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RESEARCH ARTICLE

N_2 fixation in urbanization area rivers: spatial-temporal variations and influencing factors

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Abstract

While nitrogen (N_2) fixation is an important process in nitrogen (N) biogeochemical cycling, supplying a significant portion of the N in natural ecosystems, few quantitative constraints exist concerning its contribution to the N enrichment and export from river ecosystems. This study estimates the N_2 fixation rates of urban rivers in the Yangtze Estuary area using acetylene reduction. The results demonstrate that the prominent spatiotemporal variability of river N_2 fixation rates is driven by various environmental factors. River N_2 fixation rates are significantly higher in the summer (90.57 ± 14.60 ngN·L^{-1}·h^{-1}) than in the winter (57.98 ± 15.73 ngN·L^{-1}·h^{-1}). Spatially, rivers draining urban and suburban areas have higher N_2 fixation rates than those draining rural areas. The N_2 fixation rates are positively correlated with the N_2 fixing cyanobacteria density, water temperature, light, and the water phosphorus (P) concentration, but they are negatively correlated with the dissolved N concentration (NH_4^+-N and NO_3^--N). The N_2 fixation rates annually range from 53.20 to 89.24 ngN·L^{-1}·h^{-1} for all of the sampling rivers, which is equivalent to a depth integrated (0–0.6 m) N input of 0.163–0.274 gN·m^{-2}·a^{-1}. The determined annual N input via N_2 fixation is generally higher than that of marine systems, but it is lower than that of eutrophic lakes. This study provides robust evidence that N_2 fixation can supply a substantial portion of the N input to human-impacted river ecosystems, which has not been sufficiently accounted for when determining the N mass balance of riverine ecosystems. A high N_2 fixation rate may increase the ratio of N to P input to river systems, and therefore render P the limiting factor in aquatic eutrophication.

Keywords Nitrogen fixation · Acetylene reduction assay · River · Environmental drivers

Introduction

In natural waters, nitrogen (N) mainly exists in the form of N_2, NH_4^+, NO_2^-, NO_3^-, and organic N. Complicated processes are involved in nitrogen transformation and migration, including assimilation, ammonification, nitrification, denitrification, anaerobic ammonium oxidation (anammox), and nitrogen (N_2) fixation (Kuypers et al. 2018). Biological N_2 fixation refers to the catalytic reduction of N_2 to ammonia (NH_3) by N_2 fixing organisms with intracorporal nitrogenase (N_2ase) (Howarth and Marino 1988). N_2 fixation has long been recognized as a limiting factor in the primary productivity of many ecosystems, including oceans, rivers, and lakes. Therefore, it plays an important role in the biogeochemical cycling of C and N (Falkowski et al. 2000; Galloway 1998; Schlesinger 1997).

N_2 fixation has been studied in a variety of terrestrial and aquatic ecosystems, including forests, agricultural soils, oceans, lakes, and rivers (Chang et al. 2000; Mugidde et al. 2003). It can be a dominant flux in the N input, providing up to 97% of the new N in some terrestrial ecosystems and up to 82% in some aquatic ecosystems (Kunza and Hall 2014). Several studies have concluded that N_2 fixing cyanobacteria, such as *Trichodesmium*, contribute strongly to the marine ecosystem nitrogen pool, balancing N loss through denitrification in the water column (Deutsch et al. 2007). Thus, it provides a new N source for nutrient poor seas and marine ecosystems. Similar investigations conducted in fresh water bodies have

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also shown that biological N_2 fixation is an important N source contributing to the eutrophication of some lakes (Horne and Goldman 1972; Howarth et al. 1988). N_2 fixation by plankton tends to be low in lakes with poor or moderate nutrient availability (< 0.1 $gN{\cdot}m^{-2}{\cdot}a^{-1}$), yet it is considerably higher in eutrophic lakes (0.2–9.2 $gN{\cdot}m^{-2}{\cdot}a^{-1}$; Howarth et al. 1988), representing 5.5–82% of the total "new" N inputs (Mugidde et al. 2003; Scott and Grantz 2013; Higgins et al. 2018). The annual rates of N fixation in tropical lakes and oceans can also be high (4.4–11.4 $gN{\cdot}m^{-2}{\cdot}a^{-1}$), accounting for 65–80% of the total N loading to these systems (Higgins et al. 2018; Mugidde et al. 2003).

Recent research has focused on cyanobacteria-mediated N_2 fixation, including their species composition and functional types in different ecosystems, the factors affecting their ability to fix N_2 (Smith 1995; Scott and Marcarelli 2012), and their importance in the N budgets of ecosystems (Howarth et al. 1988; Karl et al. 1997). N_2 fixation in rivers should affect the water N balance and the pollutant capacity, but it should also influence the effectiveness of water pollution prevention measures (Wei et al. 2016).

However, most studies have given little attention to the N_2 fixation rate and N_2 fixation capacity of rivers. Whether N_2 fixation is a large source of the N in river systems remains unknown. In this study, eight representative rivers in the Yangtze River estuary area were selected to better understand the temporal and spatial variation characteristics of N_2 fixation in river ecosystems and its environmental driving factors. In addition, we determined the seasonal N_2 fixation rates of these rivers and explored the contribution of N_2 fixing cyanobacteria to the N enrichment of these rivers.

Materials and methods

Study area

The study area is located in the Yangtze River Delta, Shanghai, which has a dense river network and abundant water resources consisting of 33,127 river courses extending over 24,915 km within a 569.6 km^2 area. The density of this river network is 3.93 $km{\cdot}km^{-2}$, and the river area accounts for 10.1% of the total area (Shanghai Municipal Water Bureau). Shanghai's river network mainly consists of the Huangpu River and its tributaries, which includes the Suzhou River, the Dianpu River, and the Dazhi River. Shanghai has undergone rapid economic development, with a high level of urbanization and a large amount of wastewater discharge, leading to poor water quality. According to the 2016 Shanghai municipal environmental situation bulletin, although its water environment has been improved and the pollution from N and P has largely been alleviated, N and P pollution is still the main pollution index affecting the muncipality's water quality.

Our research takes into consideration the spatial information of Shanghai's rivers. In addition, by taking into account the urbanization level, river pollution, surrounding environment, sampling feasibility, and traffic accessibility, eight rivers (Fig. 1) in the study area were used as sampling sites: the Qingpu (QP), Songjiang (SJ), Jiading (JD), Putuo (PT), Jinshan (JS), Pudong (PD), Fengxian (FX), and Chongming (CM) Rivers.

Sampling and processing

We sampled the eight rivers in the summer (July) and autumn (October) of 2015 and the winter (January) and spring (April) of 2016. Each river surface water sample was collected using a 5-L organic glass field water collector, and 1 L of each water sample placed in a brown glass bottle for determining the concentrations of N, P, and chlorophyll-a (chl-a). Another 1-L water sample was stored in a polyethylene bottle, and 15 ml of Lugol's Iodine solution was added for algae identification and algal density determinations (Stewart 1964; Capone and Carpenter 1982). The water temperature, pH (pH/Eh meter [IQ 150]), and dissolved oxygen (DO) concentration (Myratek sentry M-2) were measured simultaneously in the field using portable instruments.

Laboratory culturing

Acetylene reduction assay (ARA; Capone 1993; Flett et al. 1976) was used to estimate the N_2 fixation. The assay procedure, proposed by Hardy and Knight Jr. (1967), involves the nitrogenase (N_2ase)-catalyzed reduction of acetylene (C_2H_2) to ethylene (C_2H_4) coupled with sensitive gas chromatographic analyses. It is based on the inhibition of N_2 fixation by C_2H_2 (Schollhorn and Burris 1966) and the reduction of C_2H_2 to C_2H_4 (Dilworth 1966). The assay measures the production of C_2H_4 in a given sample after incubation with C_2H_2, which is an alternative substrate for the N_2 reducing enzyme N_2ase (Breitbarth et al. 2004). The activity of N_2ase can be quantified by measuring the C_2H_4 content produced, from which the rate of N_2 fixation can be derived (Stewart et al. 1968).

In the incubation experiment, we first poured a 185-ml water sample into a 285-ml transparent incubation flask. After sealing the aluminum cap, we injected 10 ml pure C_2H_2 into the flask to ensure that the volume of C_2H_2 accounted for 10% of the overlying gas volume in the incubation system (Capone 1993). Then, the incubation flask was shaken, to keep the gas and liquid dynamically balanced in the incubation system. The incubation apparatus was placed in natural light for 2 h, and then we extracted 2 ml of the overlying gas from the incubation flask using an airtight syringe to determine the C_2H_4 concentration.

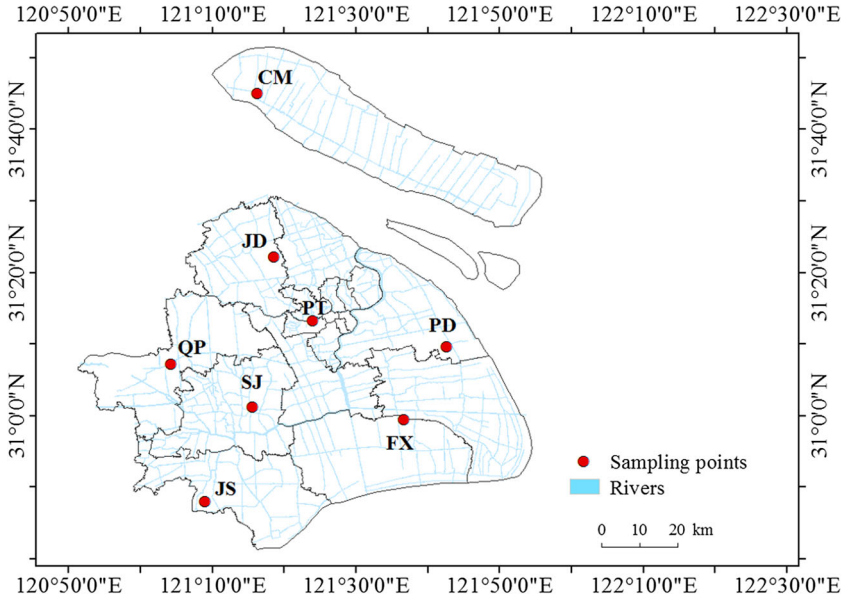

Fig. 1 Distribution of the sampling sites on the Yangtze Estuary urbanization area rivers

The light level and water temperature were recorded during the culturing phase. After culturing, the water samples in the incubation bottle were filtered through a 0.45-μm membrane and stored for the later determination of their total phosphorus (TP), total dissolved phosphorus (TDP), ammonium nitrogen ($NH4^+$-N), and nitrate nitrogen (NO_3^--N) concentrations.

Sample analysis

The C_2H_4 concentration of the overlying gas phase was detected with a high sensitivity using a gas chromatograph (GC7890A) equipped with an FID detector and a chromatographic column PLOT Q, which used He as the carrier gas (Capone 1993; Stewart et al. 1967). The determination of the TP concentration was accomplished using ammonium molybdate spectrophotometry after sample digestion using $K_2S_2O_8$. To determine the TDP, a sample was filtered through a 0.45-μm membrane and digested using $K_2S_2O_8$. Then, it was analyzed using ammonium molybdate spectrophotometry (Jin and Tu 1990). $NH4^+$-N was analyzed using Nessler's reagent spectrophotometry (Krug et al. 1979). The NO_3^--N concentration was determined using a continuous flow analyzer (Futura, Alliance Instruments, France). The Chl-a was extracted using acetone and was measured using spectrophotometry (Duan et al. 2006).

N_2 fixation rate calculation

The N_2 fixation rate was estimated from the ethylene produced using a theoretical conversion factor of 3:1 mol of C_2H_4 to moles of N_2 (Robson and Postgate 1980; Capone 1993; Fu and Bell 2003a, 2003b) based on the reaction process of biological N_2 fixation to facilitate comparisons with the results of previous studies. The solubility of C_2H_4, which is required to calculate the N_2 fixation rate, depends on the temperature and salinity at which the assay is carried out. The amount of C_2H_4 dissolved in the aqueous phase can be calculated from the C_2H_4 detected in the overlying gas phase using the Bunsen gas solubility coefficient (α). Since C_2H_4 has a different solubility under different incubation conditions, in this study, we used the Bunsen coefficient of C_2H_4 calculated by Breitbarth et al. (2004), which is based on the gas equilibrium between the gas and water phases under different conditions. Using this Bunsen coefficient, we first calculated the percentage of the total C_2H_4 dissolved in the liquid phase, and then we obtained the percentage of C_2H_4 dissolution in the on-site incubation water to correct the experimental results.

The rate of N_2 fixation, F (reported in μg of N per liter per hour ($\mu gN \cdot L^{-1} \cdot h^{-1}$) to facilitate comparison with the results of previous studies), was calculated based on the water volume and the total amount of C_2H_4 produced in the bottle during incubation:

$$V_m = 22.4 \times \frac{T}{T_0} \quad (1)$$

$$M_{C_2H_4} = \frac{M}{V_m} \times V_g + \frac{M}{V_m} \times \alpha \times V_l \quad (2)$$

$$F = \frac{M_{C_2H_4}}{V_l \times t \times n} \times M_{N_2} \quad (3)$$

where V_m is the molar volume of the gas under the experimental conditions ($L \cdot mol^{-1}$); 22.4 $L \cdot mol^{-1}$ is the molar volume of the gas under standard conditions; T and T_0 are the temperatures under the experimental and standard conditions (K), respectively; $M_{C_2H_4}$ is the total C_2H_4 produced in the incubation

flask (μmol); M is the C_2H_4 content of the overlying gas phase in the incubation flask (μmol·mol^{-1}); α is the Bunsen coefficient of C_2H_4; V_l and V_g represent the water sample volume and the overlying gas volume in the incubation flask (L), respectively; n is the conversion factor ($n = 4$); t is the incubation time (h); and M_{N_2} is the molar mass of N_2 (28 g·mol^{-1}).

Results

Spatial and temporal distribution characteristics of the water environmental parameters of the river sampling sites

The DO concentrations of the river water differed significantly during the different seasons, i.e., winter > spring > autumn > summer. The basic statistics shown in Table 1 indicate that for the eight sampling sites, the annual mean DO was highest in the QP, lowest in the PT, and ranged from 2.975 ± 1.11 to 9.02 ± 1.31 mg·L^{-1} (annual mean ± standard deviation across the four seasons). The DO values of the JS, PD, FX, and PT sites in summer were all < 2 mg·L^{-1}, indicating severe water pollution at these sites in the summer. The pH values were generally ranked as spring > autumn > winter > summer, with the annual mean maximum in the PD (7.36 ± 0.85 mg·L^{-1}) and the minimum in the SJ (6.72 ± 0.82 mg·L^{-1}).

The water properties of the sampled rivers exhibit significant temporal variations. The range of the NO_3^--N concentrations of the rivers was 0.20–3.41 mg·L^{-1} but changed significantly with the season, having higher concentrations in spring (1.79 ± 0.79 mg·L^{-1}) and winter (1.72 ± 1.21 mg·L^{-1}) and lower concentrations in summer (0.64 ± 0.18 mg·L^{-1}) and autumn (0.40 ± 0.21 mg·L^{-1}). The range of the NH_4^+-N concentration was 0.37–5.56 mg·L^{-1}. The average was highest in the winter (2.10 ± 1.70 mg·L^{-1}), followed by spring and autumn (1.79 ± 1.53 and 1.43 ± 0.40 mg·L^{-1}, respectively) and was lowest in the summer (1.15 ± 0.32 mg·L^{-1}). The concentrations of TP and TDP were both highest in the summer and lowest in the autumn. The range of the TP concentration was 0.04–0.43 mg·L^{-1}, and the concentration in autumn was significantly different from those in spring and summer ($p < 0.05$). However, the TDP concentration ranged from 0.02 to 0.34 mg·L^{-1}, with no significant difference among the seasons ($p > 0.05$).

Spatially, the NH_4^+-N, TP, and TDP concentrations of the water were not uniform. Generally, the NH_4^+-N, TP, and TDP concentrations of the urban sampling sites significantly exceeded those of the suburban and rural sampling sites ($p < 0.05$), while the NO_3^--N concentration did not exhibit a significant spatial variation ($p > 0.05$).

The density of N_2 fixing cyanobacteria

In all of the sampled rivers, among the N_2-fixing cyanobacteria only *Anabaena* was present throughout the year, while *Aphanizomenon* was only present in the summer. The densities of *Anabaena* in the surface water of the rivers exhibited obvious temporal and spatial variations. Spatially, the seasonal mean of the *Anabaena* densities of the urban areas was higher than those of the suburban and rural areas.

Table 1 Environmental and water quality parameters for the eight urbanization area river sampling sites (Mean values are shown with standard deviations, with $n = 4$)

Rivers	pH	Temperature (°C)	ρ (mg·L^{-1})				
			DO	NH_4^+-N	NO_3^--N	TP	DP
QP	7.25 ± 1.08 (5.70~8.23)	21.05 ± 9.82 (9.00~33.00)	9.00 ± 1.31 (7.56~10.31)	0.91 ± 0.25 (0.70~1.25)	0.74 ± 0.59 (0.32~1.60)	0.07 ± 0.04 (0.04~0.12)	0.03 ± 0.03 (0.01~0.07)
SJ	6.73 ± 0.82 (5.59~7.40)	18.55 ± 8.89 (9.20~30.60)	4.81 ± 2.62 (3.05~8.67)	1.85 ± 0.47 (1.52~2.54)	0.92 ± 0.67 (0.48~1.91)	0.25 ± 0.16 (0.10~0.41)	0.14 ± 0.10 (0.05~0.29)
JD	7.22 ± 0.80 (6.08~7.78)	17.93 ± 8.57 (8.90~29.50)	4.55 ± 2.13 (3.07~7.70)	3.20 ± 2.06 (1.09~5.56)	0.76 ± 0.60 (0.26~1.61)	0.18 ± 0.11 (0.04~0.28)	0.10 ± 0.07 (0.03~0.17)
PT	6.90 ± 0.56 (6.08~7.30)	21.15 ± 10.04 (11.60~34.4)	2.98 ± 1.11 (1.63~4.14)	2.39 ± 1.63 (0.51~3.79)	1.65 ± 1.51 (0.15~3.41)	0.24 ± 0.12 (0.12~0.40)	0.15 ± 0.13 (0.05~0.34)
JS	7.19 ± 0.15 (6.98~7.29)	20.53 ± 9.96 (11.10~34.60)	4.57 ± 3.46 (1.31~8.96)	1.16 ± 0.36 (0.63~1.45)	1.46 ± 1.16 (0.37~2.47)	0.10 ± 0.04 (0.07~0.15)	0.06 ± 0.03 (0.03~0.11)
PD	7.36 ± 0.85 (6.10~7.88)	18.40 ± 9.82 (6.80~30.80)	4.27 ± 1.96 (1.90~6.50)	1.23 ± 0.65 (0.47~1.80)	1.42 ± 1.32 (0.20~3.26)	0.12 ± 0.05 (0.05~0.18)	0.04 ± 0.01 (0.04~0.05)
FX	7.09 ± 0.63 (6.24~7.77)	18.98 ± 9.02 (10.80~31.80)	5.77 ± 3.23 (1.69~8.60)	1.25 ± 0.35 (0.82~1.68)	1.28 ± 1.09 (0.36~2.73)	0.23 ± 0.18 (0.06~0.43)	0.12 ± 0.13 (0.03~0.31)
CM	7.12 ± 0.91 (5.76~7.73)	19.25 ± 9.56 (8.40~31.50)	8.54 ± 1.55 (6.50~10.25)	0.95 ± 0.50 (0.38~1.53)	0.89 ± 0.59 (0.21~1.63)	0.07 ± 0.02 (0.04~0.10)	0.03 ± 0.01 (0.01~0.04)
Mean value	7.11	19.48	5.56	1.62	1.14	0.16	0.08

For example, in a downtown area (PT), the total *Anabaena* density was 1.06×10^5 cell·L^{-1}, while in a rural area (CM), it was only 5.39×10^4 cell·L^{-1} (Fig. 2). The *Anabaena* density per sampling site was highest in the summer with a summed density value reached 2.33×10^5 cell·L^{-1} and an average density per site of 2.91×10^4 cell·L^{-1}. However, in the spring and autumn, the summed density values were lower, 1.48×10^5 and 1.56×10^5 cell·L^{-1}, respectively. The genus was least abundant in winter with a summed density value of 1.15×10^5 cell·L^{-1} and an averaged value of 1.44×10^4 cell·L^{-1} for the eight sampled rivers. The mean *Anabaena* density of the river surface water was twice as high in summer as in winter.

Spatial and temporal distribution of the nitrogen fixation rate in the surface river water

Overall, the N_2 fixation rates of surface river water ranged from 33.99 ± 14.39 ngN·L^{-1}·h^{-1} to 109.53 ± 14.31 ngN·L^{-1}·h^{-1}, showing significant regularity over time and space (Fig. 3). The N_2 fixation rate was highest in summer (90.57 ± 14.60 ngN·L^{-1}·h^{-1}), followed by spring and autumn, which were similar (75.21 ± 12.95 ngN·L^{-1}·h^{-1}, 76.52 ± 12.91 ngN·L^{-1}·h^{-1}, respectively), and was lowest in winter (57.98 ± 15.73 ngN·L^{-1}·h^{-1}), which also matches the cyanobacteria trend described above. The average N_2 fixation rate in summer is 1.56 times that in winter. Rivers, lakes, and reservoirs commonly experience an epilimnetic decrease in dissolved inorganic N (DIN) during the growing season (Scott and Grantz 2013). This decrease induces phytoplankton N limitation and results in substantial N_2 fixation (Scott et al. 2008, 2009). In some cases, biological N_2 fixation can compensate for the N supply deficit (relative to P) in a single summer (e.g., Lake Waco, Texas; Scott et al. 2008). Comparisons of the seasons were examined using a one-way ANOVA and follow-up LSD (least significance difference) testing. These analyses revealed that the N_2 fixation rate in summer differed significantly from

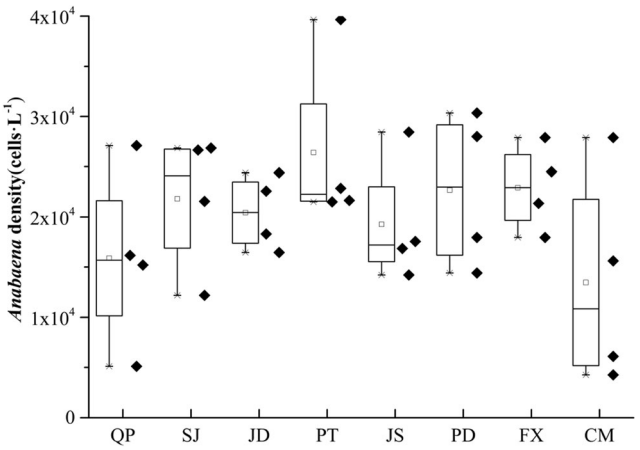

Fig. 2 Boxplot showing the Anabaena density distribution of the eight sampled rivers

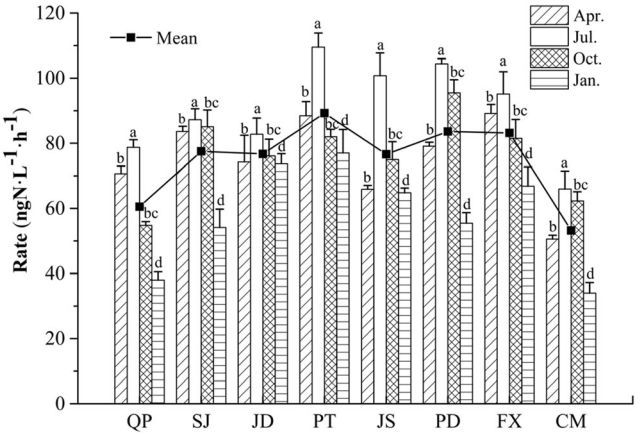

Fig. 3 The N_2 fixation rates of eight sampled rivers during four seasons. **a**, **b**, and **c** denote significant seasonal differences, one-way ANOVAs ($p < 0.01$)

those of the rest of the year, i.e., spring ($p < 0.01$), autumn ($p < 0.01$), and winter ($p < 0.001$). Likewise, winter was distinctly different from spring ($p < 0.01$), summer ($p < 0.001$), and autumn ($p < 0.01$). Consequently, seasonal variation has an obvious influence on the N_2 fixation rate.

Spatially, the N_2 fixation rates of rivers are also higher in urban areas than in suburban and rural areas. The N_2 fixation rates of rivers in the urban centers and suburban areas were significantly higher than those in rural areas ($p < 0.01$). The highest N_2 fixation rate occurred at an urban center river (PT) sampling site (89.24 ± 14.31 ngN·L^{-1}·h^{-1}) is 1.68 times the lowest value, which occurred at a rural river sampling site (CM; 53.20 ± 14.39 ngN·L^{-1}·h^{-1}). Rivers located in the city center are more polluted by industry and human activities, so the N_2 fixation rates of these rivers are much higher. In contrast, the N_2 fixation rates are lower in the less polluted rivers sampled in the suburban and rural areas. Different degrees of river pollution are known to affect the N_2 fixation rates of surface water, such that polluted rivers with low DO values have more adequate anaerobic conditions, which favor N_2 fixation (Staal et al. 2003; Gallon 1981).

Discussion

The effect of environmental factors on the N_2 fixation rate

N_2 fixation is a complex process, which is influenced by various environmental factors, including abiotic factors, e.g., temperature (Robarts and Zohary 1987), light (Hardy et al. 1973), salinity (Fu and Bell 2003a, 2003b; Hafeez et al. 1988), inorganic nutrients (such as N, P, Fe, and Mo) (Smith 1992; Karl et al. 2002; Sohm et al. 2011), trace elements (Mills et al. 2004; Howarth and Marino 1988), oxygen concentration (Staal et al. 2007; Fay 1992), and biotic factors,

e.g., the biomass of N_2 fixing organisms and the presence of organisms such as fish that feed on these N_2 fixing organisms (Wilkinson 1983).

Light and temperature

Light is essential for $N_2[C_2H_2]$ fixation by photosynthetic bacteria and algae (Levine and Lewis 1987). Its intensity, period, and wavelength all have different effects on the N_2 fixation rate. The effects of light intensity have been measured in cultured organisms (Cox and Fay 1969; Duong and Tiedje 1971). The average N_2 fixation rates of reservoirs were found to be light dependent and positively correlated with the primary productivity of the reservoirs (e.g., Lakes Elmdale, Fayetteville, and Wedington; Scott and Grantz 2013).

The ambient stream water temperature directly affects the N_2 fixation (Kunza and Hall 2014) and explains much of the temporal variation in our N_2 fixation estimates for the eight sampled rivers. Temperature mainly affects the N_2 fixation rate by affecting the metabolism of the N_2 fixing microorganisms and the activity of the N_2ase. Our findings are consistent with the known sensitivity of N_2ase activity to temperature, which has been identified in other oligotrophic streams (Marcarelli and Wurtsbaugh 2006), terrestrial ecosystems (Houlton et al. 2008), and marine N_2 fixers (Staal et al. 2003). A moderate increase in temperature can accelerate the growth rates of N_2 fixing organisms (Canale and Vogel 1974), thus promoting N_2 fixation and increasing the N_2 fixing rate (McQueen and Lean 1987). Most plankton activity is temperature dependent, with a peak activity rate occurring at 25–40 °C (Robarts and Zohary 1984). N_2ase activity also increases with temperatures (Robarts and Zohary 1987).

The study area is located in a subtropical monsoon climate zone, and thus, both light and water temperature have distinctive seasonal changes. This would explain why we found significant positive linear correlations between the N_2 fixation rate and the light ($r = 0.586$, $p < 0.01$) and the water temperature ($r = 0.669$, $p < 0.01$; Fig. 4a and b).

N_2 fixing cyanobacteria density and chlorophyll-a

Cyanobacteria, such as *Anabaena* and *Aphanizomenon*, are the main N_2 fixing organisms in freshwater ecosystems (Capone and Carpenter 1982; Gruber and Sarmiento 1997). We chose to estimate chl-a because it is a good indicator of photoautotrophic biomass (Marcarelli and Wurtsbaugh 2007), and it can reflect the density of phytoplankton in the water. In this study, both the N_2 fixing cyanobacteria density and chl-a are positively correlated with the N_2 fixation rate ($r = 0.880$, $p < 0.01$ and $r = 0.490$, $p < 0.01$, respectively; Fig. 4c and d). The N_2 fixing cyanobacteria density of the water represents the number of algae involved in N_2 fixing activities, so a higher concentration indicates a greater N_2 fixation rate (Stewart et al. 1968).

Comprehensive effects of environmental factors on the N_2 fixation rate

As a nutrient crucial to water algae growth, P has obvious promoting effects on nitrogen-fixing algae and their N_2 fixation rate (Elmetri and Bell, 2004). The importance of the P load has been demonstrated in a mesocosm experiment using samples collected from the Baltic Sea (Rydin et al. 2002). Similarly, a survey of 38 lakes (Hellström 1996) showed that N fixation is positively correlated with the TP concentration (Vrede et al. 2009). In this study, both the TP and the TDP were positively correlated with the N_2 fixation rate ($p < 0.01$; Fig. 4e and f), suggesting that they enhance the water N_2 fixation rate. By experimentally adding P to N_2 fixing algae under field and laboratory conditions, Elmetri and Bell (2004) demonstrated that the N_2 fixation rate increases linearly with increasing P-PO_4 concentration up to 0.27 μM, but after that, it does not increase further. Stewart and Alexander (1971) also showed that the acetylene reduction rate of freshwater diazotrophs is proportional to the P-PO_4 concentration up to approximately 0.6 μM, but there is no further increase beyond 1.6 μM. Physiologically, N_2 fixation is a very energetically demanding process, requiring 12–16 molecules of ATP for the reduction of 1 molecule of N_2 (Postgate 1982). Therefore, the strong dependence of N_2 fixation on the concentration of P-PO_4 is not surprising (Elmetri and Bell, 2004).

To verify our experimental results and to further investigate the significant influence of P on the N_2 fixation rate, we conducted experiments on the effects of the addition of different amounts of P on chl-a and the N_2 fixation rate. The results indicate that the addition of P stimulated chl-a 65.75%, 175.15%, 195.08%, and 34.80% above the control values in spring, summer, autumn, and winter, respectively. It also stimulated N_2 fixation by 105.86% in spring, 32.69% in summer, 56.80% in autumn, and 63.01% in winter. Similar field experiments by Lian et al. (2007) on the Beibu Gulf and Amy and Wayne (2007) on 19 stream sites in three catchments in the Sawtooth Mountains of central Idaho have also demonstrated that the addition of P stimulated the river N_2 fixation rates. However, the effects of different sampling sites, different seasons, and different P concentrations were inconsistent.

DIN (NO_3^--N, NO_2^--N, and NH_4^+-N) is recognized as a critical controlling factor of the stream N_2 fixation rates (Hiatt et al. 2017; Howarth and Marino 1988). N_2 fixation is high only in streams where the ambient NO_3^- concentration is low (Kunza and Hall 2014). In this study, the NH_4^+-N and NO_3^--N concentrations were both linearly negatively correlated with the N_2 fixation rate ($p < 0.01$; Fig. 4g and h). The rivers with high NH_4^+-N and NO_3^--N concentrations had low N_2 fixation rates. On the one hand, among the three N species, NH_4^+-N is

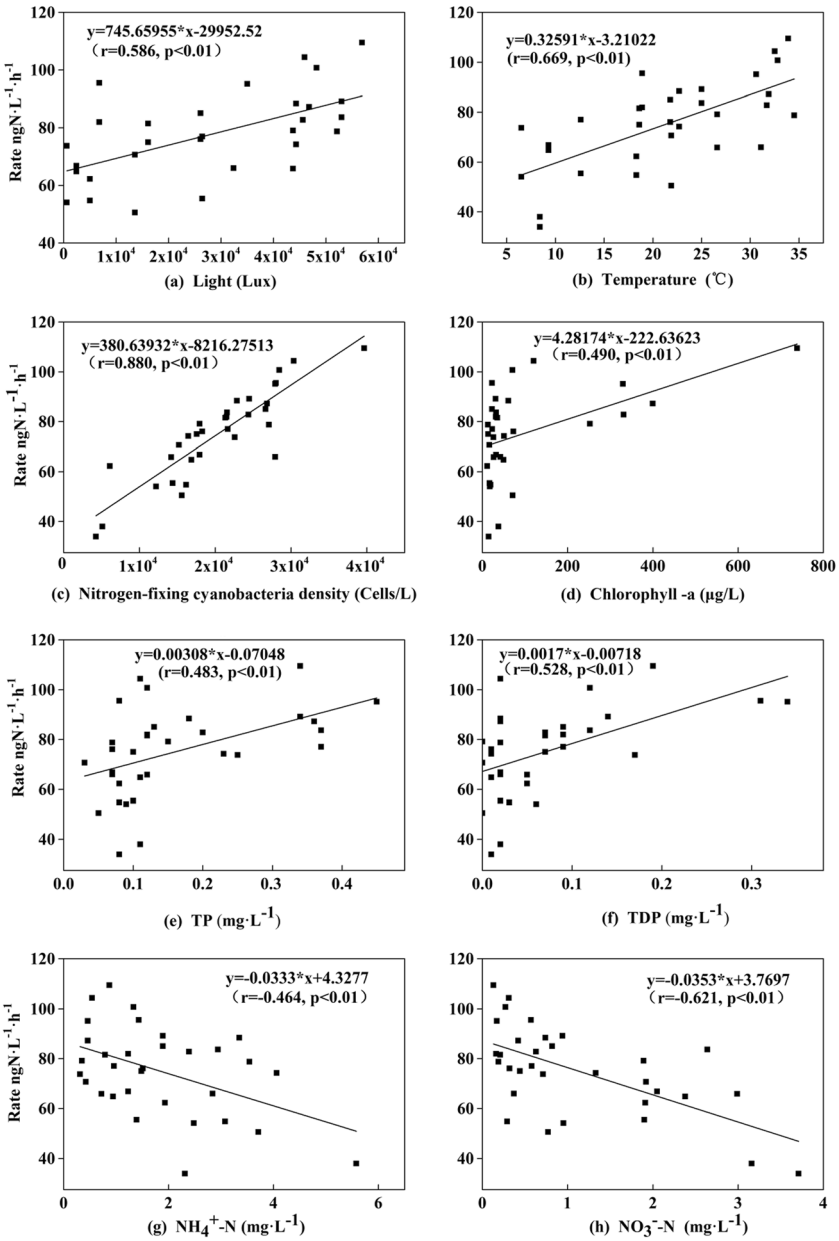

Fig. 4 Relationships between the environmental factors and the N_2 fixation rates

the most effective at inhibiting N_2 fixation genes (*nif* gene) (Gallon et al. 1996; Dominic et al. 1998). The presence of NH_4^+-N or NO_3^--N can inhibit the synthesis of N_2ase and control the N_2 fixing rate to some extent (Horne and Goldman 1972). On the other hand, cyanobacteria N_2 fixation is a highly energy consuming biological process, which occurs when $NH4^+$ and $NO3^-$ are not readily available (Kalff 2002). Acquiring N via N_2 fixation is energetically costly compared to inorganic N uptake (Howarth and Marino 1988; Vitousek et al., 2002), so low DIN concentrations strongly favor microbial N_2 fixers and increase the aquatic N_2 fixation rate (Howarth and Marino 1988; Marcarelli and Wurtsbaugh 2006; Scott et al. 2009; Kunza and Hall 2014; Hiatt et al. 2017).

A variety of environmental factors have important effects on N_2 fixation in rivers, but the relative importance of these factors is not clear (Howarth and Marino 1988). Based on our redundancy analysis of the N_2 fixation rates and environmental factors (Fig. 5), we found that several environmental factors, i.e., light, water temperature, the N_2 fixing cyanobacteria density, and the N and P concentrations of the water, are all closely related to the N_2 fixation rate. Among these factors, light, temperature, the N_2 fixing cyanobacteria density, chl-a, TP, and TDP can promote N_2 fixation and increase its rate, whereas the N concentration is the main factor inhibiting N_2 fixation and decreasing its rate. Thus, the spatiotemporal variation of these factors determines the temporal and spatial characteristics of the river water's N_2 fixation rate.

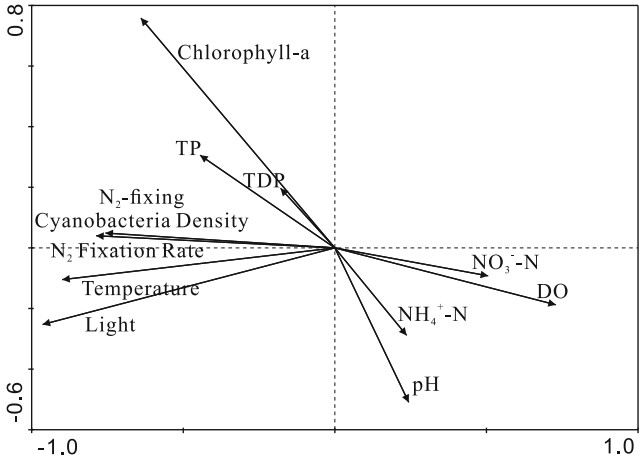

Fig. 5 Redundancy analysis of the N_2 fixation rates and the environmental factors

Contribution of N_2 fixation in surface river water

Studies over the last 25 years have shown that the N_2 fixation rate can vary significantly among different aquatic ecosystems. Generally, the rates for ocean surface water are lower than those of most lakes. For example, the Arabian Sea (7–10°N) has a rate of 0.13 $gN{\cdot}m^{-2}{\cdot}a^{-1}$ (Capone et al. 1998); the Beibu Gulf of China has a rate of 0.12 $gN{\cdot}m^{-2}{\cdot}a^{-1}$ (Lian 2009); the Atlantic Ocean has a rate of 0.011 $gN{\cdot}m^{-2}{\cdot}a^{-1}$ (Staal et al. 2007); and the Pacific Ocean has a rate of 0.002 $gN{\cdot}m^{-2}{\cdot}a^{-1}$ (Carpenter and Capone 1983). Reported area rates of N_2 fixation in eutrophic lakes range from 0.20 to 16.0 $gN{\cdot}m^{-2}{\cdot}a^{-1}$, e.g., Clear Lake 2.60 $gN{\cdot}m^{-2}{\cdot}a^{-1}$ (Horne and Goldman 1972; Higgins et al. 2018). If we regard the 0.6 m transmittance layer of a river's surface as the generating layer for N_2 fixation and set the daily N_2 fixation time as 14 $h{\cdot}day^{-1}$ (Stewart et al. 1971), the annual N_2 fixation quantities of the Shanghai rivers are estimated to be 0.163–0.274 $gN{\cdot}m^{-2}{\cdot}a^{-1}$, with corresponding mean values of 0.23 ± 0.037 $gN{\cdot}m^{-2}{\cdot}a^{-1}$.

Since the contribution rates of N_2 fixation to nitrogen pools are not the same for different types of ecosystems, the proportion of N fixed by the phytoplankton in the total N input of the entire system often differs (5.5–82%) among different ecosystems (Howarth and Marino 1988). Indeed, studies have confirmed that contributions of new N input from N_2 fixing cyanobacteria will vary according to the aquatic ecosystem studied. Some believe that new N contributes less than 2% to the phytoplankton biomass (Ferber et al. 2004), or no more than 5% to the N input (Marcarelli et al. 2008). However, others have suggested that biological N_2 fixation can contribute 58–75% of the N in water blooms (Gu and Alexander 1993), or account for 77% of the water N input (Patoine et al. 2006).

N makes its way into streams from watersheds via point sources and non-point sources (Bellmore et al. 2018; Dumont et al. 2005). Tan et al. (2014) calculated and modeled the nitrogen input load of the Shanghai river network based on a river nutrient flux model GLOBSL-NEWS2 (Mayorga et al. 2010). They divided the river nitrogen pollution input into three parts: the point source input, the non-point source input, and the upstream input. The estimated total nitrogen input of the Shanghai river network was 68.39 $Gg{\cdot}a^{-1}$, of which the point source nitrogen input contributed 15.43 $Gg{\cdot}a^{-1}$, the non-point source nitrogen input contributed 41.29 $Gg{\cdot}a^{-1}$, and the upstream nitrogen inputs contributed 11.67 $Gg{\cdot}a^{-1}$. The non-point source nitrogen input accounted for the largest proportion (60.37%). The total N input of atmospheric precipitation, including wet deposition and dry deposition, into Shanghai's regional rivers was 11.06 $Gg{\cdot}a^{-1}$. In our study, the annual N_2 fixation quantity of the surface water (0.6-m depth) in the Shanghai river network is ~0.15 $Gg{\cdot}a^{-1}$, which is approximately 1.36% of the total atmospheric deposition N input. Although compared to other N source inputs, the contribution of the annual N_2 fixation to river water N storage is not particularly high, it is a N source that cannot be ignored. Further research is needed to quantify how the importance of N_2 fixation as a source of N to aquatic ecosystems will change as other N inputs increase (Kunza and Hall 2014; Scott and Marcarelli 2012; Sobota et al. 2013).

Significance of the study of N_2 fixation in river surface water

Due to the rapidly developing economy and accelerating urbanization, a large amount of nitrogen-containing substances from production and domestic sewage are discharged into urban rivers. This has led to severe pollution of the water in urban area river networks, i.e., the eutrophication of the rivers. The large-scale problem of the water cyanobacteria phenomenon is also getting worse (Scott and Marcarelli 2012). Biological N_2 fixation can add new N to ecosystems to support primary production. Fixed N helps sustain primary productivity, particularly in years with high rates of N_2 fixation (Hayes et al. 2019). Given enough time, ecosystems can potentially accumulate sufficient fixed N to drive the system to P limitation (Scott and Grantz 2013). Biological N fixation and the subsequent remineralization provide sufficient N to support a high phytoplankton biomass, including non-diazotrophic species (Higgins et al. 2018).

N circulating bacteria play a vital role in the migration and transformation of N in eutrophic aquatic ecosystems. Therefore, the determination of river surface water N_2 fixation rates and the determination of the contribution of N_2 fixing cyanobacteria to the N sources in water bodies further our understanding of this process in N cycling and provides insight into the N conversion mechanism and its transformation effect in urban area river networks. It also provides support for the N pollution control, remediation, and management of these river channels. For eutrophic rivers in urban areas, separate control methods targeting N and P cannot effectively

solve the problem. N control combined with P control (Paerl 2009) deserves consideration for eutrophication management in these systems. With robust estimates of the rivers' N_2 fixation rates, we can more satisfactorily predict and monitor the rivers' N input and output processes. In addition, we can provide baseline information for use in eutrophication treatment and nutrient salt control strategies for urban area rivers.

Conclusions

To some extent, N_2 fixation occurs in the water of river networks in urban areas. The average annual N_2 fixation rates of the rivers in Shanghai are 53.20–89.24 $ngN \cdot L^{-1} \cdot h^{-1}$ with annual fixed rates of 0.163–0.274 $gN \cdot m^{-2} \cdot a^{-1}$, which are greater than those of some oceans but are lower than those of some eutrophic lakes. The annual N_2 fixation of the Shanghai river network is approximately 0.15 $Gg \cdot a^{-1}$, accounting for about 1.36% of the total atmospheric deposition N input.

The N_2 fixation rates of rivers have obvious temporal and spatial characteristics. First, the N_2 fixation rates of the eight rivers investigated in this study exhibit seasonal differences, i.e., they are highest in the summer, followed by spring and autumn, and they are lowest in the winter. Second, the N_2 fixation rates of rivers in different regions are significantly different due to the surrounding environmental conditions, land use types, and water quality. The concentration of dissolved N in the water was negatively correlated with the N_2 fixation rate, whereas P was positively correlated with the N_2 fixation rate.

The N_2 fixation rates of rivers are affected by various environmental factors, i.e., the density of N_2 fixing cyanobacteria, the light level, and the temperature. Temperature, which affects N_2 fixation by changing the metabolism of the N_2 fixing microorganisms and the activity of N_2ase, is the main cause of the seasonal variation in the rivers' N_2 fixation rates ($p < 0.01$).

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