HAYATI
Journal of Biosciences

Vol. 31 No. 4, July 2024 630-640
DOI:10.4308/hjb.31.4.630-640
ISSN: 1978-3019
EISSN: 2086-4094

Denitrifier Still Has the Important Role in Nitrate Reduction to N_2 Although It is Not the Predominant Population in the Estuarine Bacterial Community of Nitrate Reducing Bacteria

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ARTICLE INFO

Article history:
Received November 29, 2022
Received in revised form February 21, 2024
Accepted March 6, 2024

KEYWORDS:
denitrification,
estuary,
nitrate-ammonification,
nitrate reduction,
nitrite respiring

ABSTRACT

Denitrification and nitrate-ammonification are the responsible processes for nitrate removal in the estuaries. Temperature, nitrate and organic carbon availability are key factors controlling a rate of the nitrate reduction processes. This mixed cultures chemostat study investigates the competition ability and their nitrate reduction end-products of the bacteria isolated from an estuary at different temperatures. This study will help us to understand the seasonal nitrate reduction processes in an estuary. The experiments showed that a nitrate-ammonifier was the predominant process in the steady-state chemostat at high temperature. While a facultative denitrifier-nitrate ammonifier was the predominant process at low temperature. However, the main end products of nitrate reduction at high temperature were up to 61% N_2 indicating a denitrifier still had an important role in the end products of nitrate reduction in the estuary. The data also showed that a nitrite respiring bacterium reduced nitrite to N_2, that responsible for approximately 6-9% of total N_2 produced in the culture. This study confirmed that nitrate ammonifiers out-compete denitrifiers at high temperature, however, denitrifiers still had an important role in end products of nitrate reduction.

1. Introduction

Denitrification and nitrate-ammonification are the competing processes of microbial nitrate-reduction processes in the estuaries. These processes are the responsible processes for nitrate removal in the estuarine. Nitrate and organic carbon availability are key factors controlling rates of these processes (Dong *et al.* 2000; Fulweiler and Heiss 2014; Zhang *et al.* 2023; Murgulet *et al.* 2024). Denitrification is a reducing process of nitrate to N_2O or N_2 gas. And nitrate ammonification is a process of reducing nitrate to nitrite and subsequently to ammonium. This process accumulates high nitrite and also produces N_2O as a byproduct. Some fermenting bacteria such as *Bacillus licheniformis*, *Citrobacter freundii*, *Klebsiella oxytoca*, *K. pneumoniae*, and *Escherichia coli* have been reported having the ability to perform this process. However some strains of *Shewanella* sp. and *Serratia* sp. can possess both the denitrification and nitrate ammonification (Yoon *et al.* 2015).

The temperature could also affect the competitive ability of these bacterial processes. Denitrification is the dominant process at low temperature (winter), and nitrate reduction to ammonium at high temperature (summer). While in tropical estuaries, nitrate ammonification dominates over denitrification (Dong *et al.* 2011). In sandy tidal sediment increasing of carbon to nitrate ratio, the supply of nitrite relative to nitrate and the microbial generation time determine whether nitrate is reduced to gaseous end products or to ammonium (Kraft *et al.* 2014). Only when nitrate was added instead of nitrite at a relatively high generation time, nitrate ammonification availed; otherwise, denitrification dominated (Bu *et al.* 2017). Relationship between nitrate ammonifiers and organic matter and NO_2^-, suggesting that these

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substrates stimulated the metabolism of nitrate ammonifying bacteria and most of the converted ammonium was retained in the estuarine ecosystem (Yin *et al.* 2017).

Some denitrifiers and nitrate ammonifiers have been isolated from estuarine sediment i.e. a nitrate ammonifier (*K. pneumoniae*), a facultative denitrifier-nitrate ammonifier (*S. liquefaciens*), a nitrite-respiring bacterium (*Alcaligenes faecalis*) and a denitrifier (*Comamonas testosteroni*) (Rusmana 2006, 2007). Therefore this pure culture study investigates the growing competition between the bacteria carrying out these processes. It could help us to understand the growth and end-products of nitrate reduction in the estuaries.

2. Materials and Methods

2.1. Bacterial Isolates

Four bacteria representing a denitrifier (*C. testosteroni*), nitrate ammonifier (*K. pneumoniae*), a facultative denitrifier-nitrate ammonifier (*S. liquefaciens*), and a nitrite-respiring bacterium (*A. faecalis*) were used to determine their competition ability for nitrate in steady state at 5, 10, 15, and 20°C of nitrate-limiting chemostat with mixed cultures of (1) a denitrifier and a nitrate ammonifier, (2) a denitrifier, a nitrate ammonifier, and a facultative denitrifier-nitrate ammonifier (3) a denitrifier, a nitrate ammonifier, a facultative denitrifier-nitrate ammonifier, and a nitrite respiring bacterium.

2.2. Chemostat Conditions

The chemostat experiments were conducted using a nitrate-limiting medium. The culture was conducted at four different temperatures at 5, 10, 15, and 20°C, using a 500 ml glass vessel sealed with a rubber bung (Rusmana 2006). A peristaltic pump (multipex; LKB, Broma, Sweden) was adjusted to pump a sterile medium with dilution rate 0.02 h^{-1} for incubation for temperatures of 10, 15 and 20°C, and 0.01 h^{-1} for a temperature of 5°C. An anaerobic condition was maintained by gassing the vessel and medium with sterile oxygen-free nitrogen (OFN). The OFN flow rate was 6 ml min^{-1} and the OFN was passed through a chromous acid trap to remove out any trace amount of O_2. The temperature in the glass vessel was adjusted and maintained by circulating a coolant liquid passed through a glass jacket around the growth vessel that regulated by a thermo-circulator (Grant CFC25, Grant Instruments, Cambridge Ltd.) attached to a FH15 flow heater (Grant Instruments, Cambridge Ltd.).

The medium was used with addition of 5560 µM glycerol, as *K. pneumoniae* and *S. liquefaciens* could not use acetate as their carbon source. The microbial growth was monitored by measuring the OD with a spectrophotometer at 550 nm. Steady state was achieved when the standard deviation (SD) at least six OD readings was < 2% of the mean OD value. The culture samples at steady state were collected aseptically for measuring the total number of bacteria, nitrate, nitrite, ammonium, glycerol, and acetate concentrations. Headspace gas samples were also taken for analyzing N_2O concentrations.

2.3. Counting of Bacterial Numbers

The total number of bacteria was determined using a plate counts technique on nutrient agar, supplemented with 10 mM KNO_3 and 15‰ NaCl. The steady state samples were diluted serially with ¼ strength Ringer's solution, and 0.1 ml of suitable dilutions were plated in triplicate on the agar plates and incubated at 20°C for 3 days. The bacterial pure cultures of *C. testosteroni, K. pneumoniae, S. liquefaciens*, and *A. faecalis*, were also plated on the agar plates, and used as colony morphology references to determine the colonies for each species. Each bacterium could be distinguished from others; *C. testosteroni* developed cream, translucent, circular, pulvinate colonies; *K. pneumonaie* developed whitish cream, opalescent, circular, convex colonies; *S. liquefaciens* developed reddish cream, translucent, round, convex colonies; and *A. faecalis* developed whitish cream, circular, translucent pinhead colonies.

2.4. Chemical Analysis

Nitrate, nitrite, and ammonium were analyzed colorimetrically (Parson *et al.* 1984). Nitrate was analyzed as nitrite after the samples were passed through a copper-cadmium reduction column (Parsons *et al.* 1984). Ammonium was measured using a modified indophenol blue method with dichloroisocyanurate as chlorine donor and developed in the dark (Harwood and Kuhn 1970; Gravitz and Gleye 1975; Krom 1980).

The N_2O concentration was analyzed using a gas chromatograph (Shimazu GC 14A, Dyson, Instruments, Washington, UK) with a 36Ni electron capture detector (ECD) at 340°C, with an argon-methane carrier gas (50 ml min^{-1}, 95% Ar, 5% CH_4).

The temperature of the internal main stainless steel column (4 m × 2 mm i.d.) packed with Porapak Q (60-80 mesh, Millipore Corporate, Milliford, UK) was set up at 25°C.

3. Results

3.1. Bacterial Growth Competition

Bacterial cell concentration in mixed cultures of nitrate limited chemostats showed that the nitrate ammonifier (*K. pneumonia*) was always dominant at high temperature in all mixed cultures of nitrate limited chemostats and increased by increasing the temperatures (Figure 1). The percentages of the population dominance were also increased by increasing the temperatures. However, the percentages of its dominance were decreased by increasing the number of bacterial types of nitrate metabolisms in the mixed culture chemostats. At the highest temperature 20°C, its population percentage was 98% in the mixed culture of a nitrate ammonifier and a denifier, decreasing to 80% in the mixed culture of a nitrate amonifier, a denifier and a facultative denitrifier-nitrate ammonifier, the to 49% in the mixed culture of a nitrate ammonifier, a denifier, and a facultative denitrifier-nitrate ammonifier (Figure 2 and 3). In the contrary, the denifier (*C. testosteroni*), was always at the lowest bacterial population (for about 1-3% in the population) in all conditions of temperatures and bacterial types number of nitrate metabolisms in the mixed culture chemostats.

The facultative denitrifier-nitrate ammonifier (*S. liquefaciens*) was dominant at low temperature. Its cell density and percentage of bacterial dominance was decreased by increasing the temperatures. At the lowest temperature (5°C). its population percentage was 89% in the mixed cultures of a nitrate ammonifier, a denifier and a facultative denitrifier-nitrate ammonifier and 61.9% in the mixed culture of a nitrate amonifier, a denifier, a facultative denitrifier-nitrate ammonifier, and a nitrite respiring bacterium (Figure 2 and 3). Moreover, bacterial cell density and population percentage of an obligate nitrite utiliser (*A. faecalis*) was increased by increasing the temperatures. It was similar trend with that of the nitrate ammonifier, although with lower bacterial cell density and population percentage (Figure 3).

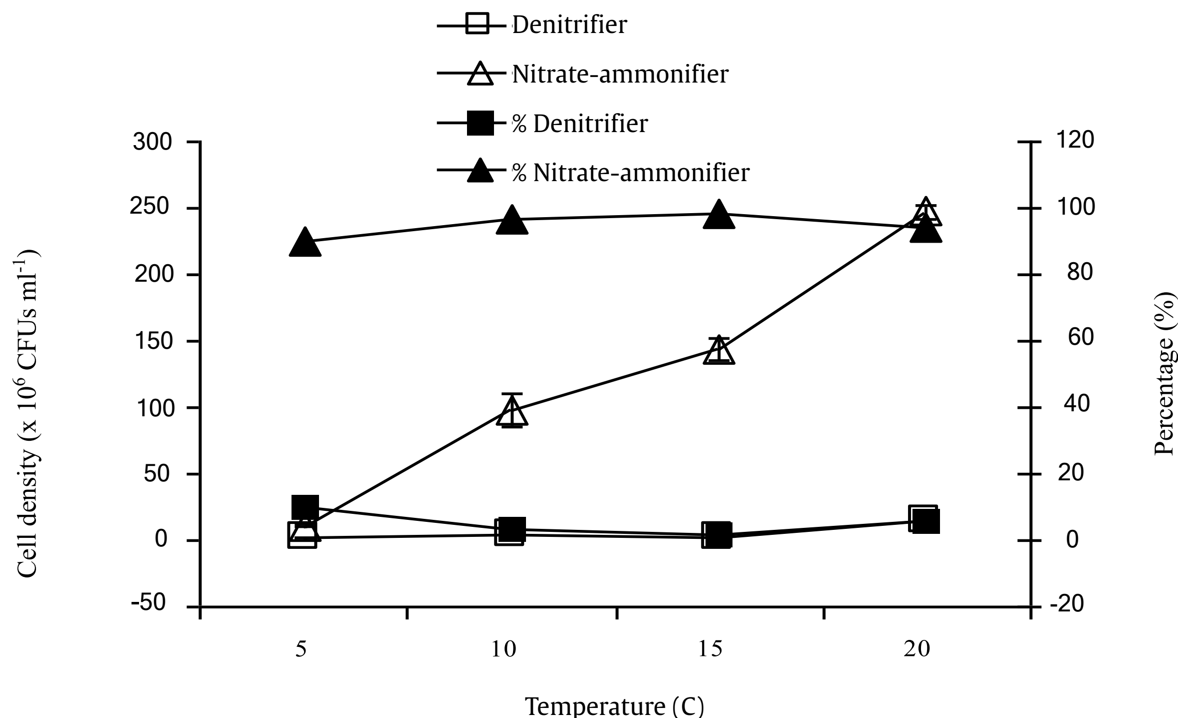


Figure 1. Cell density and percentage of a denitrifier and a nitrate-ammonifier, from the steady state of mixed culture chemostats at 5, 10, 15, and 20°C. Bars indicate standard errors (n = 3)

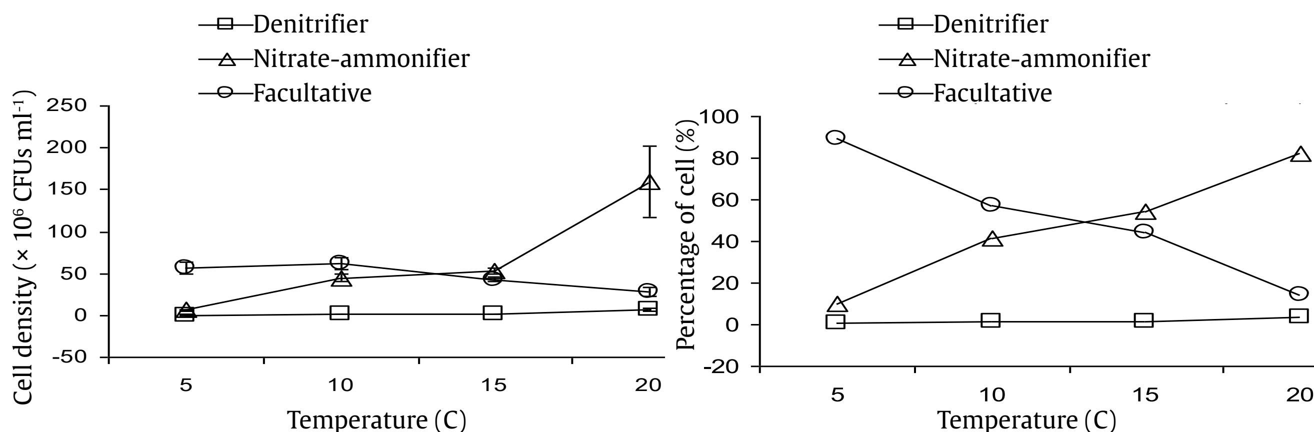


Figure 2. Cell density and percentage of a denitrifier, a nitrate-ammonifier, and a facultative denitrifier-nitrate ammonifier from the steady state of mixed culture chemostats at 5, 10, 15, and 20°C. Bars indicate standard errors (n = 3)

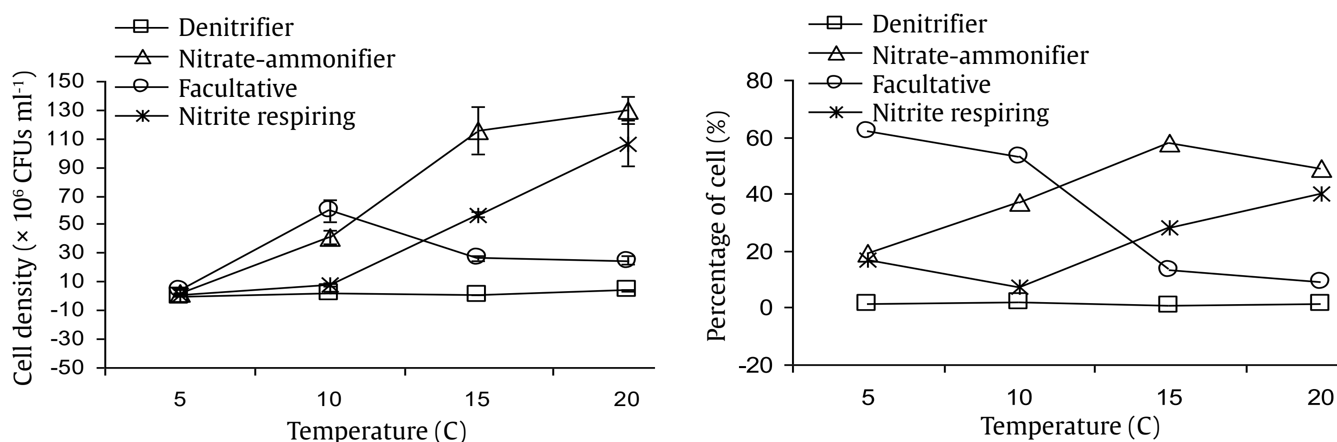


Figure 3. Cell density and percentage of a denitrifier, a nitrate-ammonifier, a facultative denitrifier-nitrate ammonifier and a nitrite respiring bacterium from the steady state of mixed culture chemostats at 5, 10, 15, and 20°C. Bars indicate standard errors (n = 3)

3.2. End-Products of Nitrate Reduction

The all mixed cultures utilized all nitrate (100%) in the steady state chemostat at the temperature of 10, 15, and 20°C. However at low temperature (5°C), 88% nitrate was utilized in the mixed culture of f a nitrate amonifier and a denifier, increasing to 91.5% in the mixed culture of a nitrate amonifier, a denifier and a facultative denitrifier-nitrate ammonifier, then to 97.2% in the mixed culture of a nitrate amonifier, a denifier, a facultative denitrifier-nitrate ammonifier and a nitrite respiring bacterium. Nitrite accumulation was high at low temperature (5°C), and the highest nitrite accumulation (5.9% of reduced nitrate) in the mixed culture of a nitrate ammonifier. However nitrite accumulation at the other temperatures was low (0.01-0.022% of reduced nitrate) in all mixed cultures (Table 1, 2, and 3). Similarly, ammonium production was high at low temperature (5°C) and the highest ammonium production (51.2% of reduced nitrate) was found in the mixed culture of a nitrate amonifier, a denifier and a facultative denitrifier-nitrate ammonifier. The trend of ammonium production was decreased by increasing temperature in all mixed cultures. The nitrogen assimilated to be organic compounds of bacterial cells was increased by increasing the temperatures. Percentages of assimilated nitrogen were 3-6% of reduced nitrate at 5°C, and 39-49%, 44-62% and 38-48% at 10 °C, 15°C and 20°C respectively (Table 1, 2, and 3).

Table 1. Concentrations of nitrate reduced and percentage recovery of nitrate reduction end-products during mixed cultures of a denitrifier, and a nitrate-ammonifier in steady state nitrate-limited chemostats at 5, 10, 15, and 20°C (Average + standard error; n = 3)

Temperature (°C)	Steady state concentrations of								NO_3^- recovery (%)	Cellular N (µM)	N_2 (%)
	NO_3- reduced		NO_2- produced		NH_4+ produced		N_2O produced				
	(µM)	(%)	(µM)	(%)	(µM)	(%)	(µM)	(%)			
5	968.25±78.4	88.02	57.30±16.68	5.92	361.53±59.32	37.34	0.10±0.005	0.01	47.67	42.65±4.48	52
10	1100±0	100.00	0±0	0.00	319.09±17.08	29.00	0.25±0.06	0.02	39.66	17.08±6.89	60
15	1100±0	100.00	1.93±0.04	0.17	301.97±43.46	27.45	0.19±0.005	0.02	44.11	181.18±2.21	56
20	1100±0	100.00	1.47±0.65	0.13	344.92±28.61	31.35	1.41±0.03	0.13	48.36	184.28±4.78	52

Table 2. Concentrations of nitrate reduced and percentage recovery of nitrate reduction end-products during mixed culture of a denitrifier, a nitrate-ammonifier, and a facultative denitrifier-nitrate ammonifier in steady state nitrate-limited chemostats at 5, 10, 15, and 20°C (Average + standard error; n = 3)

Temperature (°C)	Steady state concentrations of								NO_3^- recovery (%)	Cellular N (µM)	N_2 (%)
	NO_3- reduced		NO_2- produced		NH_4+ produced		N_2O produced				
	(µM)	(%)	(µM)	(%)	(µM)	(%)	(µM)	(%)			
5	1006.5±65.6	91.50	2.25±0.72	0.22	515.36±96.07	51.20	0.24±0.01	0.02	51.84	64.53±3.80	42
10	1100±0	100.00	0±0	0.00	319.72±22.33	29.06	0.23±0.01	0.02	48.87	217.74±18.7	51
15	1100±0	100.00	2.07±0.16	0.19	428.02±43.46	38.91	0.31±0.003	0.03	62.16	253.30±9.01	38
20	1100±0	100.00	1.85±0.34	0.17	274.54±23.80	24.96	1.35±0.02	0.12	44.64	213.35±6.76	55

Table 3. Concentrations of nitrate reduced and percentage recovery of nitrate reduction end-products during mixed cultures of a denitrifier, a nitrate-ammonifier, a facultative denitrifier-nitrate ammonifier and a nitrite respiring bacterium in steady state nitrate-limited chemostats at 5, 10, 15, and 20°C (Average + standard error; n = 3)

Temperature (°C)	Steady state concentrations of								NO_3^- recovery (%)	Cellular N (µM)	N_2 (%)
	NO_3- reduced		NO_2- produced		NH_4+ produced		N_2O produced				
	(µM)	(%)	(µM)	(%)	(µM)	(%)	(µM)	(%)			
5	1069.3±36.8	97.21	14.16±4.85	1.32	513.38±41.33	48.01	0.54±0.02	0.05	52.68	35.32±5.28	47
10	1100±0	100.00	0±0	0.00	273.50±18.74	24.86	0.65±0.02	0.06	39.03	155.22±18.4	61
15	1100±0	100.00	1.78±0.08	0.16	380.00±30.69	34.54	0.17±0.005	0.01	53.84	210.53±0.94	46
20	1100±0	100.00	1.35±0.11	0.12	239.22±20.05	21.74	0.29±0.005	0.02	38.55	183.41±1.99	62

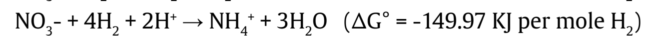
N_2O gas was produced in a small amount, 0.01–0.13% of utilized nitrate. However there was indication that N_2O gas production was increased by increasing temperatures in the mixed cultures without a nitrite respiring bacterium, but the opposite trend was found in the mixed culture with a nitrite respiring bacterium (Table 1, 2, and 3). By calculation, the nitrogen recovery from the reduced nitrate was 38 up to 62% that estimation of the nitrate reduced into N_2 was 38 up to 62%. There was no trend indication of percentage of the nitrate reduced into N_2 regarding temperature changes. The average of the estimated percentage for all temperatures in each mixed culture was 46.6-55.0%.

4. Discussion

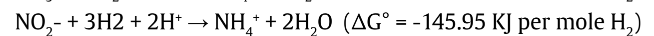
The competition experiments between a denitrifier and a nitrate ammonifier showed that nitrate ammonifier was the dominant species in the steady state chemostats at all temperatures. In substrate limited condition the ability of one species to compete to others depend on the ability of the species to sequester a substrate/nutrient. The indicator of species ability to sequester a substrate is the specific affinity (a_A), termed as μ_{max}/K_s (Nedwell 1999). The specific affinity for nitrate of *Klebsiella pneumoniae* (a nitrate ammonifier) with glycerol as a substrate is more than 10 times

that of *Comamonas testosteroni* (a denitrifier) with acetate (Table 4). Specific affinity for nitrate is affected by the environmental temperature and the type of carbon sources used (Nedwell 1999). The big differences of the a_A values of the nitrate ammonifier made it out compete for the denitrifier. However, the nitrate ammonifier did not completely exclude the denitrifier. The main end products from nitrate reduction in the chemostat mixed cultures of a denitrifier and a nitrate-ammonifier were average 55% N_2, 30% ammonium and 0.01% nitrite at 10–20°C; and 52% N_2, 37% ammonium, and 6% nitrite at 5°C. Ammonium production in the mixed culture chemostat at 10, 15, and 20°C was lower than that of a culture of a nitrate-ammonifier alone (Lloyd 2000) indicating that the denitrifier has an effect in the balance of end products when in co-culture. One possible way was that the denitrifer denitrified nitrite to N_2 (Rusmana 2006). The same indication was also shown in nitrite production at 5°C. Nitrite production in mixed culture chemostat was decreased up to 77% of than that of a culture of a nitrate-ammonifier alone (Lloyd 2000). These results indicated that the denitrifier still has an important role in influencing the end product of nitrate reduction, although it was not a dominant species in the community. The denitrifier could maintain in the steady state probably by using nitrite accumulated from nitrate reduction by the nitrate-ammonifier. Nitrate acculation by the nitrate ammonifier due to thermodynamically, nitrate reduction to nitrite is more efficient to gain energy than to ammonium. The ΔG° per mole of H_2 at pH 7 of nitrate reduction to nitrite is more negative than nitrate reduction to ammonium or nitrite reduction to ammonium as shown below:

$$NO_3^- + H_2 \rightarrow NO_2^- + H_2O \quad (\Delta G^\circ = -163.03 \text{ KJ per mole } H_2)$$

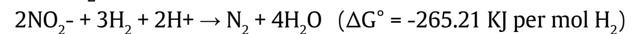


$$NO_3^- + 4H_2 + 2H^+ \rightarrow NH_4^+ + 3H_2O \quad (\Delta G^\circ = -149.97 \text{ KJ per mole } H_2)$$

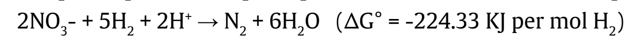


$$NO_2^- + 3H2 + 2H^+ \rightarrow NH_4^+ + 2H_2O \quad (\Delta G^\circ = -145.95 \text{ KJ per mole } H_2)$$

Nitrite, on the other hand, is also more favorable as an electron acceptor for denitrification than nitrate as shown on the following free energy (ΔG°) values per mole H_2 at pH 7 as shown below:



$$2NO_2^- + 3H_2 + 2H+ \rightarrow N_2 + 4H_2O \quad (\Delta G^\circ = -265.21 \text{ KJ per mol } H_2)$$



$$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O \quad (\Delta G^\circ = -224.33 \text{ KJ per mol } H_2)$$

N_2O production at low temperature was higher than that of high temperature. The production was only a small proportion of the nitate reduced, between 0.01–0.13%.

The next experiment competing a denitrifier, nitrate-ammonifier and facultative denitrifier-nitrate ammonifier (*S. liquefaciens*) showed that the nitrate-ammonifier was again the dominant species in the steady state chemostat at high temperature (20°C) but facultative denitrifier-nitrate ammonifier was the dominant species at low temperature (5°C). While the denitrifier was still out competed, but it was not excluded completely from the chemostat. Probably the denitrifier could maintain its presence in the chemostat by using nitrite with similar indication as described above. The specific affinity for nitrate of the facultative denitrifier-nitrate ammonifier at low temperature was higher (10°C, $a_{A(NO3-)}$ = 0.00265 l μmol^{-1} h^{-1}) than that of the nitrate ammonifier (10°C, $a_{A(NO3-)}$ = 0.00114 l μmol^{-1} h^{-1}) (Nedwell 1999). At high temperature, on the contrary, the nitrate ammonifier had higher specific affinity for nitrate (20°C, $a_{A(NO3-)}$ = 0.00221 l μmol^{-1} h^{-1}) than the facultative denitrifier-nitrate ammonifier (20°C, $a_{A(NO3-)}$ = 0.00005 l μmol^{-1} h^{-1}) (Nedwell 1999). The environmental temperature affects the specific affinity for substrate due to its effect on active transport of substrates (Nedwell 1999). Nitrate is taken into cells by active transports whether using nitrate/nitrite antiporter mechanism via NarK2 or nitrate/proton symporter mechanism via NarK1 (Wood *et al.* 2002). Temperature affects these transporters probably on a charge energetic

Table 4. Specific affinity values for nitrate of Klebsiella pneumonaie with glycerol as a substrate and *Comamonas testosteroni* with acetate as a substrate

Species	Specific affinity for nitrate (1 μmol^{-1} h^{-1}) at			Reference
	10°C	15°C	20°C	
Klebsiella pneumoniae	0.00114	0.00177	0.00221	(Lloyd 2000)
Comamonas testosteroni	0.00006	0.00009	0.00021	(Rusmana 2007)

state for powering conformational change, and the fluidity of the membrane (Nedwell 1999). Therefore the specific affinity for nitrate in bacteria decreases along with decreasing environmental temperature (Nedwell 1999). The present data support that hypothesis as $a_{A(NO3-)}$ generally decreases with temperature. However, the facultative denitrifier-nitrate ammonifier has a high specific affinity for nitrate than the other bacteria at low temperature, possibly because the bacterium has two different nitrate reductase enzymes; Nar and Nap, that were determined the presence of *nar*G and *nap*A genes (unpublished data). The activity of Nar depends on nitrate transport by NarK1 or NarK2 because the active site of this enzyme is in the cytoplasm (Moreno-Vivian *et al.* 1999; Richardson 2000). The repression by low temperature on nitrate uptake is probably due to this transport enzyme activity, similar to that inhibition shown in the nitrate ammonifier. Molecular detection of the nitrate reductase gene presence showed that the bacterium has only *nar*G (unpublished data). Nap, in contrast, does not need nitrate transporters for its activity, because the active site of this enzyme is in the periplasm (Moreno-Vivian *et al.* 1999; Richardson 2000). Nap activity is more dominant in low nitrate conditions than that of Nar (Potter *et al.* 1999; Richardson 2000). Therefore the high specific affinity for nitrate of the facultative denitrifier-nitrate ammonifier at low temperature than that of the nitrate ammonifier may due to this Nap activity.

The main end products from nitrate reduction in this chemostat mixed culture were approximately 37-55% N_2, 35-49% ammonium and low nitrite 0.0-0.19% at 10-20°C, and 42% N_2, 51% ammonium and 0.22% nitrite at 5°C. Ammonium production in this mixed culture chemostat at 10, 15, and 20°C was lower than in the mixed culture of the nitrate ammonifier and the facultative denitrifier-nitrate ammonifier (Lloyd 2000) indicating that the denitrifier denitrified nitrite to N_2 (Rusmana 2006, 2007). At low temperature, nitrite productions in the three bacterial components mixed culture chemostats were decreased by between 40-90% of that that of in the mixed cultures of the nitrate ammonifier and the facultative denitrifier-nitrate ammonifier (Lloyd 2000) at 5 and 10°C respectively. These results indicated that the denitrifier still had an important role influencing the end product of nitrate reduction although it was not a dominant species in the community, as described previously. N_2O production was only in a small amount, 0.02% for all temperature.

Similar trends population dominance for the denitrifier, the nitrate ammonifier and the facultative denitrifier-nitrate ammonifier were found in the in the steady state chemostat mixed culture of the denitrifier, nitrate ammonifier, the facultative denitrifier-nitrate ammonifier and the nitrite respiring bacterium (*A. faecalis*). While the nitrite respiring bacterium had similar trends with the nitrate ammonifier that the bacterial cells were increased by increasing temperature indicating that there was a commensal interaction between the nitrate ammonifier and the nitrite resipiring bacterium. The the nitrite respiring bacterium used nitrite produced by the nitrate ammonifier.

The main end products from nitrate reduction in this chemostat mixed culture were approximately 46-61% N_2 and 22-34% ammonium at 10-20°C, and 47% N_2 and 48% ammonium at 5°C. Compared with previous the mixed culture experiment without the nitrite respiring bacterium, N_2 production was increased by approximately 6-9% at 10-20 °C. In contrast, ammonium production in the mixed culture chemostats at 10, 15, and 20°C was decreased by approximately 13-15% compared to mixed cultures without the nitrite respiring bacterium. These results again suggested that the nitrite respiring bacterium reduced nitrite to N_2. Molecular analyses of the nitrate reduction genes showed that the nitrite respiring bacterium (*A. faecalis*) has *Nap*A, *Nir*K, and *Nos*Z (unpublished data) indicating that this nitrite-respiring bacterium can reduce nitrite into N_2. Nitrite productions at low temperature were higher than that of high temperature. The nitrite production in steady-state chemostats was between 0.12–1.32%. N_2O production at low temperature was higher than that of high temperature. The production was only in a small amount, between 0.01–0.06%. So that the nitrate reduction mechanisms and end products of this mixed culture are probably like the scheme in Figure 4.

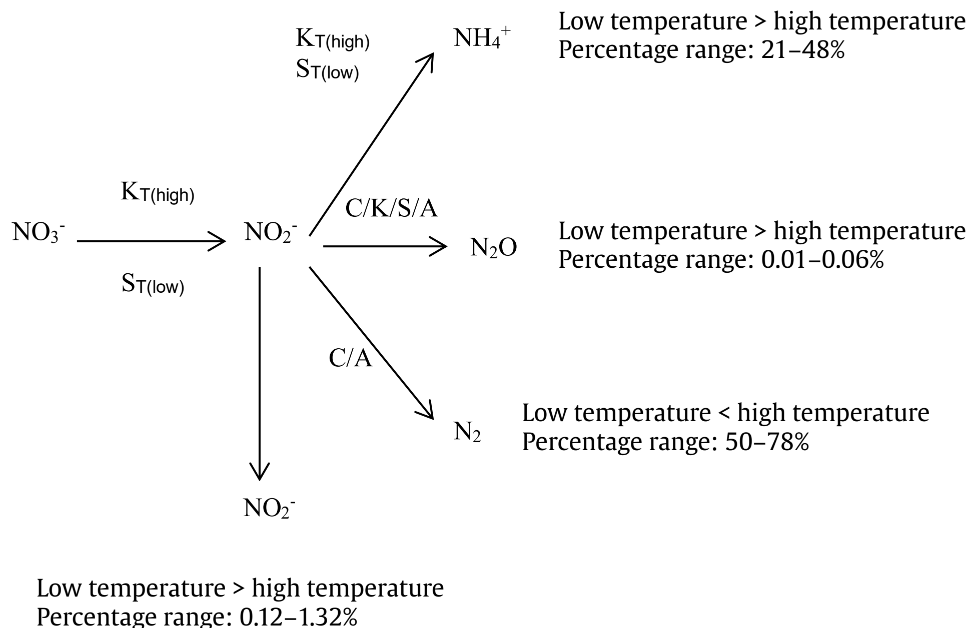


Figure 4. The illustration scheme of nitrate reduction mechanisms and end products of the mixed culture of estuarine nitrate reduction bacteria. Where K is a reduction by the nitrate ammonifier, C is a reduction by the denitrifier, S is a reduction by the facultative denitrifier-nitrate ammonifier, A is a reduction by the nitrite respiring bacterium, KT(high) is a reduction by the nitrate ammonifier at high temperatures, and ST(low) is a reduction by the facultative denitrifier-nitrate ammonifier at low temperatures

Predictions of seasonal dissimilative nitrate reduction process outcome that occur in natural environments are important to anticipate future implications of global warming as denitrification and nitrate ammonification can produce N_2O. This competition experiments for nitrate by pure cultures demonstrated that the nitrate ammonifier was a predominant species in the steady-state chemostat at high temperature (20°C). In contrast, the facultative denitrifier-nitrate ammonifier was the predominant species at low temperature (5°C). Specific affinity for nitrate $a_{A(NO3-)}$ is a suitable parameter to predict the competition ability of one species to others. The specific affinity for nitrate of the nitrate ammonifier was higher at high temperature than that of the facultative denitrifier-nitrate ammonifier. On the contrary, the facultative denitrifier-nitrate ammonifier had a higher $a_{A(NO3-)}$ value at low temperature. Environmental temperature can affect the specific affinity for nitrate due to its effect on active transport of nitrate probably on a charge energetic state for powering conformational change, and the fluidity of the membrane (Nedwell 1999), so that nitrate transport into cytoplasmic cells via NarK1, a nitrate/proton symporter, or NarK2, a nitrate/nitrite antiporter is repressed (Wood *et al.* 2002). As a result, the specific affinity values decrease along with decreasing environmental temperature.

Interestingly, the facultative denitrifier-nitrate ammonifier had a higher specific affinity for nitrate than the other bacteria at low temperature. Molecular study of dissimilative nitrate reductase genes of the bacteria (unpublished data) demonstrated that this bacterium had narG and napA genes which encode membrane-bound nitrate reductase (Nar) and periplasmic nitrate reductase (Nap) respectively, meanwhile the nitrate ammonifier had the only *nar*G gene. Smith *et al.* (2015) suggested that nitrate reduction might correlate with genetic potentials. Correspondence between rate processes and gene abundances and expression is likely to be close. Furthermore, other physical-chemical factors such as temperature may control key enzyme activity, and in-situ rates, without necessarily directly affecting the genes expression (Smith *et al.* 2015), especially nap and nar genes.

The active site of Nar is in the cytoplasm, therefore Nar activity depends on nitrate transport by NarK1 or NarK2 (Moreno-Vivian *et al.* 1999; Richardson 2000; Wood *et al.* 2002). The low-temperature repression

of nitrate uptake is probably due to this Nar activity, similar to that repression in the nitrate ammonifier. However, Nap does not need nitrate transporters for its activity, because the active site of this enzyme is in the periplasm (Moreno-Vivian *et al.* 1999; Richardson 2000), so that the high specific affinity for nitrate at low temperature in the facultative denitrifier-nitrate ammonifier might be due to this Nap activity. This result indicated that during the winter Nap activity may be higher than Nar, and it can be expected that bacteria carrying out Nap such as *S. liquefaciens* and *Shewanella* will be the predominant species in the winter season. It also becomes understandable why N_2O production was higher in the winter, probably because bacteria like Shewanella which produced N_2O as the end product of nitrate or nitrite reduction (Rusmana 2006) was dominant in the community. However, it is still unclear whether there is any physiological correlation between Nap activity and N_2O production in the winter (low temperature).

Some researchers (Berk *et al.* 1995; Richardson and Watmough 1999; Richardson 2000; Richardson *et al.* 2001) believe that Nap is a dissimilatory enzyme to regulate redox balancing, so that the bacteria can maintain and support optimal growth under some physiological conditions, such as during fermentative process in enteric bacteria and oxidative metabolism of highly reduced carbon substrates (Zumft 1997; Richardson 2000). Other proposed roles of Nap are for a transition adaptation to anaerobic metabolism from aerobic conditions (Richardson *et al.* 2001), or for a self-defense mechanism by forming high nitrite levels to inhibit the growth of potential competing bacteria (Richardson 2000; Richardson *et al.* 2001). In the estuaries, the organic carbon content of the sediment was higher in winter than summer (Dong *et al.* 2000). This may suggest that in the winter Nap acts to control redox balancing, and the reduction step of N_2O to N_2 will be repressed in order to dissipate excess reducing power and control the cyclic electron-transport system in balance. The reduction of N_2O to N_2 yields more standard free energy compared to the reduction of nitrate or nitrite to N_2O. This strategy can also reduce the potential toxicity of high accumulation of nitrite during the winter because winter temperatures might stimulate nitrite production. High concentrations of nitrite have been reported to inhibit N_2O reductase activity (Firestone and Tiedje 1979; Kaldorf *et al.* 1993), and can lead to high production of N_2O during the winter. Moreover Yoon *et al.* (2015) suggested that denitrification dominated at low carbon-to-nitrogen (C/N) ratios (that is, electron donor-limiting growth conditions), whereas nitrate ammonification was predominant at high C/N ratios (that is, electron acceptor-limiting growth conditions), and at intermediate C/N ratios, denitrification and nitrate-ammonification occurred concomitantly. Nitrate-ammonifying bacteria with high nitrate concentration produced ammonium and nitrite simultaneously during anaerobic growth on nitrate (Streminska *et al.* 2012). However, the nitrite to ammonium ratio was dependent on the bacterial species and the electron donor. The ratio of electron donor to acceptor can influence the pathway and fate of nitrate. Nitrate ammonification has a higher affinity for nitrate than denitrification and may be favored in nitrate-limited, carbon-rich environments (King and Nedwell 1985; Burgin and Hamilton 2007; Kraft *et al.* 2014). This is due to the requirement of only 5 electrons to reduce nitrate in denitrification verses the eight required for nitrate ammonification (Tiedje 1988). Nitrate ammonification may therefore, outcompete denitrification in nitrate-limited environments where these organisms gain more energy from nitrate ammonification than denitrifiers can from denitrification. Many studies in the estuaries have shown that nitrate ammonification is the dominant process of nitrate reduction. Nitrate ammonification was greater than denitrification in 30% (Giblin *et al.* 2013) and responsible for 44–74% of nitrate reduction in the estuarine sediments (Song *et al.* 2014). Therefore this pure culture study confirmed the importance of nitrate ammonification as a significant process of nitrate reduction in the estuaries, however, denitrifiers still had an important role in end products of nitrate reduction in the estuaries.

Competing of denitrification and nitrate-ammonification for nitrate removal in the estuary is affected by temperature. The experiments showed that the nitrate-ammonifier (*K. pneumonia*) was the predominant species at high temperature. In contrast, a facultative denitrifier-nitrate ammonifier (*S. liquefaciens*) was the predominant species at low temperature. There was also a commensal interaction between nitrate-ammonifier (*K. pneumonia*) and a nitrite-respiring bacterium (*A. faecalis*) at high temperature. However the main end products of nitrate reduction at high temperature were 65-78% N_2 indicated that a denitrifier (*C. testosteroni*) still had an important role in end products of nitrate

reduction, although it was not a dominant species in the estuary. The data also confirmed that a nitrite respiring bacterium (*A. faecalis*) reduce nitrite to N_2, that responsible for approximately 6-9% of total N_2 produced in the cultures. This study shows that nitrate ammonifiers out compete denitrifiers at high temperature, however denitrifiers still had an important role in end products of nitrate reduction in the estuaries.

Acknowledgements

This study was supported by visiting researcher grant from Prof. David B. Nedwell, Department of Biological Sciences, University of Essex, United Kingdom.

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