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ORIGINAL PAPER

Use of quantitative real-time PCR to monitor population dynamics of ammonia-oxidizing bacteria in batch process

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Abstract A quantitative real-time PCR (QPCR) assay with the TaqMan system was used to quantify 16S rRNA genes of β-proteobacterial ammonia-oxidizing bacteria (AOB) in a batch nitrification bioreactor. Five different sets of primers, together with a TaqMan probe, were used to quantify the 16S rRNA genes of β-proteobacterial AOB belonging to the *Nitrosomonas europaea*, *Nitrosococcus mobilis*, *Nitrosomonas nitrosa*, and *Nitrosomonas cryotolerans* clusters, and the genus *Nitrosospira*. We also used PCR followed by denaturing gradient gel electrophoresis (DGGE), cloning, and sequencing of their 16S rRNA genes to identify the AOB species. Seed sludge from an industrial wastewater treatment process controlling high-strength nitrogen wastewater (500 mg/L NH_4^+–N) was used as the inoculum for subsequent batch experiment. The *Nitrosomonas nitrosa* cluster was the predominant AOB (2.3×10^5 copies/mL) in the start-up period of the batch experiment. However, from the exponential growth period, the *Nitrosomonas europaea* cluster was the most abundant AOB, and its 16S rRNA gene copy number increased to 8.9×10^6 copies/mL. The competitive dominance between the two AOB clusters is consistent with observed differences in ammonia tolerance and substrate affinity. Analysis of the DGGE results indicated the presence of *Nitrosomonas europaea* ATCC19718 and *Nitrosomonas nitrosa* Nm90, consistent with the QPCR results.

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Introduction

Biological nitrification, the conversion of ammonia to nitrate via nitrite, is primarily performed by a unique group of autotrophic microorganisms, the nitrifying bacteria. In nitrification processes, ammonia-oxidizing bacteria (AOB) play a key role in oxidizing ammonia by participating in the first step of nitrification. Because AOB are responsible for the rate-limiting step in most nitrification processes, considerable attention has been given to investigating their ecology and physiology [3, 13].

Based on the phylogenetically conserved 16S rRNA gene sequences, all known aerobic AOB isolates are restricted to two evolutionarily distinct lineages of the class Proteobacteria. With the exception of two marine species, *Nitrosococcus oceani* and *Nitrosococcus halophilus*, within the γ-subclass of the Proteobacteria, all members of the genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* represent closely related organisms of the β-subclass of the Proteobacteria. On the basis of 16S rRNA sequence homology, it has been proposed that the latter three genera should be combined into one genus, *Nitrosospira* [3, 7, 12, 21].

Although the basic metabolism is uniform within the defined groups of AOB, which use ammonia as the sole energy source, ecophysiological differences exist between the distinct representatives [11]. In particular, a number of phylogenetically definable subgroups exist within the genus *Nitrosomonas* [2, 14, 19, 21, 26], each with different ecophysiological characteristics [11]. Members of the

Nitrosomonas europaea lineage exhibit high half-saturation constant of ammonia oxidation (between 30 and 61 μM) and are insensitive to extremely high ammonia concentrations of up to 600 mM. In contrast, members of the *Nitrosomonas nitrosa* lineage have low maximum tolerance for ammonium salts, at about 100 mM, and relatively low half-saturation constant, ranging between 14 and 46 μM [19, 25]. Despite their distinct physiological characteristics, evaluating the activities of AOB is largely process-oriented in almost all engineered nitrification systems. However, an understanding of AOB community structures and the accurate quantification of each population in an engineered nitrification system would greatly assist the prediction and effective control of the process operations [4, 22].

Quantitative real-time PCR (QPCR) is widely used for microbial quantification in a variety of environmental research areas [8, 9, 17, 29]. Using a QPCR assay, a wide dynamic quantification range of 7–8 logarithmic decades and highly reliable quantification can be achieved. This assay is also faster and easier than hybridization techniques. The QPCR assay with the TaqMan probe system is highly specific because three oligonucleotide sequences are used (i.e., forward and reverse primers, and a TaqMan probe) that are complementary to the target sequence. In subsequent discussions, the 'primer and probe set' denotes the three oligonucleotides comprising the forward and reverse primers and a dually labeled fluorescent TaqMan probe.

Group-specific primer and probe sets targeting 16S rRNA genes of *β*-proteobacterial AOB were recently developed to facilitate their sensitive detection and quantification, and the specificities of the sets were verified both in silico and in vitro [16]. However, to ensure the reliability of these group-specific sets in a natural environment, it is essential that the microbial concentrations estimated with QPCR and these sets should agree well with the operating conditions and the performance of the nitrification process.

Therefore, this study focused on the application of group-specific primer and probe sets targeting the 16S rRNA gene sequences of *β*-proteobacterial AOB, which are likely to be found in most engineered nitrification processes [20, 23]. Five separate primer and probe sets were used in the QPCR assay to quantify the 16S rRNA genes of *β*-proteobacterial AOB belonging to the *Nitrosomonas europaea*, *Nitrosococcus mobilis*, *Nitrosomonas nitrosa*, and *Nitrosomonas cryotolerans* clusters, and the genus *Nitrosospira*, denoted NSMeur, NSMmob, NSMnit, NSMcry, and NSS, respectively. Qualitative and quantitative determinations of the diversity and population dynamics of AOB in a batch nitrification process treating high-strength nitrogen wastewater were investigated using group-specific QPCR assays. We also used PCR followed by denaturing gradient gel electrophoresis (DGGE), cloning, and sequencing of the 16S rRNA genes to confirm the specificity of the group-specific quantification method.

Materials and methods

Operation of the nitrification bioreactor

Aerobic return sludge from a domestic wastewater treatment plant located in Pohang, Korea, was cultivated in a completely stirred tank reactor (CSTR) with a working volume of 7 L to produce a constant source of inoculum for the biological nitrification reaction. Medium for the inoculum feed, made by the dilution of an industrial nitrogen wastewater, contained 504 mg/L NH_4^+–N, 496 mg/L SCN^-, and other inorganic compounds that are necessary for the growth of nitrifying bacteria. The inoculum system was maintained at pH 8.0 and 30 °C, with a hydraulic retention time (HRT) of 10 days. The steady-state effluent from this system was used to provide seed cultures for the subsequent batch experiment.

Another CSTR with a working volume of 6.5 L, equipped with temperature, pH, and dissolved oxygen (DO) controllers, was used in the batch mode to monitor the population dynamics of AOB. The composition of the medium used in the batch experiment was as follows: 2,360 mg/L $(NH_4)_2SO_4$, 129 mg/L KH_2PO_4, 5 mg/L $CaCl_2{\cdot}2H_2O$, 385 mg/L K_2HPO_4, 50 mg/L $MgSO_4{\cdot}7H_2O$, 500 mg/L $NaHCO_3$, 5 mg/L $FeSO_4{\cdot}7H_2O$, 7 mg/L KCl, and 5 mg/L $MnSO_4{\cdot}H_2O$. Steady-state effluent from the inoculum system was seeded at an initial volatile suspended solid (VSS) concentration of 13 mg/L. Pure air was supplied to maintain a DO concentration of up to 4 mg/L. The reactor was operated at pH 8.0 and 30 °C.

Ammonia, nitrite, and nitrate ion concentrations were measured in duplicate with ion chromatography (790 Personal IC, Metrohm, Switzerland). The VSS and thiocyanate concentrations were measured with the procedure described in standard methods [1]. Total genomic DNA was extracted in duplicate from 1.5 mL of mixed liquor suspended solid samples using an automated nucleic acid extractor with a commercial DNA purification kit (Magtration System 6GC, Precision System Science, Chiba, Japan), as described previously [28].

Microbial quantification using QPCR assays

Five previously reported group-specific primer and probe sets [16] were used to separately detect four subgroups of *Nitrosomonas* (the *Nitrosomonas europaea*, *Nitrosococcus mobilis*, *Nitrosomonas nitrosa*, and *Nitrosomonas cryotolerans* clusters) and the genus *Nitrosospira* (Table 1). The term '-set' was suffixed to the microbial group abbreviations described earlier to denote the corresponding primer and probe sets used to detect the target microbial groups. The primer and probe set used to detect the *Nitrosomonas europaea* cluster (NSMeur), for example, was the NSMeur-set.

Table 1 Primer and probe sets used in this study

Name	Function	Target group	Sequence (5′ → 3′)	*E. coli* numbering
NSMeur-828F	F primer	*Nitrosomonas europaea* cluster	GTTGT CGGAT CTAAT TAAG	828–846
NSMeur-984T	Probe		CCTAC CCTTG ACATG CTTGG AATC	984–1007
NSMeur-1028R	R primer		TGTCT TGGCT CCCTT TC	1028–1044
NSMmob-988F	F primer	*Nitrosococcus mobilis* cluster	GCTTG GAATT TTACG GAGAC	988–1017
NSMmob-1243T	Probe		AGTGT ACAGA GGGTA GCCAA CCC	1243–1265
NSMmob-1282R	R primer		CTACG AAGTG CTTTG TGAG	1282–1300
NSMnit-438F	F primer	*Nitrosomonas nitrosa* cluster	TTCGG TCGGG AAGAW ATAG	438–456
NSMnit-483T	Probe		CGGTA CCGAC ATAAG AAGCA CCGG	483–506
NSMnit-633R	R primer		CTAGT YATAT AGTTT CAAAC GC	633–654
NSMcry-211F	F primer	*Nitrosomonas cryotolerans* cluster	AGACC TTRTG CTTTT GGAG	211–229
NSMcry-270T	Probe		CCAAC TACTG ATCGT YGCCT TGGT	270–293
NSMcry-434R	R primer		TTTTC TTCTC RACTG AAAGA G	434–454
NSS-209F	F primer	*Nitrosospira* genus	CAAGA CCTTG CGCTY TT	209–225
NSS-432T	Probe		TTTCG TTCCG GCTGA AAGAG CT	432–453
NSS-478R	R primer		TCTTC CGGTA CCGTC AKT	478–495

All QPCR assays were performed using a LightCycler 1.2 (Roche Diagnostics, Mannheim, Germany) in 20 μL reaction capillary tubes. Each capillary tube was separately loaded with 2 μL of template DNA, followed by 1 μL (final concentration, 500 nM) of the forward and reverse primers, together with 1 μL (final concentration, 100 nM) of the TaqMan probe corresponding to each primer and probe set, 4 μL of the LightCycler TaqMan Master mix (Roche Diagnostics), and PCR-grade sterile water to a final volume of 20 μL. All experiments were performed in duplicate.

A two-step amplification of the target DNA, combining the annealing and extension steps, was performed under the following conditions: an initial 10 min incubation at 94 °C for *Taq* DNA polymerase activation; 45 cycles of denaturation at 94 °C for 10 s, and simultaneous annealing and extension at 60 °C for 30 s. The fluorescence response data were obtained during the annealing and extension period and the threshold cycle (C_T) was generated with an automated method of absolute quantification analysis using LightCycler Software (version 4.0). Previously reported values for the slope and intercept of each primer and probe set [16] were used to quantify the copy numbers of the 16S rRNA genes of the target AOB clusters in the samples.

PCR–DGGE analysis

Three DNA samples extracted from the inoculum system were used as the templates for PCR. Bacterial 16S rRNA gene fragments were amplified with forward primer 338f (5′-ACTCCTACGGGAGGCAG-3′; with a GC clamp at the 5′ terminus) and reverse primer 805r (5′-GACTACCAGGGTATCTAATCC-3′). Partial 16S rRNA gene amplifications were performed in 50 μL reaction mixtures using a thermal cycler (PTC-100, MJ Research, Watertown, MA, USA). PCR conditions were 94 °C for 10 min; 20 cycles consisting of 94 °C for 30 s, 65 °C to 55 °C in 0.5 °C decrements/cycle for 30 s, and 72 °C for 45 s; 20 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 7 min.

DGGE was performed on 8% polyacrylamide gel with a 25–55% denaturant gradient (100% denaturant corresponded to 7 M urea and 40% formamide). Electrophoresis was run at 80 V for 13 h in 1× TAE buffer at a constant temperature of 60 °C, using the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The PCR products separated on the gel were stained with ethidium bromide for 25 min and destained for 20 min before UV transillumination. The major DGGE bands were cut from the gel and subcloned with the pGEM-T Easy Vector System (Promega, Madison, WI, USA). The plasmids were extracted from transformed and cultured cells using a plasmid DNA purification kit (Plasmid Quick, General Biosystem, Seoul, Korea), and the cloned 16S rRNA genes were sequenced by a commercial sequencing service (Macrogen, Seoul, Korea). The clone sequences were compared with those deposited in the GenBank database, using the BLASTn program.

Results and discussion

Performance of nitrification inoculum system

Table 2 summarizes the performance and nitrogen balance of the inoculum system at steady-state operation. Steady-state was assumed when the effluent NH_4^+ and NO_2^-

Table 2 Chemical performance and nitrogen balance at steady-state operation of the inoculum system

Composition	Concentration (mg/L)
Influent	
NH_4^+–N	504.0 ± 14
SCN^-–N	119.7 ± 0.7
Effluent	
NH_4^+–N	0 ± 0
NO_2^-–N	26.5 ± 2.7
NO_3^-–N	590.1 ± 17.6
SCN^-–N	0.7 ± 0.4
Nitrogen balance	
Reduced NH_4^+–N	504.0
Reduced SCN^-–N	119.0
Produced NO_x^- –N	616.5
Recovery (%)	99.0%

concentrations did not vary by more than 10%. The steady-state data were obtained from eight consecutive samples collected during a period of 50 days of steady-state operation. At 10 days HRT, the ammonia nitrogen (504 mg/L) and thiocyanate (496 mg/L) contained in the influent industrial wastewater were almost completely oxidized, with removal efficiencies of 100 and 99.4%, respectively. Thiocyanate oxidation results in the accumulation of ammonia, thus contributing to the elevation of ammonia in the system. Therefore, the 119.7 mg of nitrogen included in the 496 mg of thiocyanate per liter of influent was considered in the nitrogen balance of the system. The overall mass-balance recovery in the conversion of nitrogen at steady-state was 99.0% (Table 2).

Three consecutive samples were analyzed at steady-state using DGGE to monitor and identify the bacteria in the inoculum system. DGGE analysis of the PCR products amplified using the bacterial universal primer pair (i.e., 338f and 805r) showed the presence of four bands (Fig. 1). Sequences derived from bands A, B, C, and D were similar to the 16S rRNA gene sequences of *Nitrosomonas nitrosa* Nm90 (AJ298740; 99%), uncultured bacterium clone GZKB17 (AJ853512; 99%), *Thiobacillus denitrificans* NCIMB9548 (AJ243144; 98%), and uncultured bacterium BIfcii9 (AJ318122; 95%), respectively. The band migration patterns were identical for all samples analyzed. This result indicates that the bacterial community structure was stable and that *N. nitrosa* Nm90 was the predominant AOB in the inoculum system. The first isolates of *N. nitrosa* originated from industrial sewage disposal plants, as reported in previous studies [10, 25], and this organism has also been found in other industrial wastewater treatment plants [15, 22]. Therefore, the occurrence of *N. nitrosa* Nm90 in the inoculum system fed with high-strength industrial wastewater is consistent with previous studies.

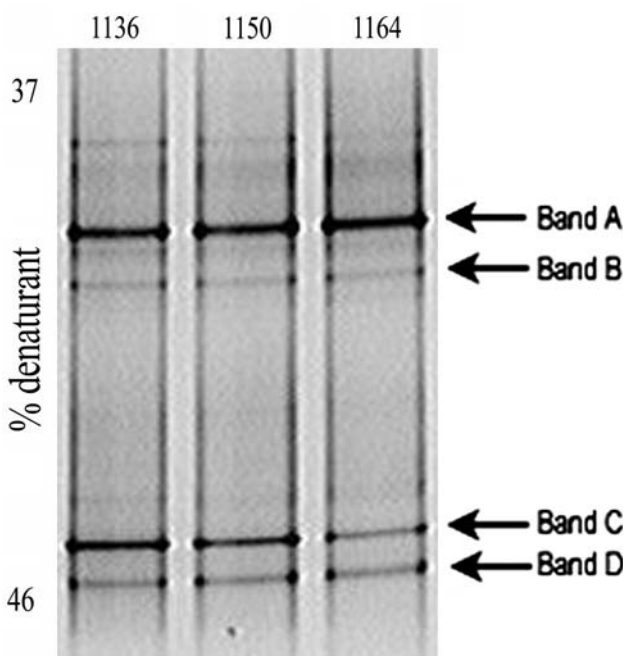

Fig. 1 DGGE image of inoculum samples amplified with the 338f and 805r primers. Three consecutive samples were analyzed and the excised bands are indicated by *arrows*. *Numbers* shown at the top of each *lane* represent the time of sample collection (days)

Performance of batch experiment

Figure 2 shows how the residual concentrations of ammonia, nitrite, and nitrate, as the nitrogen balance in the batch reactor changed over time. It is clear that ammonia oxidation was followed by nitrite oxidation. The initial concentration of ammonia nitrogen, 488.5 mg/L NH_4^+–N, was completely oxidized to nitrite (489.6 mg/L NO_2^-–N) during 11.1 days of incubation. The concentration of the ammonia nitrogen decreased linearly from 398.1 to 25.9 mg/L nitrogen in the period between 7.3 and 10.6 days of incubation at a rate of 112.8 mg/L nitrogen per day. The nitrite concentration remained at steady-state for about 6 days after ammonia oxidation was complete. The accumulated nitrite was completely oxidized to nitrate after 21 days of incubation. The nitrite oxidation rate was 257 mg/L NO_2^-–N per day. This was 2.3-fold higher than the ammonia oxidation rate. This result is consistent with the fact that ammonia oxidation is frequently the rate-limiting step in the nitrification process [5, 13, 18].

Population dynamics of AOB in the batch experiment

Figure 3 shows the changes in the 16S rRNA gene copy numbers for the *Nitrosomonas europaea* and *Nitrosomonas*

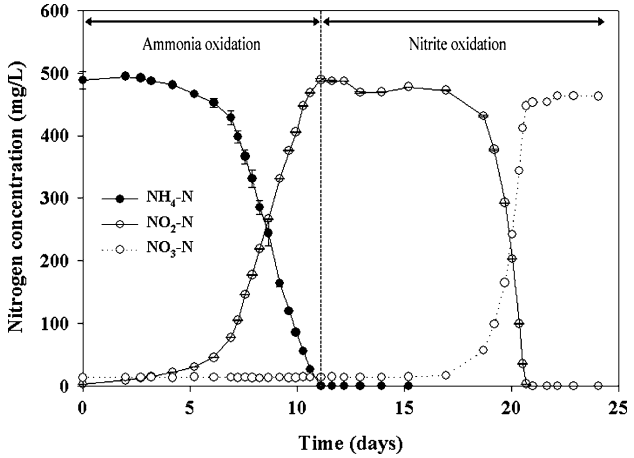

Fig. 2 Temporal changes in the concentrations of ammonia, nitrite, and nitrate in the batch experiment

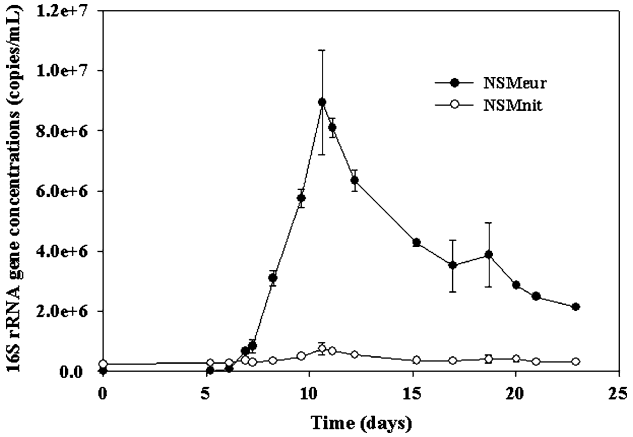

Fig. 3 Temporal changes in the 16S rRNA gene copy numbers of the *Nitrosomonas europaea* and *Nitrosomonas nitrosa* clusters in the batch experiment

nitrosa clusters, quantified using QPCR assays with the NSMeur- and NSMnit-sets, respectively, in the batch experiment. There were no positive amplification results in the QPCR tests for the NSMmob-, NSMcry-, and NSS-sets. The initial 16S rRNA gene copy numbers for the *N. europaea* and *N. nitrosa* clusters were 7.5×10^3 and 2.3×10^5 copies/mL, respectively. This result is in good agreement with the expectation that *N. nitrosa*-like AOB would be the most abundant AOB in the start-up period in the batch reactor because *N. nitrosa* Nm90 was the predominant AOB in the seed inoculum. There is a significant possibility of false negative detection of *N. nitrosa*-like AOB because of the insufficient coverage of this microbial group by some AOB-specific primers [11, 21]. However, in this study, the *N. nitrosa* cluster was successfully quantified using QPCR assays with the NSMnit-set throughout the period of the batch experiment. Therefore, the NSMnit-set can be used to selectively detect and quantify changes in the 16S rRNA gene concentrations of the *N. nitrosa* cluster in environments where other AOB coexist.

A lag period of approximately 6 days was observed with respect to the increase in the copy numbers quantified with the NSMeur- and NSMnit-sets. After the lag period, the 16S rRNA gene concentrations quantified with the NSMeur- and NSMnit-sets increased and reached maxima of 8.9×10^6 and 7.4×10^5 copies/mL, respectively, after 10.6 days of incubation. The rate of increase for the *N. europaea* cluster was much higher than that for the *N. nitrosa* cluster. *Nitrosomonas europaea*-like AOB are the most commonly isolated and best-investigated AOB because they outcompete other AOB in environments that are rich in ammonia [6, 17, 24]. The *N. europaea*-like AOB are known to be less sensitive to high ammonium salt concentrations (i.e., 600 mM) than are *N. nitrosa*-like AOB (i.e., 100 mM) [19, 25]. Hence, the competitive dominance of the *N. europaea* cluster in the batch experiment was probably the result of their tolerance of high concentrations of ammonia. This result indicates that the changes in the 16S rRNA gene copy numbers quantified with the group-specific QPCR assays reflected the physiological characteristics of each AOB group. The competitive dominance shift between the two AOB clusters in the batch reactor indicates that *N. nitrosa*-like AOB can be regarded, in ecological terms, as typical K-strategists compared with *N. europaea*-like AOB, which display a rapid growth strategy under feast substrate-loading conditions.

A similar tendency in the population dynamics was observed with DGGE. DGGE analysis of the PCR products amplified using an AOB-specific primer pair produced a single band (data not shown). The band intensity gradually increased during the exponential phase of ammonia oxidation and decreased after ammonia oxidation was complete. The sequence derived from the DGGE band was similar to the 16S rRNA gene sequence of *N. europaea* ATCC19718, with 98% similarity. This result implies that the QPCR assay with the NSMeur-set successfully quantified the target AOB group.

The specific growth rates of the two clusters could be estimated in the exponential growth period based on the 16S rRNA gene quantification together with curve fitting. The estimated specific growth rates for the *N. europaea* and *N. nitrosa* clusters were 0.76 and 0.34 day^{-1}, respectively. These values are consistent with the range of growth rates cited in the literature [5, 27], which is another indication of the specificity and applicability of the primer and probe sets to nitrification systems.

In conclusion, QPCR assays with the AOB group-specific primer and probe sets successfully quantified the AOB 16S rRNA gene copy numbers in a laboratory-scale nitrification bioreactor. The *N. nitrosa* cluster was the predominant

AOB group in the industrial wastewater treatment process controlling high-strength nitrogen wastewater (500 mg/L NH_4^+–N). In the batch experiment, the *N. europaea* and *N. nitrosa* clusters were detected with the corresponding QPCR assays and the 16S rRNA gene copy numbers of each cluster were quantified in the ranges 7.5×10^3 to 8.9×10^6 copies/mL and 2.3×10^5 to 7.4×10^5 copies/mL, respectively. The competitive dominance between the two AOB clusters is consistent with the physiological properties of each AOB group reported in previous studies. The QPCR method, together with group-specific primer and probe sets, is a powerful approach to the study of AOB dynamics, providing a better understanding of the microbial communities and control strategies in the nitrification process.

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