 detoxification of hydroxylamine (Koch *et al.*, 2015; Ushiki *et al.*, 2018).

140 As for aerobic nitrite oxidation, orthologue of *nxrABC* was found in the NPIRA4 bin,
141 whereas one or two genes among the *nxrABC* were often missing in other NPIRA bins (the
142 NPIRA01, NPIRA02, NPIRA03, and NPIRA06 bins). This is likely due to the incomplete
143 nature of the NPIRA bins (**Table 1**). As shown in **Table 1**, the obtained NPIRA bins showed
144 high strain heterogeneity, which became a bottleneck at the assembly and binning of contig
145 sequences, and the genes highly conserved among *Nitrospira* genomes (*i.e.*, *nxr*) were often
146 not assigned into specific NPIRA bins. Indeed, the *nxrA* of the NPIRA03 bin, the *nxrB* of the
147 NPIRA01 and NPIRA04 bins, and the *nxrC* of the NPIRA03 and NPIRA04 bins were
148 annotated as the fragmented genes located at the end of the contigs.

149 As a component of respiratory chain, *nuo*-operons (*nuoNMLKJIHGFECDBA* and
150 *nuoNMMLKJIGDCBA*) encoding NADH:ubiquinone oxidoreductase (complex I) were found
151 from the NPIRA bins except for NPIRA06 bin in which the *nuoNMLKJIHGFECDBA* operon
152 was missing. The *NuoNMMLKJIGDCBA* is a 2M-type complex I (Chadwick *et al.*, 2018)
153 and likely involved in the reverse electron flow from quinol to ferredoxin which is required
154 for CO₂ fixation via rTCA cycle (Lawson *et al.*, 2021). Two copies of *qcr*-operon encoding
155 quinol-cytochrome *c* reductase (complex III) were found from the NPIRA01, NPIRA02,
156 NPIRA03, and NPIRA04 bins, and alternative complex III module (ACIII) previously found

157 in the *Nitrospira marina* genome was located in the NPIRA01, NPIRA03, and NPIRA06
158 bins. *Nitrospira* use a novel cytochrome *bd*-like heme-copper oxidase as a terminal oxidase
159 (Lücker *et al.*, 2010; Simon and Klotz, 2013). Multiple copies of the genes encoding putative
160 cytochrome *bd*-like oxidase were found in the NPIRA bins, and the heme *b* and copper-
161 binding sites conserved in canonical cytochrome *bd* oxidase were found in the
162 NPIRA01_31930, NPIRA02_18030, NPIRA03_03640, and NPIRA04_28020 proteins. The
163 recently determined *Nitrospira marina* genome showed that this *Nitrospira* has an additional
164 putative terminal oxidase with high O₂ affinities, *cbb3*-type terminal oxidase (Bayer *et al.*,
165 2021). The gene encoding *cbb3*-type terminal oxidase was found from the NPIRA01,
166 NPIRA03, and NPIRA04 bins. In *Nitrobacter*, soluble and membrane bound monoheme
167 cytochrome *c550* transfer the electrons released from Nxr to terminal oxidase (Nomoto *et al.*,
168 1993). The orthologue of the genes encoding *Nitrobacter winogradskyi* soluble and
169 membrane-bound *c550* (the Nwi_2582 and Nwi_0712 genes) (Starkenburger *et al.*, 2006) was
170 not found in the NPIRA bins. The NPIRA bins had the 57 to 72 genes encoding monoheme
171 cytochrome proteins as well as the *Nitrospira defluvii* genome (Lücker *et al.*, 2010), and those
172 cytochrome proteins supposedly participate the electron transferring from *Nitrospira* Nxr to
173 the above terminal oxidase(s). The H⁺-translocating F₁F₀-ATPase was found in the NPIRA
174 bins, and putative Na⁺-translocating ATPase previously found in the *Nitrospira marina*
175 genome was located in the NPIRA03, and NPIRA06 bins.

176 The NPIRA bins had the genes encoding 2-oxoglutarate:ferredoxin oxidoreductase
177 (OGOR), five- and/or four-subunit pyruvate:ferredoxin oxidoreductase (POR), and ATP-
178 citrate lyase (ACL) that are key enzymes in the rTCA cycle (Lücker *et al.*, 2010; Bayer *et al.*,
179 2021) (**Table S2**). Four-subunit POR is thought to be more oxygen sensitive but with higher
180 specific activity (Bayer *et al.*, 2021). The gene encoding four-subunit POR was only found
181 from the NPIRA03, NPIRA04 and NPIRA06 bins, whereas the genes encoding the five-
182 subunit POR were well conserved in the NPIRA bins.

183 *Nitrospira marina* (Bayer *et al.*, 2021) and *Nitrospira moscoviensis* (Koch *et al.*,
184 2015) are capable of the growth utilizing formate, and the gene encoding formate
185 dehydrogenase (Fdh) was located in the NPIRA03 and NPIRA06 bins affiliated into the
186 *Nitrospira marina* clade (**Fig. 2**). On the other hand, the NPIRA bins affiliated into the
187 *Nitrospira salsa* clade were commonly lacking the gene encoding formate dehydrogenase
188 (**Table S2**), suggesting those *Nitrospira* are incapable of utilizing formate or just reflecting
189 incomplete nature of the NPIRA bins. It would be of interest to examine formate utilization of
190 *Nitrospira salsa* because the formate utilization would be a niche determinant for *Nitrospira*
191 *salsa* and *Nitrospira marina* who are capable of formate utilization.

192 As an oxidative stress defense system, the NPIRA bins carried the genes encoding
193 superoxide dismutase (SOD), catalase, glutathione peroxidase, and cytochrome *c* peroxidase.
194 Sulfite oxidation coupled with the reduction of cytochrome *c* by *Nitrospira* remains

195 controversial due to lack of experimental demonstration, whereas the *sorAB* encoding
196 sulfite:cytochrome *c* oxidoreductase was conserved among the NPIRA bins.

197 **2-2) Metabolic potentials of the NMNS bins**

198 The *amoCAB* gene cluster was found in the NMNS01 bin (**Table S4**). The *amoCAB* gene
199 cluster (NMNS01_40000 to NMNS01_40020 genes) was located in the short contig (2.6 kb),
200 and no neighbor gene was located in the contig. In the NMNS02 bin, *amoCAB* gene cluster
201 was not found, and only *amoC* was found (NMNS02_32030 gene). Hydroxylamine oxidation
202 to NO and electron transferring to ubiquinone molecules are carried out by a hydroxylamine
203 ubiquinone redox module (Simon and Klotz, 2013) composed of hydroxylamine
204 dehydrogenase (Hao), cytochrome *c554*, and cytochrome *c552*. The *haoAB*, *c554*, and *c552* genes
205 are composed of a gene cluster in *Nitrosomonas* sp. Nm143 and *Nitrosomonas aestuarii*
206 genomes, and the *haoAB*, *c554*, and *c552* gene cluster was found in the NMNS02 bin but not in
207 the NMNS01 bin. Hydroxylamine oxidation reaction of *Nitrosomonas europaea* Hao
208 produces nitric oxide (NO), which is subsequently oxidized to NO₂⁻. Nitrosocyanin (Arciero
209 *et al.*, 2002) is a candidate of NO oxidase (Lancaster *et al.*, 2018), and the gene encoding
210 nitrosocyanin was found from both the NMNS01 and NMNS02 bins. Apart from the above
211 genes, the NMNS01 and NMNS02 bins had the genes encoding copper-containing NO-
212 forming nitrite reductase (NirK), cytochrome-containing nitric oxide reductase (cNor),

213 cytochrome *c*' beta (Cyt b'), and cytochrome P460 (Cyt_{P460}), and those enzymes may
214 contribute to nitrogen oxide transformation other than aerobic ammonia oxidation.

215 The electrons released from hydroxylamine (and NO) oxidation enter into a
216 respiratory chain in the form of ubiquinol for the generation of proton motive force across a
217 membrane and also of reducing power (*i.e.*, NAD(P)H) required for CO₂ fixation. The *pet*-
218 operon (*petABC*) encoding cytochrome *bc₁* complex (complex III) was found in both the
219 NMNS01 and NMNS02 bins. The electrons released from ubiquinol oxidation are transferred
220 from the complex III to terminal oxidase using a soluble cytochrome *c* (*c₅₅₂*) (Simon and
221 Klotz, 2013), and the *c₅₅₂* gene was located in both the NMNS01 and NMNS02 bins. The
222 NMNS01 and NMNS02 bins had the genes encoding an *aa₃*-type terminal oxidase with low
223 O₂ affinity but not a *cbb₃*-type terminal oxidase with high O₂ affinity and putative high-O₂-
224 affinity terminal oxidase sNor (Sedlacek *et al.*, 2020). The NMNS01 and NMNS02 bins had
225 the two copies of *nuo*-operon (*nuoABCDEFGHIJKLMN* and *nuoABB/C/DEFGHIJKLMN*)
226 encoding NADH:quinone oxidoreductase (complex I) as well as the *Nitrosomonas aestuarii*
227 genome.

228 **References**

229 Arciero, D.M., Balny, C., and Hooper, A.B. (1991) Spectroscopic and rapid kinetic studies of
230 reduction of cytochrome *c₅₅₄* by hydroxylamine reductase from *Nitrosomonas*
231 *europaea*. *Biochemistry* **30**: 11466–11472.

232 Bayer, B., Saito, M.A., McIlvin, M.R., Lucker, S., Moran, D.M., Lankiewicz, T.S., et al.
233 (2021) Metabolic versatility of the nitrite-oxidizing bacterium *Nitrospira marina* and
234 its proteomic response to oxygen-limited conditions. *ISME J* **15**: 1025-1039.

235 Carantoa, J.D., and Lancaster, K.M. (2017) Nitric oxide is an obligate bacterial nitrification
236 intermediate produced by hydroxylamine oxidoreductase. *Proc Natl Acad Sci U S A*
237 **114**: 8217–8222.

238 Chadwick, G.L., Hemp, J., Fischer, W.W., and Orphan, V.J. (2018) Convergent evolution of
239 unusual complex I homologs with increased proton pumping capacity: energetic and
240 ecological implications. *ISME J* **12**: 2668–2680.

241 Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M. *et al.* (2015)
242 Complete nitrification by *Nitrospira* bacteria. *Nature* **528**: 504–509.

243 Koch, H., Lucker, S., Albertsen, M., Kitzinger, K., Herbold, C., Spieck, E. *et al.* (2015)
244 Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus
245 *Nitrospira*. *Proc. Natl. Acad. Sci. U. S. A.* **112**: 11371–11376.

246 Lancaster, K.M., Caranto, J.D., Majer, S.H., and Smith, M.A. (2018) Alternative bioenergy:
247 updates to and challenges in nitrification metalloenzymology. *Joule* **2**: 421–441.

248 Lawson, C.E., Munding, A.B., Koch, H., Jacobson, T.B., Weathersby, C.A., Jetten, M.S.M.,
249 et al. (2021) Investigating the chemolithoautotrophic and formate metabolism of

250 *Nitrospira moscoviensis* by constraint-based metabolic modeling and ¹³C-tracer
251 analysis. *mSystems* ; 6: e00173-21.

252 Lückner, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B. *et al.* (2010) A
253 *Nitrospira* metagenome illuminates the physiology and evolution of globally
254 important nitrite-oxidizing bacteria. *Proc Natl Acad Sci U S A* 107: 13479–13484.

255 Nomoto, T., Fukumori, Y., and Yamanaka, T. (1993) Membrane-bound cytochrome *c* is an
256 alternative electron donor for cytochrome *aa3* in *Nitrobacter winogradskyi*. *J Bacteriol*
257 **175**: 4400–4404.

258 Sedlacek, C.J., Giguere, A.T., Dobie, M.D., Mellbye, B.L., Ferrell, R.V., Woebken, D. *et al.*
259 (2020) Transcriptomic response of *Nitrosomonas europaea* transitioned from
260 ammonia- to oxygen-limited steady-state growth. *mSystems* **5**: e00562-19.

261 Simon, J., and Klotz, M.G. (2013) Diversity and evolution of bioenergetic systems involved
262 in microbial nitrogen compound transformations. *Biochim Biophys Acta* **1827**: 114–
263 135.

264 Starkenburg, S.R., Chain, P.S.G., Sayavedra-Soto, L.A., Hauser, L., Land, M.L., Larimer,
265 F.W. *et al.* (2006) Genome sequence of the chemolithoautotrophic nitrite-oxidizing
266 bacterium *Nitrobacter winogradskyi* Nb-255. *Appl Environ Microbiol* **72**: 2050–2063.

267 Ushiki, N., Fujitani, H., Shimada, Y., Morohoshi, T., Sekiguchi, Y., Tsuneda, S. (2018)
268 Genomic analysis of two phylogenetically distinct *Nitrospira* species reveals their
269 genomic plasticity and functional diversity. *Front. Microbiol.* **8**: 2637.

270 van Kessel, M.A.H.J. van Speth, D.R., Albertsen, M., Nielsen, P.H., Camp, H.J.M.O. den,
271 Kartal, B. *et al.* (2015) Complete nitrification by a single microorganism. *Nature* **528**:
272 555–559.