Kinetics of Ammonia-Oxidizing Microorganisms and Nitrite-Oxidizing Bacteria Enriched at High and Low Ammonia Concentrations

Tarnpetch Charoanwoodtipong[1], Tawan Limpiyakorn[2], Benjaporn Boonchayaanant Suwannasilp[3]

***Abstract*—**In this study, the kinetics of ammonia-oxidizing microorganisms (AOM) and nitrite-oxidizing bacteria (NOB) enriched at high and low ammonia concentrations were investigated. Two moving-bed biofilm batch reactors were used for the enrichment of AOM and NOB. The first reactor (Reactor A) was intermittently fed with 50 mg-N/l ammonia whereas the second reactor (Reactor B) was intermittently fed with 1 mg-N/l ammonia. Biocarriers in these reactors, were subsequently used for kinetic analysis in series of batch reactors. The results show that ammonia oxidation kinetics were greatly different in these reactors. Maximum specific substrate utilization rates (q_{max}) of ammonia in Reactor A and B were 0.128 and 0.0089 mg-N/mgMLVSS.hr, respectively. Half saturation constants (K_s) for ammonia in Reactor A and B were 8.78 and 1.19 mg-N/l, respectively. The results suggested the presence of low ammonia affinity AOM in Reactor A and high ammonia affinity AOM in Reactor B. However, nitrite oxidation kinetics in these reactors were not much different, especially for K_s values, which were 2.57 and 1.34 mg-N/l for Reactor A and B, respectively. The results suggested that the NOB communities in both reactors might be similar.

***Keywords*—**nitrification, ammonia-oxidizing microorganisms, nitrite-oxidizing bacteria, microbial kinetics.

I. Introduction

AQUACULTURE is one of the most important business in Thailand. On these days, there are a lot of aquaculture breeding types, such as freshwater and seawater fish or shrimp ponds, which take places all over the country for supporting domestic consumption and exporting. However, due to the fact that the competition in this business has greatly increased, the producers aim to maximize the aquaculture amount as much as possible. In other words, they have to breed the aquaculture in high density of population to maximize the profit. As a result, the high density of waste excretion from the aquaculture can severely harmful to the living organisms in the systems, which can cause massive loss in productivity and profitability for the producers.

Normally, the aquaculture excretes the waste in the form of ammonia (NH_4^+) and this ammonia can cause a lot of adverse effects to the aquaculture. For examples, the unionized form of ammonia (NH_3) in the amount of 0.43 mg-N/l can reduce the weight of the fish by 25 percent and can also increase the death rates of the fish in the system [1]. Aside from the toxicity of ammonia, the intermediate product of ammonia oxidation, nitrite (NO_2^-), can increase the death rates of the aquaculture as well [2]. As a result, ammonia and nitrite must be treated and controlled properly in the systems.

According to the nitrogen removal mechanisms, ammonia can be converted to nitrate (NO_3^-), which is the non-toxic form, via nitrification [3]. Nitrification can be divided into 2 steps. First, ammonia is converted to nitrite (NO_2^-) by ammonia-oxidizing microorganisms (AOM), including ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Next, the nitrite can be further oxidized to nitrate by nitrite-oxidizing bacteria (NOB).

As a result, both AOM and NOB are being used to treat ammonia in the aquaculture systems. In order to start up the treatment system, one simplest way is to obtain the ammonia-oxidizing inoculum from other ponds or treatment systems and directly add into the new systems [4]. However, inappropriate inoculum might cause the start-up period to be too long or even make the system fail due to the inappropriate biological kinetic characteristics of the microorganisms [5]. The differences in ammonia-oxidizing and nitrite-oxidizing communities have been reported to be linked to different ammonia and nitrite oxidation kinetics [6,7] which might be the main reason of the ineffective start-up for ammonia removal in aquaculture breeding system.

In this study, the effects of ammonia concentrations on ammonia-oxidizing and nitrite-oxidizing kinetic characteristics were investigated. Moving-bed biofilm reactors operated as semi-batch systems were used in this study to enrich ammonia-oxidizing communities at low (1 mg-N/l) and high (50 mg-N/l) ammonia concentrations. Maximum specific substrate utilization rates (q_{max}) and half-saturation constants (K_s) were determined to differentiate the kinetic characteristics of the microorganisms in the systems.

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II. Materials And Methods

Reactor operation

Two 10 liter moving-bed biofilm reactors were used in this experiment for the enrichment of AOM and NOB. Both reactors were operated in the semi-batch operation. Reactor A and Reactor B were fed with the synthetic wastewater with the ammonia concentration of 50 mg-N/l and 1 mg-N/l, respectively. The synthetic wastewater was intermittently fed to both reactors 6 days per week. The pH in both reactors was controlled to be in the range of 7-8. The 0.5 M NaOH was used for adjusting the pH for both reactors. Plastic media, BCN-012 KLL (Wassecare Thailand) were used as biocarriers for microorganisms to attach to and form biofilms. The amount of 5 liters of plastic media was added into each reactor.

Synthetic wastewater

Ammonium chloride (NH_4Cl) was used as a source of ammonia in the synthetic wastewater. The synthetic wastewater consisted of 12.6 g/l Marinium reef sea salt, 0.1 g/l KBr, 5g/l $MgSO_4.7H_2O$, and 400 g/l KH_2PO_4. In addition, 1 ml/l of non-chelated trace element, 1 ml/l of the selenite tungstate solution, 1ml/l of mixed vitamin, 1ml/l vitamin B1, and 1ml/l of vitamin B12 were added into the synthetic wastewater. The non-chelated trace element consisted of 5g/l $MnCl_2.6H_2O$, 24mg/l $NiCl_2$, 30mg/l H_3BO_3, 2.1g/l $FeSO_4.7H_2O$, 36 mg/l $CuCl_2.2H_2O$, 144 mg/l $ZnSO_4.7H_2O$, 40 mg/l $MnSO_4$, 190 mg/l $CoCl_2.6H_2O$, 36 mg/l $NaMoO_4.2H_2O$, and 12.5 ml/l HCl solution. The selenite tungstate solution consisted of 400 mg/l NaOH, 8 mg/l $NaWO_4.2H_2O$ and 6 mg/l $NaSeO_3.5H_2O$. Sodium bicarbonate of 0.25 M was used as a pH buffer and the carbon source for microorganisms in the systems. The salinity was controlled at 15-20 ppt [6].

Analytical measurement

Ammonia concentration was measured by the salicylate-hypochlorite method [8]. Nitrite concentration was measured by colorimetric method using the sulfanilamide solution and NED solution [9]. Nitrate concentration was measured by UV spectrophotometer [10]. The salinity was measured by the refractometer and pH was measured by the pH meter. Dissolved oxygen concentration was measured by using a DO probe meter.

Kinetic experiment

Kinetic experiment was carried out in series of batch reactors (300 ml) with the addition of 100 ml of biocarriers obtained from Reactor A and Reactor B after 145 d and 154 d of operation, respectively. The synthetic wastewater used in the kinetic experiment was the same as that in the reactor operation. NH_4Cl and $NaNO_2$ were used as a source of ammonia and nitrite, respectively. The pH was controlled at 7-8. The samples were collected and measured over time. For each ammonia and nitrite initial concentration, the maximum specific substrate utilization rate was analyzed by finding the initial slope of the ammonia/nitrite consumption with respect to time. The maximum specific substrate utilization rates that were obtained for each ammonia and nitrite concentration were used to construct Monod kinetics. Half-saturation constants (K_s) and maximum specific substrate utilization rates (q_{max}) were determined using SigmaPlot® version11. The amount of biomass attached on the plastic media was analyzed via MLSS/MLVSS, which was then used to estimate specific substrate utilization rates (q). The Monod equation can be written as shown in (1).

$$q = \frac{q_{max} S}{K_s + S} \quad (1)$$

where, q = specific rate of substrate utilization (mg-N/hr. mgMLVSS)
q_{max} = maximum specific rate of substrate utilization (mg-N/hr. mgMLVSS)
S = substrate concentration (mg-N/l)
K_S = half saturation constant (mg-N/l)

MLSS/MLVSS measurement

The biomass attached on the plastic media were sonicated by a probe sonicator to wash out the biomass from the plastic media for 30 minutes. Then, the samples were filtered. MLSS and MLVSS were measured using the gravimetric method. The amount of biomass in MLSS and MLVSS per volume of plastic media was then used in kinetic estimation.

III. Results And Discussion

Reactor operation

The results of reactor operation of both reactors are shown in Figure 1-4. Figure 1 and 2 show ammonia concentrations of both reactors. For Reactor A, the results show that the average ammonia concentration in the influent was 47.95±8.89 mg-N/l and the average remaining ammonia after 1 day operation was at 0.033±0.081 mg-N/l. For Reactor B, the results show that the average ammonia in the influent was 1.044±0.206 mg-N/l and the average remaining ammonia after 1 day operation was at 0.058±0.063 mg-N/l. The remaining ammonia after each batch was nearly zero for the entire operation for both reactors, suggesting that ammonia oxidation occurred effectively in these systems. It should be noted that ammonia was added into the systems almost every day although the ammonia concentrations were not measured every day.

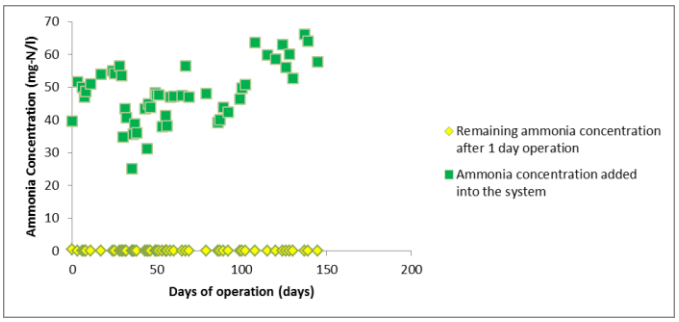

Fig. 1 Ammonia concentration in reactor A (50mg-N/l).

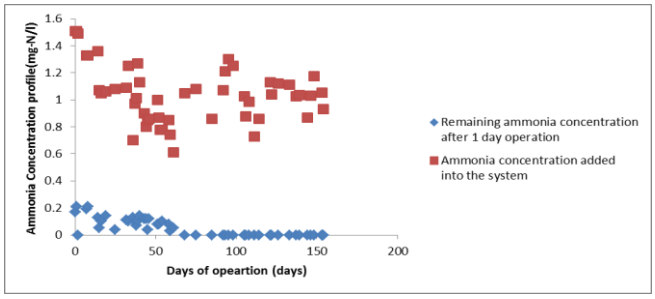

Fig. 2 Ammonia concentration in reactor B (1 mg-N/l).

Figure 3 and 4 show the concentrations of nitrogen species in both Reactor A and B, including the influent ammonia concentrations, remaining nitrite concentrations, and nitrate concentrations. The dash lines represent the days that the fresh synthetic wastewater was replaced to prevent the accumulation of toxic substances in the systems. The remaining nitrite concentrations of both reactors were 0.043 ± 0.039 mg-N/l and 0.0015 ± 0.0017 mg-N/l, respectively, suggesting that nitrite oxidation occurred effectively in the systems. As a result, AOM and NOB are expected to be successfully enriched in the systems, and ammonia was completely oxidized to nitrate. The other parameters related to the reactor operation are shown in Table 1 and 2.

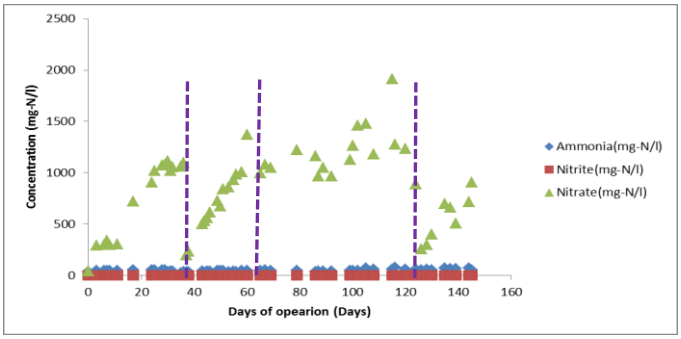

Fig. 3 Concentrations of nitrogen species in Reactor A.

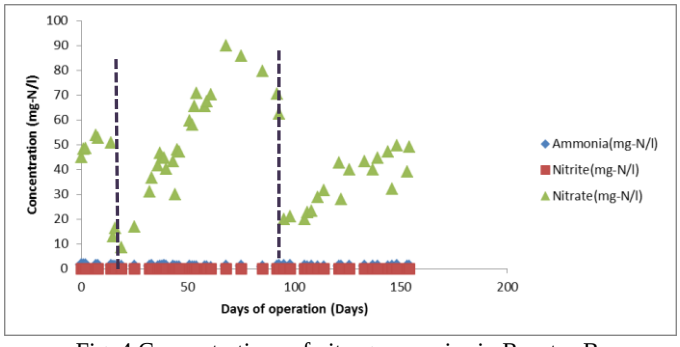

Fig. 4 Concentrations of nitrogen species in Reactor B.

TABLE I
THE CONTROLLED PARAMETERS IN REACTOR A.

Parameters	Average ± S.D.
pH	7.93 ± 0.28
Temperature (^{o}C)	29.64 ± 0.90
Dissolved oxygen (mg O_2/l)	7.55 ± 0.61
Salinity (ppt)	16.87 ± 2.66

TABLE II
THE CONTROLLED PARAMETERS IN REACTOR B.

Parameters	Average ± S.D.
Initial pH	7.61 ± 0.19
pH after 1 d of batch operation	6.95 ± 0.26
Temperature (^{o}C)	29.64 ± 0.90
Dissolved oxygen (mg O_2/l)	7.64 ± 0.47
Salinity (ppt)	15.65 ± 1.63

Kinetic experiment

The results of ammonia oxidation kinetics of the biocarriers obtained from Reactor A and Reactor B are shown in Figure 5 and 6, respectively. The results show that the AOM enriched in Reactor A fed with high concentrations of ammonia had the maximum specific substrate utilization rate (q_{max}) and half-saturation constant (K_S) of 0.121 mg-N/hr.mgMLVSS and 8.79 mg-N/l, respectively. These values were higher than the AOM enriched in Reactor B, which had the q_{max} and K_s of 0.0089 mg-N/hr.mgMLVSS and 1.19 mg-N/l, respectively.

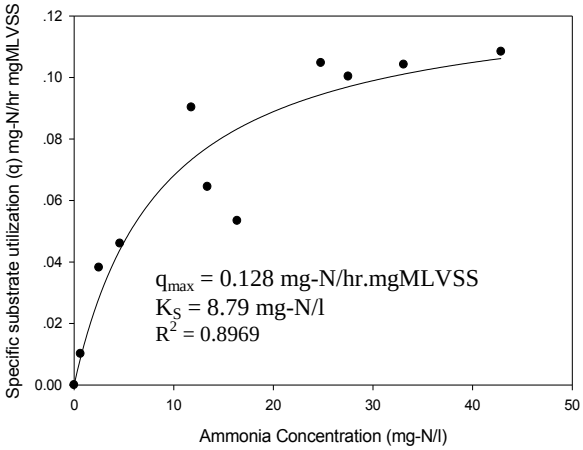

Fig. 5: Kinetic characteristics of ammonia-oxidizing microorganisms enriched in Reactor A (50 mg-N/l ammonia).

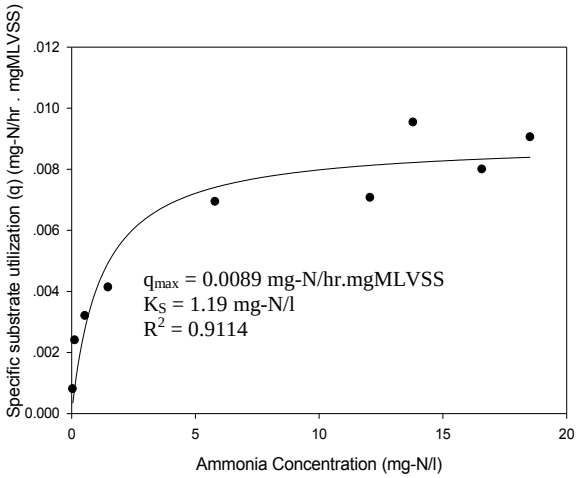

Fig. 6 Kinetic characteristics of ammonia-oxidizing microorganisms enriched in Reactor B (1 mg-N/l ammonia).

The results of ammonia oxidation kinetics clearly show the differences in the kinetics of AOB enriched at high and low ammonia concentrations. The AOB in Reactor A enriched at 50 mg-N/l of ammonia appeared to have lower affinity to

ammonia compared to those enriched in Reactor B at 1 mg-N/l of ammonia, according to their K_s values (8.79 mg-N/l ammonia for Reactor A and 1.19 mg-N/l ammonia for Reactor B.

Previous studies have reported the K_s values of low ammonia affinity AOB. Jung et al. [11] reported the K_s values of *Nitrosomonas europaea* in the range of 7.72-18.2 mg-N/l. Martens-Habbena et al. [12] also reported the K_s values of *Nitrosomonas europaea*, *Nitrosomonas communis*, and *Nitrosomonas eutropha*, in the range of 0.7-14 mg-N/l, 14-70 mg-N/l, and 11.2-70 mg-N/l, respectively. In addition, Chodanon [7] investigated the kinetics of AOM enriched at a high ammonia concentration (25 mg-N/l) in a continuous system. It was found that *Nitrosomonas europaea* was the dominant AOM and the K_s value for ammonia of this enrichment was 9.23 mg-N/l. Despite the mentioned previous studies, Park and Noguera [13] reported the lower values of K_s of *Nitrosomonas europaea*, which were in the range of 0.65-2.59 mg-N/l. These values were in the same range as the Ks reported by Keen and Prosser [14] which was 3.65 mg-N/l.

On the other hand, previous studies have reported the K_s values of high ammonia affinity AOB, such as *Nitrosomonas oligotropha*, *Nitrosomonas marina*, and *Nitrosospira* group. Martens-Habbena et al. [12] reported the K_s values of *Nitrosomonas oligotropha* and *Nitrosospira* sp. in the range of 0.28-1.4 mg-N/l and 1.26-9.8 mg-N/l, respectively. Park and Noguera [13] reported K_s value of *Nitrosomonas oligotropha* in the range of 0.79-1.65 mg-N/l. Limpiyakorn et al. [15] also reported the K_s of *Nitrosospira* spp. within the range of 1-7 mg-N/l. In addition to AOB, there are some studies that reported the K_s values of AOA, which were in the range of 0.0014-0.00182 mg-N/l [11,12].

According to the K_s values from previous research, Reactor A was likely to consist of low ammonia affinity AOB, such as *Nitrosomonas europaea*, *Nitrosomonas communis*, and *Nitrosospira* spp. since the K_s value of the AOM in Reactor A (8.79 mg-N/l) fell into the range of K_s values of these species. For Reactor B, *Nitrosomonas oligotropha* and/or *Nitrosospira* spp. might probably dominate the AOM community in the reactor. Despite the low K_s (1.19 mg-N/l) in Reactor B, AOA might hardly be found in Reactor B since the K_s values of AOA was in the extremely low range.

For the maximum specific substrate utilization rates (q_{max}), there are not many studies showing clearly differences in q_{max} for the high and low ammonia affinity AOM. Nevertheless, the results obtained in this study show that the q_{max} of AOM in Reactor A (0.128 mg-N/hr.mgMLVSS), which had lower affinity to ammonia, were higher than those in Reactor B (0.0089 mg-N/hr.mgMLVSS).

The results of nitrite oxidation kinetics of the biocarriers obtained from Reactor A and B are shown in Figure 7 and 8, respectively. From the results, the NOB enriched in Reactor A had the q_{max} and K_s of 0.048 mg-N/hr.mgMLVSS and 2.57 mg-N/l, respectively whereas the NOB enriched in Reactor B had the q_{max} and K_s of 0.0086 mg-N/hr.mgMLVSS and 1.34 mg-N/l, respectively.

The results of nitrite oxidation kinetics show that despite the great differences in the q_{max}, the K_s values of NOB from both reactors were not much different (2.57 and 1.34 mg-N/l for Reactor A and B, respectively). It should be noted that nitrite accumulation was not observed in both reactors, suggesting the very low nitrite concentrations in both reactors throughout the operation. Since NOB in both reactors were exposed to low nitrite, it was not surprising that the kinetics of NOB in both reactors would be similar, unlike the case of ammonia oxidation.

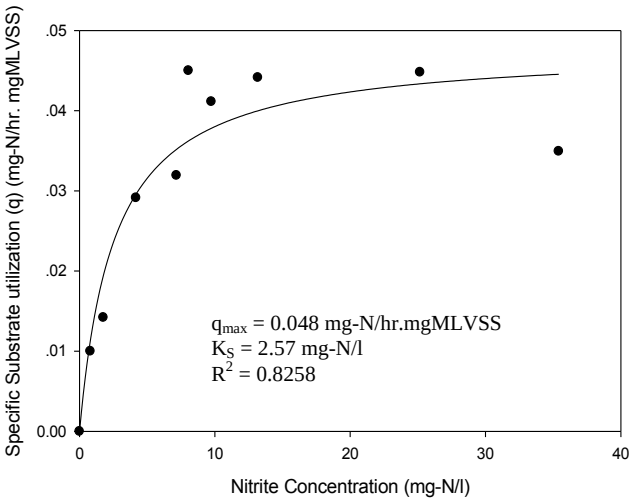

Fig. 7 Kinetic characteristics of nitrite oxidizing bacteria enriched in Reactor A (50 mg-N/l ammonia).

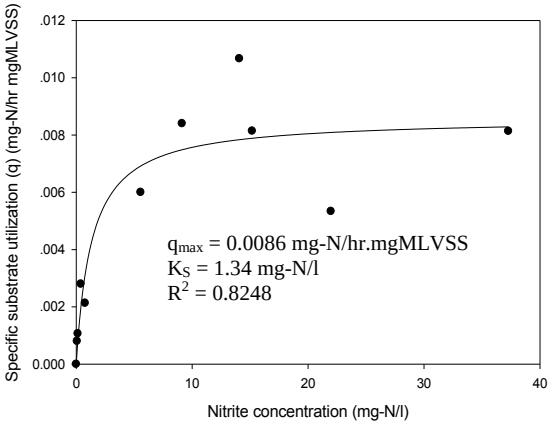

Fig. 8 Kinetic characteristics of nitrite-oxidizing bacteria enriched in Reactor B (1 mg-N/l ammonia).

From a previous study, the K_s of *Nitrobacter*, low nitrite affinity NOB, was reported to be 1.3 mg-N/l [16]. Schramm et al. [17] also reported the K_s of *Nitrobacter* to be in the range of 0.84-8.4 mg-N/l. Moreover, according to Tangkitjawisut et al. [6], the K_s values of the NOB enriched at high nitrite concentrations (3, 20, 100 mg-N/l) were found to be in the range of 8.36-12.20 mg-N/l, in which the dominant NOB was *Nitrobacter*.

For high nitrite affinity NOB, such as *Nitrospira*, Blackburne et al. [16] reported the K_s value of *Nitrospira* to be 0.9 mg-N/l, which lies in the same range those reported by

Manser et al. [18] which was between 0.13-1 mg-N/l. In addition, Tangkitjawisut et al. [6] found that the NOB enriched at low nitrite concentrations (0.1, 0.5 mg-N/l), in which *Nitrospira* was the dominant NOB, had the K_s values in the range of 0.71-0.98 mg-N/l.

According to the results, Reactor A and B show only slight differences in the K_s values (2.57 and 1.34 mg-N/l for Reactor A and B, respectively). Moreover, from the K_s values, it was difficult to identify the dominant group of NOB in these systems.

Although this study reveals the kinetic characteristics of ammonia and nitrite oxidation in the reactors enriched at low and high ammonia concentrations, microbial communities of AOM and NOB in these systems have not yet been identified. Fluorescence *in situ* hybridization (FISH) will be further used to investigate the groups of AOM and NOB in these systems. In addition, the shifts in kinetic characteristics after switching from high ammonia concentration to low ammonia concentration and vice versa will be further investigated along with the FISH analysis to observe the changes in microbial communities in both systems.

IV. Conclusion

Ammonia-oxidizing microorganisms enriched at high and low ammonia concentrations show distinctive ammonia oxidation kinetics as shown by the great differences in half-saturation constants (K_s), which were 8.79 mg-N/l and 1.19 mg-N/l when enriched at 50 mg-N/l and 1 mg-N/l of ammonia, respectively. On the other hand, nitrite-oxidizing bacteria found in the reactors enriched with high and low ammonia concentrations were not much different in terms of nitrite oxidation kinetics. The K_s values of nitrite oxidation were 2.57 and 1.34 mg-N/l for the reactors enriched with high and low ammonia, respectively.

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