Distribution of *Nitrobacter* and *Nitrospira* Communities in an Aerobic Activated Sludge Bioreactor and their Contributions to Nitrite Oxidation

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ABSTRACT

An analysis of nitrite-oxidizing bacteria in the activated sludge process of a full-scale partially nitrifying wastewater treatment plant revealed *Nitrospira* and *Nitrobacter* averaged 10^{13} cells·L^{-1} and 10^{12} cells·L^{-1}, respectively. Correlation coefficients linking shifts in NOB community to operational or environmental variables illustrated *Nitrospira* were negatively correlated to nitrite (r = -0.45, P<0.01), while *Nitrobacter* showed no significant relationship to nitrite (P=0.1017). *Nitrospira* was negative correlation to DO (r = -0.46, P<0.01) and positively correlated to temperature (r = 0.59, P<0.0001). However, *Nitrobacter* was positively correlated to DO (r = 0.38, P<0.01) and HRT (R = 0.33, P<0.05), as well as negatively correlated to temperature (r = -0.49, P<0.001) suggesting niche adaptations within the NOB community. The positive association between *Nitrobacter* and DO supports a selective advantage over *Nitrospira* in completely nitrifying plants. Given the operational schematic at this WWTP, *Nitrospira* contributed more to nitrification than *Nitrobacter* in this WWTP.

Keywords: *Nitrospira*, *Nitrobacter*, Nitrite oxidation, NOB, Activate Sludge, WWTP

INTRODUCTION

Nitrification plays an important role in wastewater treatment plants (WWTPs). The biomass and activity of the autotrophic bacteria community, including ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), are highly influential to nitrification performance in activated sludge systems or membrane bioreactors in sewage treatment plants (Graham D W et al 2007, Lydmark P et al 2007, and Nogueira R et al 2006). Intrestingly, few investigators have quantitative data on NOB communities with even fewer reporting links to operational or environmental variables, especially under partially nitrifying conditions. Optimal and efficient operations of the nitrification process is largely dependent on maintaining nitrite-oxidation, which becomes increasingly difficult under oxygen limiting conditions due to the competition for dissolved oxygen amongst the entire microbial community. Therefore, environmental and operating factors that impact the NOB community and its function is critical to the optimization of partially nitrifying treatment plants to maximize chemical oxygen demand (COD) and biochemical oxygen demand (BOD) reduction (Park et al., 2010a; Park et al., 2010b). Instability of nitrite-oxidation can often result in excess nitrite concentrations in the effluents which may lead to a higher chlorine demand, thus increasing operating costs to the utility.

Quantitative fluorescence *in situ* hybridization (FISH) with oligonucleotide probes has previously been used to determine spatial relationships between *Nitrobacter* and *Nitrospira* as well as to determine the density of these genera in wastewater treatment systems (Schramm et al., 1998; Schramm et al., 1999; Limpiyakorn et al., 2005; Limpiyakorn et al., 2006; Graham et al., 2007; Lydmark et al., 2007). Evidence in the literature suggests that plant operating conditions plays an important role in the selection of the dominant NOB. Several studies have indicated *Nitrospira* to be the dominant nitrite oxidizer in nitrifying biofilms from wastewater treatment plants and aquaria (Daims et al., 2000; Blackburne et al., 2007; Zeng et al., 2009; Huang et al., 2010). However, *Nitrobacter* was also observed as a superior competitor when the available substrate was abundant (Schramm et al., 2000; Coskuner and Curtis, 2002; Gieseke et al., 2003; Nogueira and Melo, 2006). Alternatively, quantitative polymerase chain reaction (qPCR) has proven to be a powerful analytical approach that is effective at demonstrating the instability of nitrification in lab-scale bioreactors due to the mutual relationship between AOB and NOB (Graham et al., 2007). It has also been used to identify relationships between AOB cell abundance and operational parameters in full-scale WWTPs (Harms et al., 2003; Layton et al., 2005). However, there have been no studies on the distribution of *Nitrospira* and *Nitrobacter* over an extended period of time at a WWTP, where complete nitrification is inhibited through low concentrations of dissolved oxygen (DO).

Previous studies have been carried out to determine the kinetic and characterization of NOB, such as the maximum specific growth rate (μ_{max}), specific oxidizing activity, and cell lysis rate of NOB using both pure and mixed culture (Prosser et al., 1989; Ehrich et al., 1995; Altmann et al., 2003; Harms et al., 2003; Vadivelu et al., 2006; Fujita et al., 2010). Schramm et al. (1999) indicated that the specific ammonia-oxidizing activity of *Nitrosospira* and

nitrite-oxidizing activity of *Nitrospira* under ammonium limiting conditions measured on site were much lower than that in pure culture. It was considered that the higher activities of nitrifying bacteria in earlier studies was likely caused by an underestimation of cell concentrations using inaccurate measurement techniques (Kim and Kim, 2006). Furthermore, Kindaichi et al. (2006) found that the specific growth rate of nitrifying bacteria in batch culture was lower than in complete nitrifying biofilms using real time quantitative polymerase chain reaction technique, which was potentially attributed to the substrate transport limitation and an increase in the cell maintenance energy requirement, or the production of soluble microbial products and extracellular polymeric substances in biofilm (Kindaichi et al 2004, Okabe et al 2005). However, few reports exist on the activities of NOB in full-scale nitrifying systems since pure and mixed culture laboratory-based studies do not satisfactorily mimic the conditions in a full-scale wastewater treatment plant.

Accordingly, the primary objectives were to determine the abundance of NOB over time using quantitative polymerase chain reaction (qPCR) and by extension to assess their relative contributions to nitrite oxidation. Additionally, we identified operational and environmental variables that significantly correlated to changes in NOB lineages. Molecular-based monitoring and analysis for key nitrifying microorganisms in a WWTP is likely to permit better prediction and control of process performance and efficiency.

METHODOLOGY

Samples collection

Samples were taken from the Chiquita Water Reclamation Plant, a municipal wastewater treatment plant located in San Juan Capistrano, CA. Primary effluents were biologically treated using the activated sludge process in aeration tanks with a capacity of 2.3×10^7 liters per day. Activated sludge treatment was operated for BOD as well as suspended solids removal and achieved partial nitrification with a typical SRT of 4.27 days and an operational DO set point of 1.0 $mg\cdot L^{-1}$. The aeration basin was divided into two zones, an anoxic selector zone located at the front of the aeration basin followed by the aerobic zones in the tanks. Approximately 250 mL of sample were collected from the primary clarifier effluent, selector zone, middle of the aerobic zone, and secondary clarifier effluent in sterile bottles on a weekly basis over the course of one year. Samples were transported on ice to the laboratory and processed within 24 h.

DNA Extraction

Genomic DNA was extracted from samples using a modified bead beating protocol (Yu and Mohn, 1999; Gedalanga and Olson, 2009) with the exception that the physical cell lysis was performed using a Fast-Prep-24 Homogenizer (MP Biomedicals, Solon, OH) at 4.0 m/s for 20s. All concentrations of genomic DNA were measured by spectrophotometry at 260/280 nm (Beckman DU7400 Spectrophotometer, Fullerton, CA).

16S rDNA qPCR for Nitrite-Oxidizing Bacteria

A 10-fold dilution series was prepared from the genomic DNA of *Nitrospira* and *Nitrobacter*.

NOB 16S rDNA gene fragments were qPCR amplified in triplicate on a Mastercycler ep realplex (Eppendorf, Hamburg, Germany) using primers listed in Table 1. All qPCR reactions were performed in a total volume of 25µl solution containing: 5µl template DNA, 3.5mM $MgCl_2$, 1X PCR buffer, 200nM dNTP, 0.75U AmpliTaq DNA polymerase, 250nM dual-labeled probe, 50nM forward primer, and 50nM reverse primer. Cycling parameters for qPCR included an initial denaturation at 95ºC for 2mins, followed by 40 cycles of 95ºC for 20s and annealing at 60ºC for 40s (*Nitrospira*) or 68 ºC for 40s (*Nitrobacter*). A temperature gradient analysis indicated optimal annealing conditions using 60ºC and 68ºC for the *Nitrospira* and *Nitrobacter* primer sets, respectively (data not shown), therefore the protocol described in Graham et al (2007) was modified. Additionally, thermodynamic evaluation of the *Nitrobacter* primers suggested 68ºC to be ideal for efficient and stringent DNA amplification (personal communications Graham and Knapp, 2009). Cell numbers for *Nitrobacter* and *Nitrospira* were calculated using 1 copy of the 16S rDNA gene per cell based on studies by Harms et al (Harms et al., 2003) and Graham et al (Graham et al., 2007), respectively.

Physical and chemical analysis

Temperature, DO, and pH measurements were obtained at the point of sample collection using a portable water-sampling device (HACH, Loveland, Colorado). 50 mL of each sample were filtered using a 1.5 µm Glass Microfiber Filters (Whatman, Piscataway, New Jersey) to remove suspended solids prior to ammonia, nitrite, and nitrate measurements to remove inhibition in the colorimetric measurement. Ammonia concentrations were determined using an ISOTemp ammonia probe (Thermo Fisher Scientific, Waltham, Massachusetts) according to the manufacturer's instructions. Nitrite and nitrate concentrations were obtained using HACH NitriVer/NitraVer reagents according to the manufacturer's instructions. Mixed liquor suspended solids (MLSS), total suspended solids (TSS), SRT, flow, total BOD, nitrogenous BOD, and carbonaceous BOD were obtained from laboratory personnel and plant operators in accordance with standard methods (APHA et al., 1998).

Kinetics of nitrification

The Monod model forms the basis of the equation used for the effect of substrate concentration on the growth rate of nitrifiers which was modified by Weismann (1994) to include the operational parameters of temperature, dissolved oxygen and pH in waste treatment systems. A similar approach was presented earlier by Barnes and Bliss (1983) where ammonia was used as the substrate. The equation of Wiesmann 1994 and Ruiz 2003 is shown below and was used in this study.

$$\mu = \mu_{\max} \frac{[HNO_2\text{-N}]}{K_{SH} \cdot e^{(A_E/T)} \cdot 10^{-pH} + [HNO_2\text{-N}] + \frac{[HNO_2\text{-N}]^2}{K_{IH} \cdot e^{(A_E/T)} \cdot 10^{-pH}}} \frac{DO}{K_o + DO} \qquad \text{(Eq. 1)}$$

Where

μ = specific growth rate (d^{-1})

μ_{max} = maximum specific growth rate (d^{-1})

$[HNO_2\text{-N}]$ = nitrite concentration (mg L^{-1})

K_{SH} = half-saturation coefficient for the unionized substrate (mg L^{-1})
K_{IH} = inhibition coefficient for the unionized substrate
K_o = oxygen half-saturation coefficient (mg L^{-1})
A_E = activation energy
T = absolute temperature (K)

A summary of values which have been determined for the kinetic coefficients above is given in Table 3. Additionally, nitrite-oxidizing activity rates were calculated using an equation adapted from Harms et al. (2003), as follows:

$$Nitrite - oxidizing\ activity = \frac{NO_3^- Effluent - NO_3^- Influent}{Biomass \times HRT} \quad \text{(Eq.2)}$$

Where
Nitrite-oxidizing activity = specific oxidizing activity of NOB ($fmol_{NO_2^-N}$ h^{-1} $cell^{-1}$)
$NO_3^-N_{Effluent}$ = Nitrate concentration in the secondary clarifier effluent (mg L^{-1})
$NO_3^-N_{Influent}$ = Nitrate concentration in the primary clarifier effluent (mg L^{-1})
Biomass = Cell concentrations of *Nitrospira* and *Nitrobacter* (Cells L^{-1})
HRT = Hydraulic retention time (hrs)

Nitrate concentrations were used as substrate values with the understanding that the nitrate produced originated from nitrite. Therefore, the difference in nitrate concentrations between the effluent and influent of the activated sludge process is representative of the amount of nitrite oxidized during treatment.

RESULTS

Key plant performance and operating parameters from August 2008 through July 2009

Chiquita Water Reclamation Plant (CWRP) is a municipal wastewater treatment plant with the capacity to treat approximately 6 mgd. Average BOD removal rates, ammonia removal rates, and DO concentrations were 87.96±0.08%, 35.06±16.06%, and 0.87±0.16 mg·L^{-1}, respectively. Influent ammonium concentrations were relatively stable with average levels of 29.36±5.36 mg NH_4^+-N·L^{-1}.

Quantitative polymerase chain reaction analysis of NOB populations

The 16S rDNA genes from *Nitrospira* and *Nitrobacter* were successfully amplified from all 48 weekly DNA extracts obtained from CWRP. Fig.1 shows nitrite concentrations and the abundance of *Nitrobacter* and *Nitrospira* in the aerobic zone for the 12-month study period. *Nitrospira* concentrations averaged 5.71 ± 2.68×10^{12} cells·L^{-1} and represented an average of 76% ± 18% of the total NOB, while *Nitrobacter* averaged 1.87 ± 1.78×10^{12} cells·L^{-1} and represented only 24% ± 18% of the NOB community at the midpoint in the aerobic zone.

To assess possible links between *Nitrospira* and *Nitrobacter*, and the contribution of nitrite concentrations to abundance of NOB, a correlation coefficient (*r*) analysis and a no correlation hypothesis test (*P*) were employed. Table 2 presents an inverse trend between the

abundance of *Nitrospira* and *Nitrobacter* (r = -0.24, P< 0.05). Correlation coefficients linking shifts in NOB community composition to nitrite concentrations illustrated *Nitrospira* were significantly and negatively correlated to nitrite concentrations (r = -0.45, P< 0.01), while *Nitrobacter* showed no significant relationship to nitrite concentrations (r = 0.24, P= 0.1). The difference between the two analyses was likely due to the variability produced by the magnitude of the changes in cell concentrations over time.

Evaluating the effects of temperature on NOB populations

To evaluate the temperature effects on the populations of *Nitrospira* and *Nitrobacter*, Fig.2 showed the relationship between monthly average temperature and monthly average abundance of *Nitrobacter* and *Nitrospira*. Temperature appeared to play an important role in lineage changes. Relatively low temperatures (24°C-25°C) were favorable conditions for *Nitrobacter*, while *Nitrospira* was more adapted to higher temperatures (29°C-30°C). Temperature selectivity was demonstrated in our study, where *Nitrospira* achieved peak concentrations during the months with the highest water temperatures (August 2008, September 2008, and July 2009) and cell abundance did not vary greater than 1 order of magnitude. The *Nitrospira* abundance (Table 2) showed a strong positive correlation to temperature (r = 0.59, P<0.0001), while *Nitrobacter* populations were negatively correlated to temperature (r = -0.49, P<0.001).

Evaluating the effects of DO concentrations on NOB populations

Fig. 3 showed the monthly average DO concentration in the aerobic zone and the effect on *Nitrospira* and *Nitrobacter* cell abundance. Increasing DO concentrations resulted in greater *Nitrobacter* abundance which peaked at approximately the same time as the DO concentration in April 2009 when the lowest concentration of *Nitrospira* was also observed. *Nitrospira* abundance showed a significantly negative correlation to DO (r = -0.46, P<0.01). However, *Nitrobacter* populations were significantly and positively correlated to DO (r = 0.38, P<0.01).

Kinetic activity analysis

The specific growth rates were used to evaluate the NOB activity affected by substrate concentration, temperature, pH and DO. Using the model from Wiesmann 1994 and Ruiz et al. (2003) (Eq.1), *Nitrospira* demonstrated a better relationship with the NOB specific growth rate than *Nitrobacter* (Fig. 4). The specific growth rate of *Nitrobacter* did not correlate significantly with any of these physicochemical parameters, however *Nitrospira* growth rates were negatively correlated with temperatures below 27°C (r =-0.44, P<0.05). Additionally, the specific oxidation rate of NOB (Total amount NOB = *Nitrobacter*+*Nitrospia*) calculated based on Eq.2 resulted in a range of 0 – 0.014 $fmol_{NO_2^-\text{-}N}\ h^{-1}\ cell^{-1}$.

DISCUSSION AND CONCLUSIONS

The results showed that *Nitrospira* was the dominant NOB in the bioreactor with the exceptions of April 2009 (Fig. 1). NOB abundance in the CWRP aeration basin was greater than other studies (Harms et al., 2003; Geets et al., 2007), however variations in NOB

abundance could be explained by differences in environmental conditions, operational control, and quantitative methodologies. Additionally, overestimations of NOB cell abundance may result from limitations in qPCR primers and probe specificities. This may arise as a result of newly sequenced gene fragments from other organisms that are highly homologous to the primers and probes currently used for specific detection. Environmental samples are subject to this bias because the infiltration of microorganisms can be limitless.

The cell abundance of NOB populations monitored at CWRP did not vary greatly over the entire study period. This result is supported by Hawkins et al. (2006) which demonstrated that *Nitrobacter* ribosomal gene (rDNA) abundance varied comparatively little (less than two fold) under both nitrite starvation and excess nitrite conditions. NOB competition was undoubtedly observed in terms of nitrite concentration. An inverse relationship between *Nitrospira* and nitrite concentration (Fig. 1) was also found most of the time in the aeration zone while *Nitrobacter* was not negatively affected by changes in nitrite concentrations. Interestingly, nitrite inhibition was only observed for *Nitrospira* growth in this bioreactor. A recent study corroborates the sensitivity of *Nitrospira* to nitrite concentrations (ter Haseborg et al., 2010).

Pure culture studies on *Nitrobacter* and *Nitrospira* have shown that these organisms thrived between 30°C-45°C and 30°C-35°C, respectively. The optimal growth temperature for these groups of organisms was well above actual wastewater temperatures. However, *Nitrospira* did not always remain the dominant NOB. We observed a gradual increase in *Nitrobacter* as water temperatures dropped with peak concentrations reached in April (Fig.2). This suggested that conditions of relatively low temperatures (<25°C) were favorable for *Nitrobacter* growth. *Nitrospira* were more adapted to conditions of this plant which included generally low DO (<1.0 $mg \cdot L^{-1}$), shorter SRT, and higher temperatures during summer months, while *Nitrobacter* increased in abundance when conditions were the opposite.

The results in Fig. 3 suggest that *Nitrospira* has a competitive advantage over *Nitrobacter* for the available oxygen in oxygen-limited environments. Subsequently, when DO concentrations fell below 1.0 $mg \cdot L^{-1}$ in May 2009, we observed a recovery of *Nitrospira* concentrations and a decrease in *Nitrobacter* concentrations. Our results demonstrate that higher DO concentrations were more suitable to the growth of *Nitrobacter* in this bioreactor, while *Nitrospira* were selectively enriched when DO concentrations were less than <1.0 $mg \cdot L^{-1}$. Our results supported the previously determined difference in oxygen affinity of *Nitrobacter* compared to *Nitrospira* because at lower oxygen concentrations it was clear that *Nitrospira* was the dominant competitor. Blackburne et al. (2007) determined the oxygen half-saturation constant values (K_o) of enriched *Nitrospira* and *Nitrobacter* cultures with negligible oxygen mass transfer resistances were overlapping with values of $0.43 \pm 0.08 mg \cdot L^{-1}$ and $0.54 \pm 0.14 mg \cdot L^{-1}$, respectively. However, the oxygen uptake rates (OUR) were very different being 32 ± 2 $mg \cdot gVSS^{-1} \cdot h^{-1}$ for *Nitrospira* and 289 ± 15 $mg \cdot gVSS^{-1} \cdot h^{-1}$ for *Nitrobacter* (Blackburne et al., 2007). Previous research also found *Nitrospira* concentration peaked at the same time oxygen disappeared in a membrane aerated biofilm (MAB), suggesting that oxygen affinity might play a role in the selection of *Nitrospira* over *Nitrobacter,* as the K_o for *Nitrospira* is lower than for *Nitrobacter* (Schramm et al., 2000). The strong relationship that

Nitrobacter showed with DO concentrations clearly demonstrated why this genus has a selective advantage over *Nitrospira* in fully nitrifying plants.

The dependency of oxygen solubility with temperature made it difficult to separate the impacts of DO concentration from temperature on NOB abundance and activity. Our study demonstrated a strong negative correlation (r= -0.66, P<0.0001) between DO concentration and temperature in the bioreactor. A Student's t-test of population means resulted in a statistically significant difference between *Nitrospira* abundance at low (DO < 0.89 $mg \cdot L^{-1}$) and high DO concentrations (DO $\geq$ 0.89 $mg \cdot L^{-1}$). Although *Nitrobacter* did not exhibit this same relationship with DO (P=0.11), comparing cell abundance at relatively high (T > 27.1°C) and low (T < 27.1°C) temperatures produced statistically significant differences for both *Nitrobacter* (P<0.01) and *Nitrospira* (P<0.01). These results suggest that temperature plays a more powerful role in the determination of NOB abundance than DO, however adjusting DO concentration can be used to increase *Nitrobacter*, whereas water temperature is not easily controlled.

The previous analysis showed the predominance of *Nitrospira* over *Nitrobacter* suggesting that *Nitrospira* was more likely to dominate nitrite oxidation under conditions with low ammonium and nitrite concentrations, which would provide a selective advantage due to the lower nitrite half-saturation constant value (K_s=0.25$mgN \cdot L^{-1}$) for this group in comparison with *Nitrobacter* (K_s=0.39$mgN \cdot L^{-1}$) (Blackburne et al., 2007; Downing and Nerenberg, 2008). The results supported the K/r hypothesis that *Nitrospira* is a K-strategist adapted to low nitrite concentrations, while *Nitrobacter* is an r-strategist mostly depending on an abundance of substrate concentration (Schramm et al., 1999; Kim and Kim, 2006).

The maximum nitrite-oxidizing activity based on the NOB 16S rDNA gene copies was 0.014 $fmol_{NO_2\text{-}N}\ h^{-1}\ cell^{-1}$. This value is 1-2 orders of magnitude lower than the most reported values (Ehrich et al 1995, Altmann et al 2003, Kindaichi et al 2006, Lydmark et al 2007, Fujita et al 2010). However, our result is within range of the lowest reported value (0.025 $fmol_{NO_2\text{-}N}\ h^{-1}\ cell^{-1}$) obtained by measuring the activated sludge samples from a municipal wastewater treatment plant (Gieseke et al., 2001). Also, Schramm et al (1999) measured the specific nitrite oxidation rate low to be 0.02 $fmol_{NO_2\text{-}N}\ h^{-1}$ cell-1 with FISH and microelectrodes technique. The lower activity value may result from unfavorable in situ conditions such as nitrite and/or oxygen limitation compared to the cultivation conditions.

The research in this plant complements and extends that of Nogueira et al (2006) by demonstrating *Nitrospira* concentrations were negatively correlated with NO_2-N. Although no significant correlation was found between NO_2-N and *Nitrobacte*r, the data did show a positive trend. Additionally, there was a sharp difference in adaptation to temperature and dissolved oxygen between these nitrifying bacteria. While ammonia is not limiting since this is a partial nitrifying plant, oxygen and temperature appear to favor *Nitrospira.* These data suggest that as ammonia oxidation increases, nitrite concentrations approach suboptimal levels for *Nitrospira* thus resulting in nitrite accumulation.

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Table1. Nucleotide sequence of primers and dual-labeled probes for *Nitrospira* and *Nitrobacter* （Graham et al 2007）.

Target organism	*Primers or probes*	*Sequence (5′ → 3′)*	T_a(℃)
Nitrospira spp.	Nspra-675f	GCGGTGAAATGCGTAGAKATCG	
	Nspra-746r	TCAGCGTCAGRWAYGTTCCAGAG	60
	Nspra-723Taq	FAM-CGCCGCCTTCGCCACCG-TAMRA	
Nitrobacter spp.	Nitro-1198f	ACCCCTAGCAAATCTCAAAAAACCG	
	Nitro-1423r	CTTCACCCCAGTCGCTGACC	68
	Nitro-1374Taq	FAM-AACCCGCAAGGAGGCAGCCGACC-TAMRA	

Table 2. Correlations between operational data and NOB abundance

	Cross-correlation coefficient		*P*	
	Nitrobacter	*Nitrospira*	*Nitrobacter*	*Nitrospira*
Nitrobacter	-	-0.2664	-	0.0672
Nitrospira	-0.2664	-	0.0672	-
NO_2-N	0.2391	**-0.4535**[*]	0.1017	**0.0012**[b]
NO_3-N	0.0004	-0.0589	0.9978	0.6909
NH_3-N	0.0335	-0.0407	0.8209	0.7837
DO	**0.3807**[*]	**-0.4560**[*]	**0.0076**[b]	**0.0011**[b]
AOB	-0.1231	-0.1137	0.4045	0.4417
pH	0.1633	0.0573	0.2674	0.6990
SRT	-0.2880	0.1986	0.0644	0.2073
HRT	**0.3346**[*]	-0.1619	**0.0201**[a]	0.2715
MLSS	-0.0858	0.2025	0.5620	0.1674
Temperature	**-0.4930**[*]	**0.5881**[*]	**0.0004**[c]	**0.0000**[d]

*For Cross-correlation coefficients, bold indicates a significant correlation (a: $P < 0.05$; b: $P<0.01$; c:$P<0.001$;d:$P<0.0001$).

Table 3 Kinetic coefficients at T=20℃ (Wiesmann 1994)

Coefficient	Definition	NO_2-oxidation
μ_{max}	Maximum specific growth rate	$1.08 day^{-1}$
K_{SH}	Half-velocity constant for the unionized substrate	3.2×10^{-5} mg HNO_2-N L^{-1}
K_{IH}	Inhibition coefficient for the unionized substrate	0.26 mgHNO_2-NL^{-1}
K_{O2}	Oxygen saturation coefficient	1.1 mgO_2L

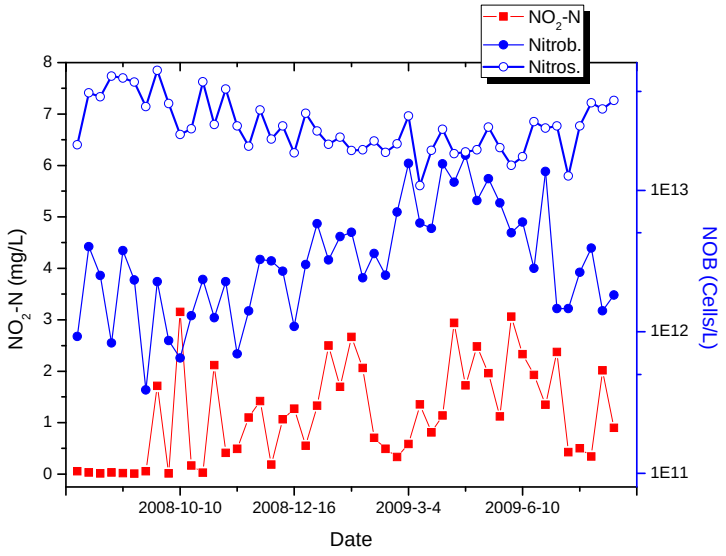

Fig. 1 *Nitrospira* and *Nitrobacter* abundance in weekly samples over a 1 year time series, as measured by qPCR comparing with NO_2-N concentration.

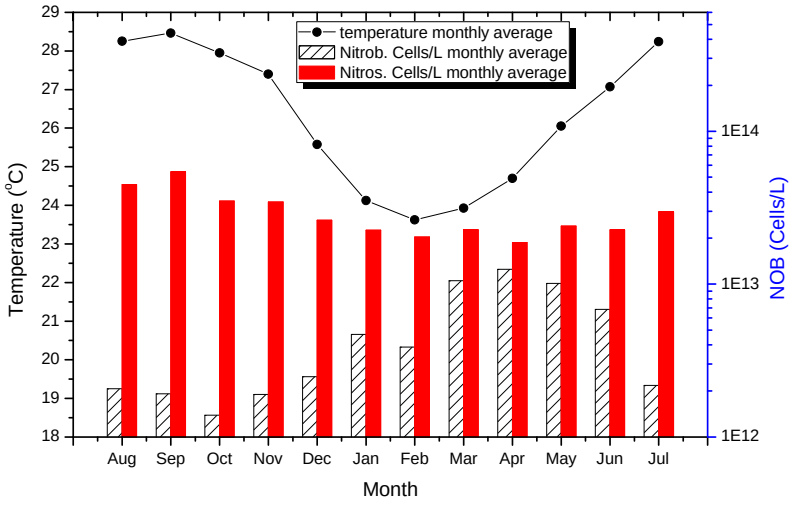

Fig. 2 The relationship between temperature and abundance of *Nitrobacter* and *Nitrospira*.

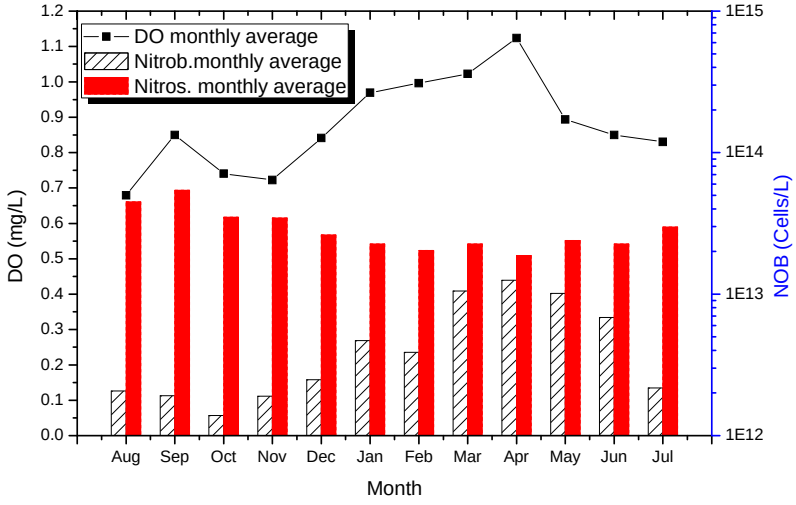

Fig. 3 The relationship between DO and abundance of *Nitrobacter* and *Nitrospira*.

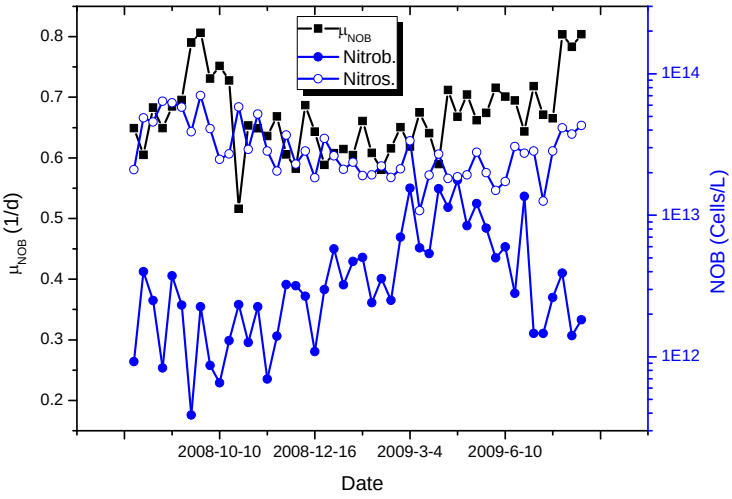

Fig.4 The relationship between the NOB specific growth rate and abundance of *Nitrobacter* and *Nitrospira*.