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# *Nitrospira* Community Composition in Nitrifying Reactors Operated with Two Different Dissolved Oxygen Levels

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***Nitrospira* is a dominant member of nitrite-oxidizing bacteria (NOB) in nitrifying bioreactors as well as in natural habitats. In this study, *Nitrospira* NOB were investigated in the two nitrifying reactors operated with high and low dissolved oxygen (DO) concentrations for a period of 300 days. Phylogenetic and terminal restriction fragment length polymorphism analyses based on 16S rRNA gene sequences revealed that the *Nitrospira* community compositions of the two reactors during the early period related to group 1 and half of the *Nitrospira* community composition shifted to group 2 in the high-DO reactor after day 179, although there was no significant change in the low-DO reactor. These results suggested that DO was an important factor affecting *Nitrospira* community compositions in the nitrifying reactors.**

**Keywords:** Nitrification, nitrite-oxidizing bacteria, *Nitrospira*, terminal restriction fragment length polymorphism (t-RFLP), dissolved oxygen

Nitrification, the conversion of ammonia to nitrate, is an important microbial process for nitrogen removal in wastewater treatments as well as for nitrogen cycling in natural habitats [9]. Nitrification is mediated by two groups of aerobic chemolithoautotrophic bacteria: ammonia-oxidizing bacteria (AOB) that oxidize ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) that oxidize nitrite to nitrate [1]. NOB have been less studied than AOB in wastewater treatment bioreactors. One of the reasons for this is the belief that AOB grow faster than NOB [16], which leads to the conclusion that ammonia oxidation to nitrite by AOB is the rate-limiting step in nitrification [16]. However, AOB are not likely to always be more active than NOB in wastewater treatment bioreactors. Hao and Chen [4] introduced conditions of nitrite build-up in the systems, such as low temperature, elevated pH, presence of unionized ammonia, low dissolved oxygen, and presence of specific chemicals.

NOB together with AOB are found in various natural and engineering habitats including soil, freshwater, seawater, and wastewater treatment bioreactors where a nitrogen source is present under aerobic or sub-aerobic conditions [17]. Most NOB strains grow chemolithoautotrophically, although some NOB strains have been known to metabolize organic matters such as yeast extract, peptone, pyruvate, and acetate [17]. NOB belong to four different groups of bacteria [3]: *Nitrobacter* (*Alphaproteobacteria*), *Nitrospina* (*Deltaproteobacteria*), *Nitrococcus* (*Gammaproteobacteria*), and *Nitrospira* (Phylum *Nitrospira*). Although *Nitrobacter* was previously believed to be the major NOB in natural and engineering habitats [17], due to easier cultivation than the other groups of NOB [5], culture-independent molecular techniques have revealed that *Nitrospira* is more frequently detected in these environments than *Nitrobacter* [14].

NOB require oxygen for their energy-generating metabolism. Therefore, when oxygen is limiting, NOB have to compete with other types of aerobic bacteria such as AOB. Low oxygen level, however, does not always seem to be inhibitory for the growth of NOB. For instance, a laboratory-scale bioreactor operated with very low levels [0.06–0.16 mg/l of dissolved oxygen (DO)] showed almost complete nitrite oxidation [13]. Although NOB activities in low oxygen environments have been published occasionally, it has not been well reported what types of NOB strains are able to survive in such low oxygen levels and how NOB are able to compete with other aerobic bacteria. Thus, the objective of this study was to investigate NOB community compositions belonging to *Nitrospira* in laboratory-scale reactors operated with high and low oxygen levels and to trace the NOB community changes over the reactor operation.

Nitrite oxidation performances were monitored from the two nitrifying chemostat reactors operated with high

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(8.5 mg/l) and low DO concentrations (0.12–0.24 mg/l). Mineral base ammonia medium (average ammonia concentration: 30 mg/l) was fed into the reactors such that a 10-day retention time was maintained for a period of 300 days [7]. As presented in the previous publication [7], in the high-DO reactor influent, ammonia was converted to nitrate nearly completely without accumulation of nitrite from the beginning of reactor operation, whereas in the low-DO reactor, ammonia and nitrite fluctuated in the middle of reactor operation (days 150–240) when DO level was changed from 0.24 to 0.12 mg/l. However, after day 240, nitrite accumulation was not observed, as in the high-DO reactor. In nitrifying bioreactors, aeration is a critical operational parameter and constitutes a significant operational cost [8]. The results demonstrate that aerobic AOB and NOB persist together, and nitrite oxidation can be possible even in low-DO conditions (0.12–0.24 mg/l), which also suggests that economic benefits would be realized by lowering the aeration rate in nitrifying reactors.

NOB community compositions in the two reactors were investigated at days 14 and 271, respectively, by constructing *Nitrospira* 16S rRNA gene clone libraries. A total of 15

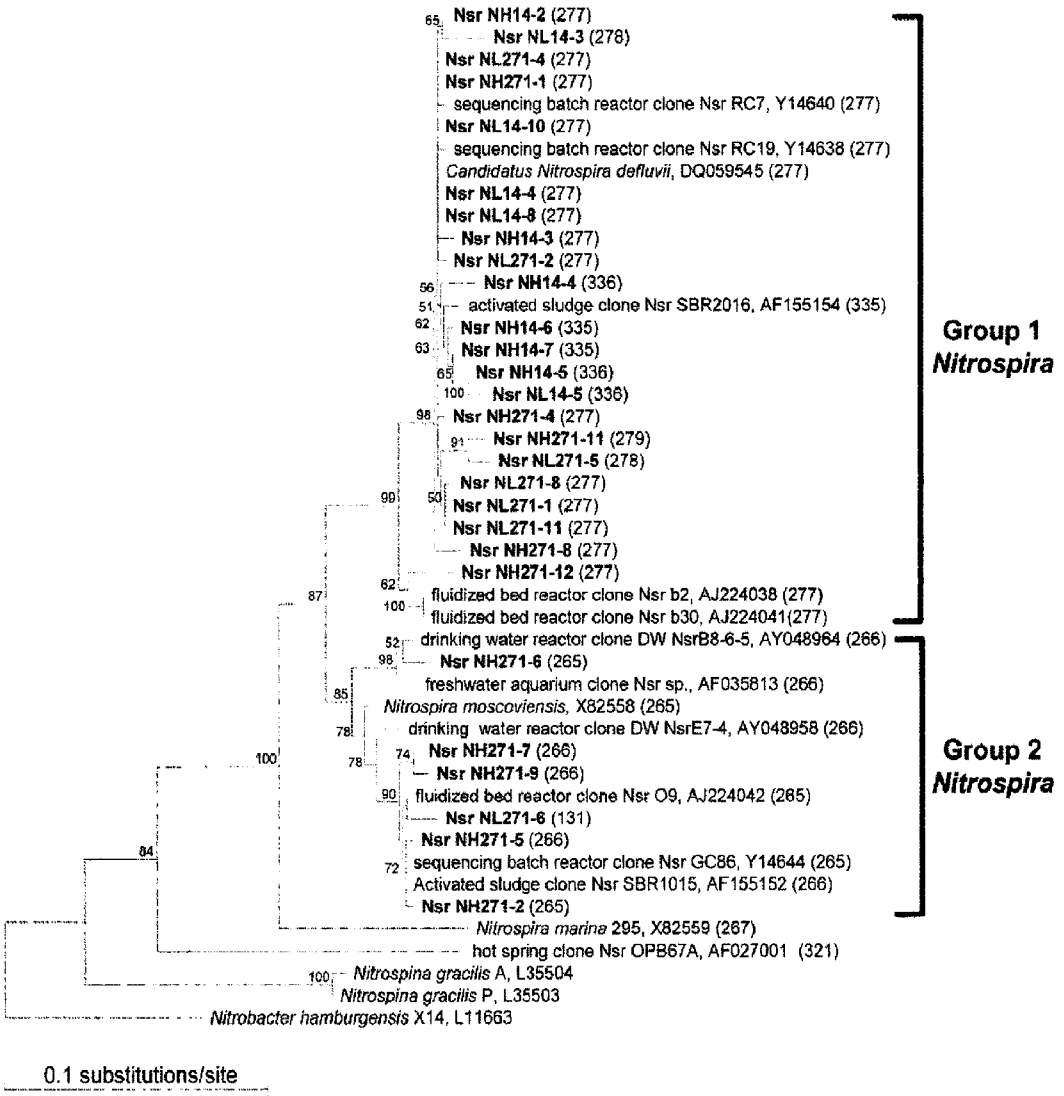


**Fig. 1.** Neighbor-joining tree based on 16S rRNA gene sequences retrieved from this study (boldface type) and other publications [2, 5, 6, 10, 11, 14].

The sizes of terminal restriction fragments digested with MspI restriction enzyme were determined *in silico* and are indicated beside sequence names in parentheses. Bootstrap values were determined based on 100 trials and shown at nodes greater than 50. Abbreviations: Nsr NL14, 271 (*Nitrospira* clones recovered from the low-DO reactor at days 14 and 271, respectively); Nsr NH14, 271 (*Nitrospira* clones recovered from the high-DO reactor at days 14 and 271, respectively). Sampling, DNA extraction, PCR, cloning, and phylogenetic analysis were conducted following protocols introduced in the previous publication [7]. *Nitrospira* 16S rRNA sequences determined in this study have been deposited in the GenBank database under accession numbers EU303243 to EU303270. PCR for *Nitrospira*-specific 16S rRNA was conducted using primers EUB338f (5'-ACTCCTACGGGAGGCAGC-3') and Ntspa0685M (5'-CGGGAATTCCGCGCTC-3') with reaction conditions introduced by Regan *et al.* [10]. The phylogenetic analysis was conducted using the ClustalX software [15].

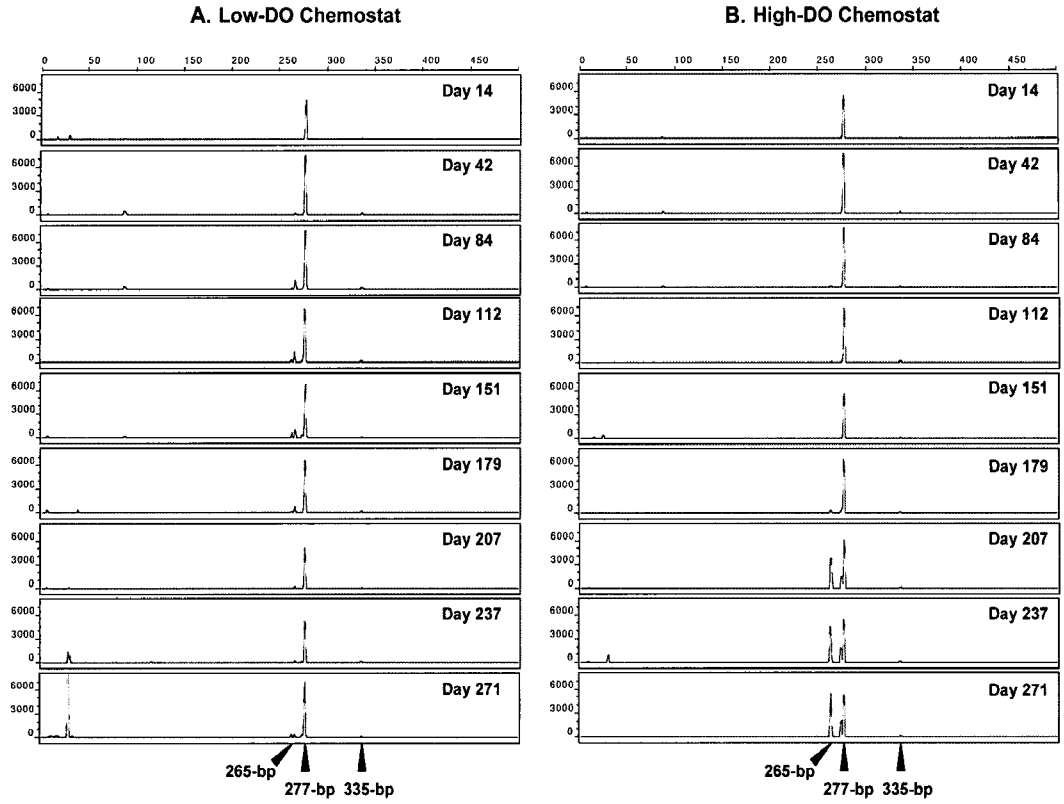


**Fig. 2.** *Nitrospira* population changes traced by t-RFLP analysis for the low-DO (**A**) and the high-DO reactors (**B**).
X- and Y-axes of each eletropherogram indicate the size of terminal fragment in base pairs and fluorescence intensity in fluorescence units, respectively. Peaks of 265–267 base pairs (bp) correspond to a signature of group 1 *Nitrospira*, and peaks of 277–279 or 335–336 base pairs matched to signatures of group 2 *Nitrospira*. Peaks less than 50 bp were not identified from the *in silico* analysis (Fig. 1) and were assumed to be noise background generated from primers [7]. Sampling, DNA extraction, PCR, restriction digestion, and analysis of terminal fragments were conducted following protocols in the previous publication [7]. Samples were run through an ABI 310 DNA sequencer (Applied Biosystems, Foster City, U.S.A.). Peak sizes were analyzed using the GeneScan software (Applied Biosystems, Foster City, U.S.A.).

clones (5 clones at day 14 and 10 clones at day 271) were retrieved from the low-DO reactor, and a total of 13 clones (6 clones at day 14 and 7 clones at day 271) for the high-DO reactor. A phylogenetic analysis based on 16S rRNA gene sequences was conducted using clones collected from this study, water treatment reactors (DW Nsr B8-6-5 and E7-2), a hot spring (Nsr OPB67A), a freshwater aquarium (Nsr sp. AF035813), clones from nitrifying reactors (SBR1015, SBR2016, Nsr b2, Nsr b30, Nsr RC7, Nsr RC19, Nsr GC86, Nsr O9), two *Nitrospina* strains (*Nitrospina gracilis* A and P), two *Nitrospira* strains (*N. moscoviensis* and *N. marina*), and a *Nitrospira* sequence of an enrichment culture (*Candidatus N. defluvii*) rooting with a *Nitrobacter* strain (*Nitrobacter hamburgiensis* X14) (Fig. 1). The analysis demonstrated that all of the 28 clones retrieved in this study were distributed to either a branch containing *N. moscoviensis* (group 2 *Nitrospira*) or a branch containing *Candidatus N. defluvii* (group 1 *Nitrospira*). The sequence identity of the two groups of *Nitrospira* ranged from 88% to 93%, and the bootstrap value for bifurcating the two groups is 87%. This demonstrates that the two groups of *Nitrospira* are different, at least on the species level. The phylogenetic differentiation of the two groups of *Nitrospira* was first suggested by Daims *et al.* [3]. Later, Maixner *et al.* [6] confirmed that these were different functionally. In the low-DO reactor, all 5 clones retrieved at day 14 belonged to group 1 *Nitrospira*, whereas 6 and 1 clones belonged to group 1 and group 2 *Nitrospira*, respectively, at day 271. This indicated that low DO (0.1–0.2 mg/l) did not significantly affect *Nitrospira* community composition. In the high-DO reactor, all 6 clones belonged to group 1 *Nitrospira* at day 14, like the low-DO reactor. On the contrary, 5 clones belonged to group 1 *Nitrospira* and 5 clones belonged to group 2 *Nitrospira* at day 271, which demonstrates that high DO (8.5 mg/l) may have exerted a selective pressure on *Nitrospira* community composition in the high-DO reactor.

Dynamic changes of *Nitrospira* community compositions were traced using terminal restriction fragment length polymorphism (t-RFLP) analysis at days 14, 42, 84, 112, 151, 179, 207, 237, and 271 for the two reactors. This analysis could be used to fingerprint two groups of *Nitrospira* based on different size of terminal restriction fragments (t-RFs): 265–267 base pairs (bp) of t-RFs correspond to group 1 *Nitrospira*, and 277–279 bp or 335–336 bp t-RFs

correspond to group 2 *Nitrospira* (refer to *in silico* analysis in Fig. 1). As shown in Fig. 2A, in the low-DO reactor, the 277 bp of t-RF (*i.e.*, signature of group 1 *Nitrospira*) was dominant from the beginning of reactor operation (days 14 and 42). Although the 265 and 335 bp of t-RFs (*i.e.*, signatures of group 2 *Nitrospira*) were found after day 84, the peak areas for these t-RFs were insignificant (<12% of total peak area). This result agrees with the phylogenetic analysis (Fig. 1) and confirms that low-DO did not significantly affect the *Nitrospira* community. In the high-DO reactor, the 277 bp t-RF was dominant up to day 179, as in the low-DO reactor. Afterwards, a 265 bp t-RF emerged and constituted 33–39% of total peak area, which confirms that high-DO gave a selective advantage to group 2 *Nitrospira*.

The work presented here is the first report regarding the effect of DO on *Nitrospira* community in nitrifying reactors. The phylogenetic analysis (Fig. 1) and the t-RFLP fingerprinting analysis (Fig. 2) clearly demonstrated that a low DO level did not affect the *Nitrospira* NOB community significantly, which is quite different from the results of the AOB community in the same reactor [7] where a low DO shifted AOB belonging to *Nitrosomonas oligotropha* to *Nitrosomonas europaea* after day 42 [7]. On the contrary, the *Nitrospira* community composition shifted from group 1 to group 2 in the high-DO reactor. It is not clear why the high-DO reactor selected group 2 *Nitrospira*. A possible explanation of this is higher oxygen affinity (or lower oxygen tolerance) of group 1 *Nitrospira* than group 2 *Nitrospira*. Comparison between the two strains belonging to each group will verify this hypothesis, although a representative of group 1 *Nitrospira* has yet to be isolated in pure culture [14]. Another possible answer is a higher ammonia sensitivity of group 2 *Nitrospira* than group 1 *Nitrospira*. In the high-DO reactor, ammonia levels in the effluent were lower (0.1±0.3 mg/l of nitrogen) than those in the low-DO reactor (3.3±6.2 mg/l of nitrogen). Ammonia level is believed to be a factor affecting activity of NOB. Simm *et al.* [12] reported that unionized ammonia inhibited the activity of *Nitrospira* in batch reactors.

The t-RFLP analysis introduced in this study is a convenient method to differentiate two groups of *Nitrospira* that are frequently found in engineering systems and natural aquatic habitats [5, 6, 14]. Previously, Regan *et al.* [10] introduced the method, but they did not recognize the importance of different sizes of t-RFs generated by digestion with the MspI restriction enzyme. Although the t-RFLP analysis cannot provide exact quantitative information, the analysis proved to be useful for fingerprinting the *Nitrospira* community structure before extensive sequence-based studies, such as clone library analysis.

In conclusion, we identified that *Nitrospira* sequences retrieved from the two reactors, operated with high and low DO concentrations, were distributed into the two phylogenetically distinct groups of *Nitrospira* and demonstrated that high DO shifted the *Nitrospira* community composition belonging to group 1 to group 2. These findings support our suggestion that DO affects the *Nitrospira* community composition, which provides a basis for future research on detailed mechanisms of how the two groups of *Nitrospira* respond to different DO levels.

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