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Dissolved Inorganic Nitrogen Input via Net Nitrogen Mineralization under Antibiotics and Warming from the Water Level Fluctuation Zone of a Three Gorges Tributary

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Abstract: The water level fluctuation (WLF) zone is one of the dominant sources of total dissolved inorganic nitrogen (TDN) export via net nitrogen (N) mineralization in the Three Gorges Reservoir (TGR). However, antibiotics pollution may impact the process of TND exports from WLF zone in the TGR, especially under drying-rewetting processes and climate warming, and thus increasing the risk of eutrophication in the tributaries of the TGR. The effects of the antibiotics Griseofulvin (GIN) and Fosfomycin (FIN) with 0, 0.2 and 0.4 g kg^{-1} net N mineralization rate (NMR) from WLF-zone soil in the Pengxi river, a typical tributary of the Yangtze River, under 25 and 35 °C were estimated in 30-day flooding and drying incubations. The results showed that GIN concentrations, temperatures and their interaction significantly affect net-nitrification rates (NNR) and NMR under drying and did not significantly affect NNR under flooding. FIN concentrations and temperatures solely influenced the NNR under flooding. The amounts of TDN exports via NMR without antibiotics from the WLF zone of Pengxi River are 6883.8 (flooding, 25 °C), 9987.3 (flooding, 35 °C), 9781.6 (drying, 25 °C), and 27,866.5 (drying, 35 °C) t $year^{-1}$, which is 21.0, 29.8, 30.4 and 84.8 times of the permissible Class A discharge in China according to (GB18918-2002). Thus, the NMR of WLF zone should be controlled whether there is antibiotics pollution or not, especially during the dry period for alleviating water eutrophication. This study will be helpful for the assessment of nitrogen budgets in the WLF zone to eutrophication in the Three Gorges Reservoir.

Keywords: antibiotic; Three Gorges Reservoir; water level fluctuating zone; total dissolved nitrogen; temperature increasing; eutrophication

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1. Introduction

The water stored in the Three Gorges Reservoir (TGR) can reach 4×10^{10} m^3/a, which is one of the world's largest reservoirs with antiseasonal flow regulation [1]. The area of the water level fluctuation (WLF) zone is 348.9 km^2 between 145 m above mean sea level (masl) and 175 masl in the TGR since 2003 [2]. Most of nutrients accumulated in the WLF zone during the dry period (summer season) may be released into the water under the anoxic condition during the submerging period from October to the next March (winter season). The relatively slow flow velocity resulted in the weakened diffusion and exchange capacity in the tributaries of the TGR [3]. The decreased diffusion speed and self-purification capacity of various pollutants raise the risk of eutrophication in the

TGR tributaries. Moreover, the water bloom frequently occurred in the tributaries of the TGR [4,5].

The 67 antibiotic compounds were represented from the bulk of literature worldwide from 2008 to 2018 [6]. However, most antibiotics cannot be fully absorbed by humans and animals [7]. About 75% of them are discharged into the environment in the form of prototype or active metabolites through domestic sewage, aquaculture wastewater, agricultural manure irrigation runoff, etc. [8]. The killing pathogens, antibiotics may inhibit the activity of beneficial microorganisms in the environment [9]. Thus, antibiotics may disturb or even destroy the ecosystem, which poses potential risks to the ecological environment.

Compared with other regions of China, the higher pollution level of antibiotics has been found in the water, soil or sediment of the TGR [10]. Total concentrations of tetracycline, sulfonamides and quinolones ranged from 21.55 to 536.86 ng/L, 3.69 to 438.76 ng/g, 15.78 to 213.84 ng/g in the water, soil and sediment of TGR, respectively [11]. The seasonal distribution of tetracycline is correlated with the flow operation in the TGR [12]. The concentration of antibiotics in water is in the dozens of µg/kg, but it is from µg/kg to mg/kg in soil/sediment due to the strong adsorption capacity of soil/sediment for most antibiotics [13]. The adsorption strength is closely related to soil pH, soil clay, organic matter and iron oxide content. The adsorption mechanism of antibiotics on soil/sediment is complicated, because they can exist in both ionic and molecular forms in aqueous solution. Antibiotics can be absorbed and held in soil by interacting with the adsorption sites on the surface of soil organic matter or inorganic colloid through intermolecular forces, such as hydrogen bond and Van der Waals force, or through cation exchange, static electricity, bond bridge, coordination or complexation, etc. [14,15].

Nitrogen (N) mineralization is a process in which organic nitrogen-containing compounds in soil are transformed into inorganic nitrogen under the action of soil microorganisms, which is one of the critical processes of terrestrial N cycle [16]. Soil N mineralization is mainly focused in temperate grassland, alpine wetland and WLF zone [17–19]. The factors, such as land use, litter, temperature and soil moisture significantly affect N mineralization in the WLF zone in the dry period [20,21]. During the flooding period, the residual mineralized N and newly mineralized N may cause a potential risk for water eutrophication associated with the slow water velocity [22]. The soil organic N is mineralized by bacteria, fungi and actinomycetes. All of them use soil organic matter as an energy source under aerobic or anaerobic conditions. The bacteria involved in the ammonization process mainly include *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus cereus*, *Clostridium putrefaciens*, *Proteus vulgaris*, *Pseudomonas fluorescens*, etc., while the fungi mainly include *Alternaria*, *Aspergillus*, *Mucor*, *Penicillium*, *Rhizopus*, etc. [23]. However, TDN inputs from net N mineralization of the WLF-zone soil under antibiotic pollution and temperature increasing in the TGR tributaries remain unclear. The objectives of this study are to: (1) investigate the effect of the types and addition of antibiotics on the TDN inputs from the net N mineralization of the WLF-zone soil under warming and drying/inundation condition; (2) estimate the contribution of TDN inputs induced by antibiotics via N mineralization processes of the WFL zone soil to water eutrophication in the TGR tributary.

2. Materials and Methods

2.1. Study Area

The WLF zone in the Pengxi River is about 48.02 km^2, accounting for 15.9% of the total WLF zone area of the TGR [24]. The region is located at the north subtropical humid monsoonal climate with average annual precipitation of 1053.15 mm and average annual temperature of 18.2 °C and annual effective solar radiation of 3650 MJ/m^2, comprised of valley slopes and bench terraces [25]. The water level rises to the highest level of 175 masl in December, and then drops to the lowest water level of 145 masl the following June for electricity generation and agricultural irrigation [26]. Thus, the WLF zone in the TGR is under flooding about half a year and drying in another half.

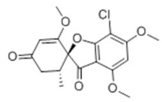
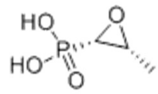
2.2. Sampling

We collected mixed surface soil samples in the upper, middle and downstream of the WLF zone in the Pengxi River in July 2017. Briefly, five soil samples were randomly collected by 100 cm^3 ring knife and mixed before further treatment from 145 masl to 175 masl at each sampling section of the WLF zone. One subsample was weighed immediately, brought back to the laboratory on the same day and dried for 24 h for calculating the water content and bulk density. The other soil samples were passed through a 2-mm sieve after being air-dried, and gravel and plant residuals were removed for the determination of soil physical and chemical properties and laboratory incubation. The properties of soil samples were shown as C: 1.35%, N: 0.09%, C/N: 0.63, pH: 7.04, NH_4-N: 10.59 mg/kg and NO_3-N: 17.99 mg/kg.

2.3. Antibiotics

Griseofulvin (GIN) is an antifungal medicine that is used to treat infections such as ringworm, athlete's foot, jock itch, and fungal infections of the scalp, fingernails, or toenails. GIN is a white or almost white fine powder, odorless and slightly bitter. It is easily soluble in dimethylformamide, slightly soluble in anhydrous ethanol and extremely slightly soluble in water. Melting point: 218–224 °C [27]. Fosfomycin (FIN) is a broad-spectrum antibacterial discovered by Merck and CEPA Company in 1967. Its molecular weight is small and its structure is unique. FIN is a white crystalline powder, soluble in water and easily deliquesced in air. The FIN solutions are relatively stable with a pH of 6.5–8.5. It is hard to decompose in the aqueous solution with a pH of 4–11, suggesting that FIN can maintain its antibacterial activity up to 30 years [28]. The characteristics of the two antibiotics were shown in Table 1.

Table 1. Antibiotics used in this study.

Antibiotics	Molecular Formula	CAS	Molecular Weight	Structure	Function
GIN	$C_{17}H_{19}ClO_6$	126-07-8	352.77		Anti-bacteria
FIN	$C_3H_7O_4P$	23155-02-4	138.06		Anti-fungal

2.4. Experiment Design

The soil was incubated in flooding and nonflooding conditions at 25 °C and 35 °C for 30 days, respectively [17]. The concentrations of FIN and GIN were set as 0, 0.2 and 0.4 g kg^{-1}, respectively, according to the mean level of antibiotic concentration in the rivers of China [13]. Each experiment condition was set with three groups of parallel to ensure experimental accuracy. Total 72 samples were included in this experiment: two temperatures × two flooding conditions × two antibiotics species × three concentration gradients × three replicates = 72 samples.

2.5. Soil Incubation and Analysis

The preincubation was proceeding at 50% WHC (maximum soil water holding capacity) for 24 h. During the incubation, 60% WHC was kept in the drying treatment by adding 5 mL antibiotic solution of 0, 0.2 and 0.4 g kg^{-1}, while 180% WHC was executed in the flooding treatment by adding 15 mL of the corresponding antibiotics solution. The moisture was kept by weighing in the three-day interval. After the incubation, NH_4-N content was measured by Nessler's reagent spectrophotometry. NO_3-N were analyzed by phenol disulfonic acid colorimetry. The net ammonification rate (NAR, mg kg^{-1} d^{-1}), net nitrification rates (NNR, mg kg^{-1} d^{-1}) and net N mineralization rate (NMR, mg kg^{-1} d^{-1})

on a dry mass basis were calculated as the changes in inorganic N (NH_4-N+NO_3-N) in the initial and incubated samples during the 30-day incubation [17]. All samples were executed alongside external quality control standards (LGC, Bury, UK) [29].

2.6. Data Evaluation

Total amount of TDN inputs was calculated considering the total area and drying/submerging time of WLF-zone soil as follows:

$$\text{TDN} = \text{NMR} \times \text{t} \times \text{H} \times \text{D} \times \text{S} \tag{1}$$

where TDN is the total amount of total inorganic N derived from the net N mineralization from the WLF-zone soil into the Pengxi River during a given time (kg year^{-1}). NMR is the soil net N mineralization rate (mg kg^{-1} d^{-1}); t was assigned as 180 d year^{-1} in the flooding or drying period. H is the depth of surface soil (20 cm); D is the soil bulk density (kg m^{-3}); S is the whole areas of the WLF zone in the Pengxi River (48.02 km^2). We assumed that TDN input via net N mineralization during the drying period will enter the water with the rising of the water level during the submerging period.

Exceeding standard multiple (MES) of TDN export was calculated as:

$$\text{ESM} = \text{TDN}_{\text{total}} \times \text{A}_{\text{permissible}} \tag{2}$$

where ESM is the exceeding standard multiple of TDN inputs into the water; $A_{permissible}$ is the annual permissible discharge amount of TDN from a municipal wastewater treatment plant with the peak flow of 60,000 m^3 d^{-1} according to Class I (A) of the Wastewater Discharge Standard of GB18918-2002 in China (COD = 50, BOD_5 =10, TSS = 10, TN = 15, NH_3-N = 5, TP = 0.5).

Significant differences between treatments were tested by Fisher's least significant difference procedure at $p < 0.05$. Linear mixed model was used to evaluate the interaction effects of antibiotic concentrations and water regimes on nitrogen mineralization. Graphs were drawn using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, USA) and statistical analyses were conducted using SPSS Statistics 20 (IBM SPSS, Inc., New York, NY, USA).

3. Results and Discussion

3.1. NH_4^+-N and NO_3^--N Variation under the Flooding and Drying Conditions

Under GIN treatment, ammonia nitrogen was affected by antibiotic concentrations, hydrological conditions and their interaction (Figure 1a,b). Compared with flooding, NH_3-N was much lower under GIN addition and drying at both 25 and 35 °C of. Moreover, GIN addition increased NH_4-N under flooding at 25 °C (Figure 1a), while decreased it under drying at 35 °C (Figure 1b). Under FIN addition, the hydrological condition significantly affected the ammonium nitrogen content at 25 °C while it had no significant effect at 35 °C. FIN addition increased NH_4-N at 25 °C, but decreased it at 35 °C under flooding. There was no variation of NH_4-N among FIN concentrations under drying at 35 °C. The NO_3-N content under drying was much higher relative to flooding at 25 and 35 °C (Figure 2). GIN decreased NO_3-N content in the drying condition, but had no significant effect on NO_3-N under flooding at both temperatures (Figure 2a,b). Moreover, FIN brought down NO_3-N content at both temperatures and hydrological conditions except for FIN of 0.4 g kg^{-1} concentration under 35 °C and drying (Figure 2c,d).

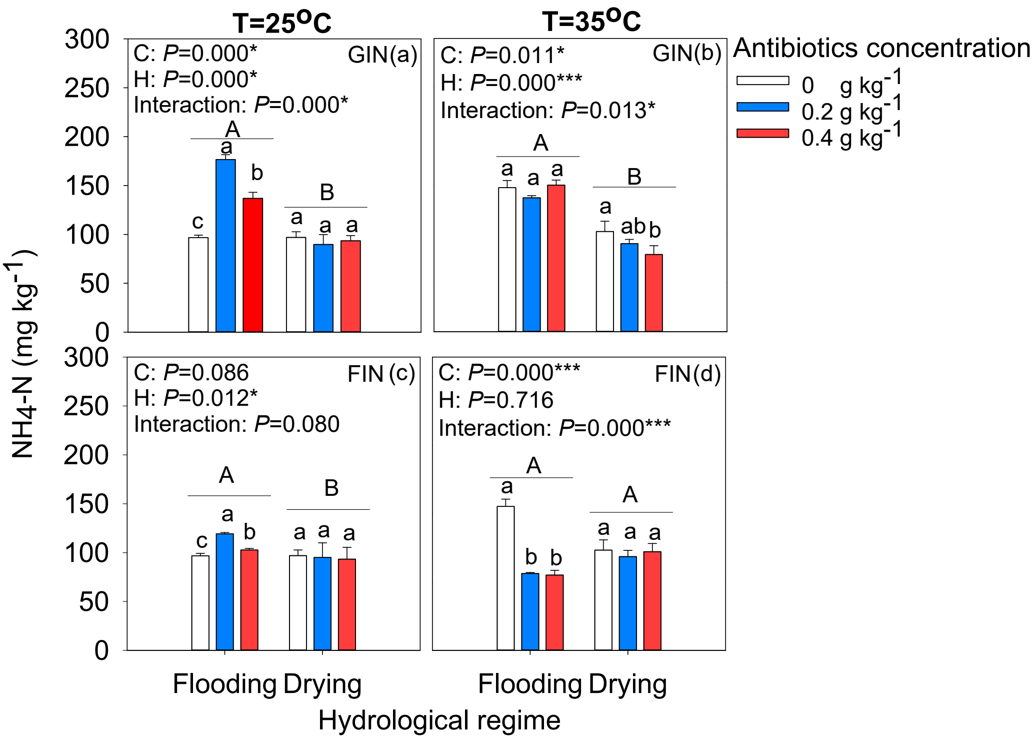


Figure 1. The variation of ammonium at 25 °C and 35 °C during 30-day flooding or drying incubation. Different uppercase letters indicate significant differences between flooding and drying. Different lowercase letters represent significant differences among antibiotics concentration under flooding or drying conditions. Different numbers of asterisks represent a significant difference (* $p < 0.05$, *** $p < 0.001$).

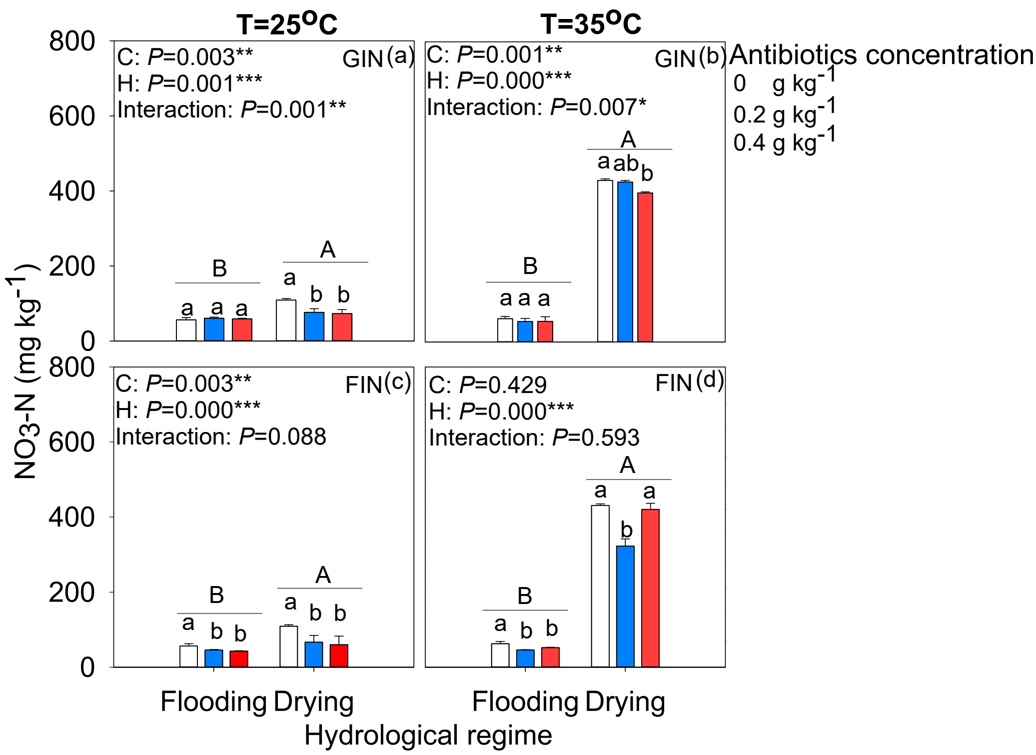


Figure 2. The variation of nitrate at 25 °C and 35 °C during 30-day flooding or drying incubation. Different uppercase letters indicate significant differences between flooding and drying. Different lowercase letters represent significant differences among antibiotics concentration under flooding or drying conditions. Different numbers of asterisks represent a significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2. NAR, NNR and NMR

NAR decreased under drying (Figure 3b), while it increased under flooding compared to the control after GIN addition (Figure 3a). On the contrary, FIN decreased the NAR under flooding (Figure 3c), but did not affect NAR under drying (Figure 3d). Antibiotics concentrations, temperatures and their interaction significantly affect NNR and NMR under drying (Figures 4b,d and 5b,d) and did not significantly affect NNR under flooding after GIN addition (Figure 4a). Antibiotics concentrations and temperatures solely influenced the NNR under flooding of FIN (Figure 4c). Antibiotics concentration and its interaction with temperature significantly influenced NMR under flooding after GIN and FIN addition (Figure 5a,c). Soil organic nitrogen transformation ability is an important indicator to measure soil microbial activity, and its dynamic change directly reflects the change of soil ecosystem function [30]. Antibiotics can kill or inhibit certain microbial groups in soil, thereby affecting soil microbial communities and causing a series of changes in ecological functions, such as soil organic matter decomposition, nitrogen transformation and enzyme activity [31]. From our results, NAR and NMR with medium GIN condition under flooding were higher than relative to the control and higher concentration (Figures 3a and 5a). There are two main reasons, Firstly, GIN with the medium concentration was continuously degraded under submerged anaerobic conditions, and became the carbon source of anaerobic microorganisms at low redox potential. This also shows that the GIN addition will stimulate soil organic N mineralization under flooding. Secondly, GIN as carbon-rich substances will be used by some resistant microorganisms in the soil as carbon sources, which promotes the growth and reproduction of microorganisms, thereby improving the mineralization of soil organic nitrogen, in turn increasing the ammonization and total net nitrogen mineralization rate.

However, NAR and NMR decreased under higher concentrations (0.4 g kg^{-1}) of GIN under drying, which might be due to the selective change of soil microbial community under the antibiotic stress, and quickly restored to the original community function, so that the soil nitrogen mineralization rate was restored [32]. The NAR, NNR, NMR decreased with the increase of GIN concentrations under drying. The possible reason was antibacterial function of GIN, which led to the inhibition of ammonifier and nitrobacteria. At the same time, FIN totally decreased the NAR, NNR, NMR under drying or flooding conditions except for NAR under drying. It is because that FIN mainly had antifungal function and had no inhibitory effect on bacteria. The results showed that the increase of temperature promoted NMR under drying after GIN and FIN addition (Figure 5b,d), which can be mainly contributed to the increasing of nitrification under warming (Figure 4b,d). However, the increase of temperature consistently decreased the NAR and NMR under GIN of 0.2 g kg^{-1} and FIN of 0.2 and 0.4 g kg^{-1} under flooding. Some studies have suggested that the application of antibiotics can kill some microorganisms in the soil and inhibit the mineralization of soil organic nitrogen [33], or the addition of antibiotics under the experimental conditions does not affect the metabolism of soil microorganisms [34]. This difference may be related to the complex soil physical and chemical properties, different microbial community composition and their tolerance to external stress.

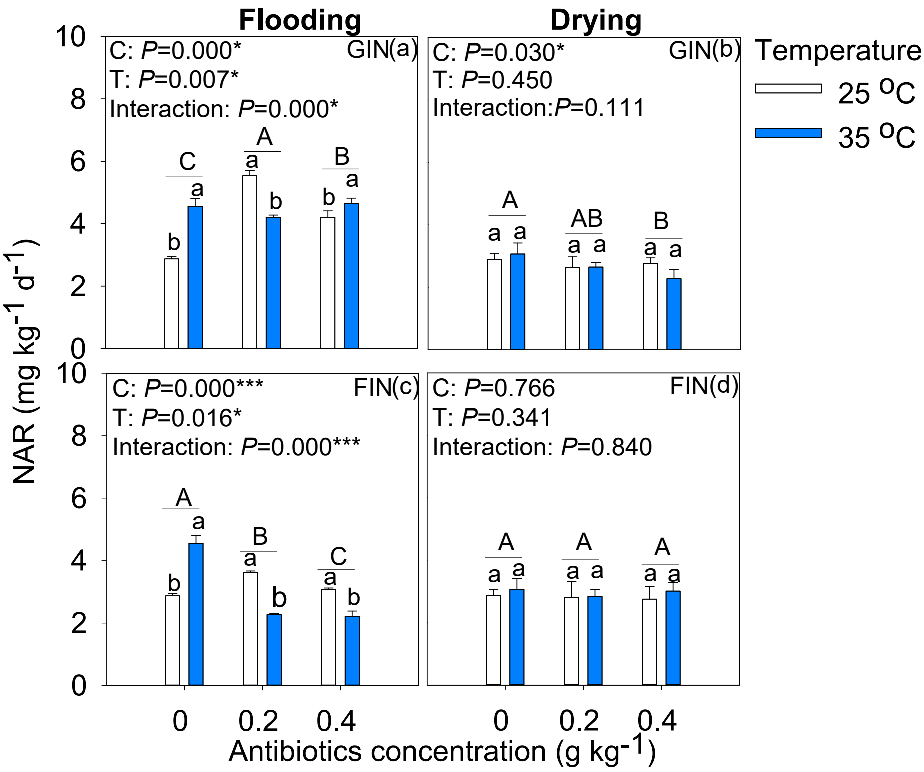


Figure 3. Net ammonification rate (NAR) with the antibiotics content of 0, 0.2 and 0.4 g kg^{-1} in the flooding and drying conditions at 25 °C and 35 °C. Different uppercase letters indicate significant differences among antibiotics concentrations of 0, 0.2 and 0.4 g kg^{-1}. Different lowercase letters represent significant differences between 25 and 35 °C at the same antibiotic concentration ($p < 0.05$). Different numbers of asterisks represent a significant difference (* $p < 0.05$, *** $p < 0.001$).

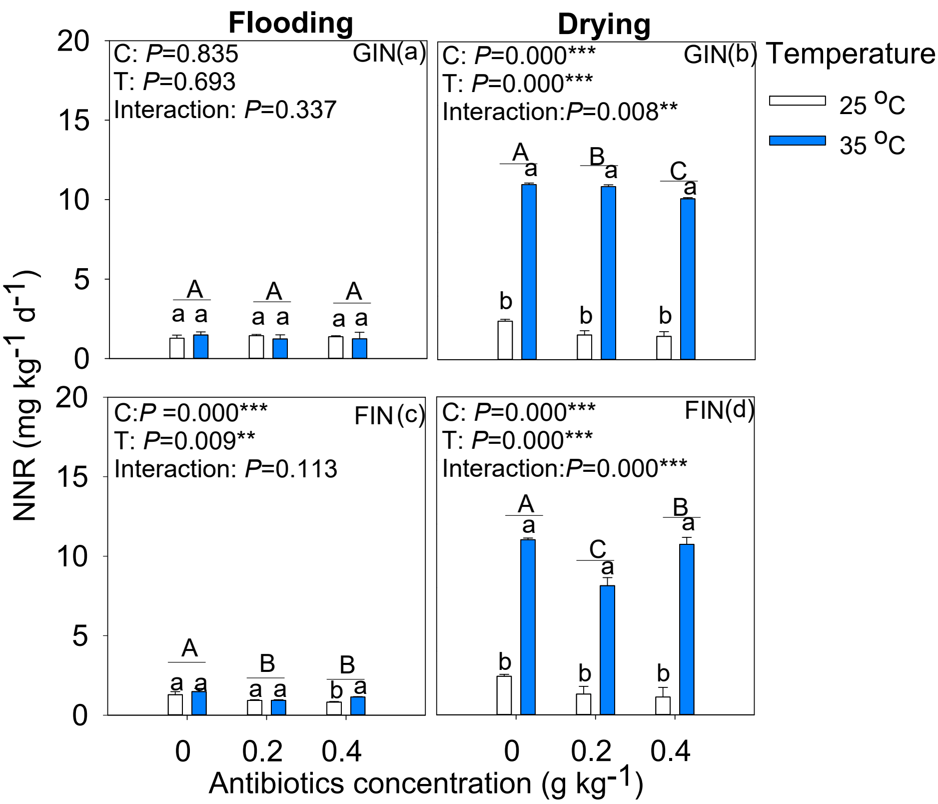


Figure 4. Net nitrification rates (NNR) with the antibiotics content of 0, 0.2 and 0.4 g kg^{-1} in the

flooding and drying conditions at 25 °C and 35 °C. Different uppercase letters indicate significant differences among antibiotics concentrations of 0, 0.2 and 0.4 g kg^{-1}. Different lowercase letters represent significant differences between 25 and 35 °C at the same antibiotic concentration ($p < 0.05$). Different numbers of asterisks represent a significant difference (** $p < 0.01$, *** $p < 0.001$).

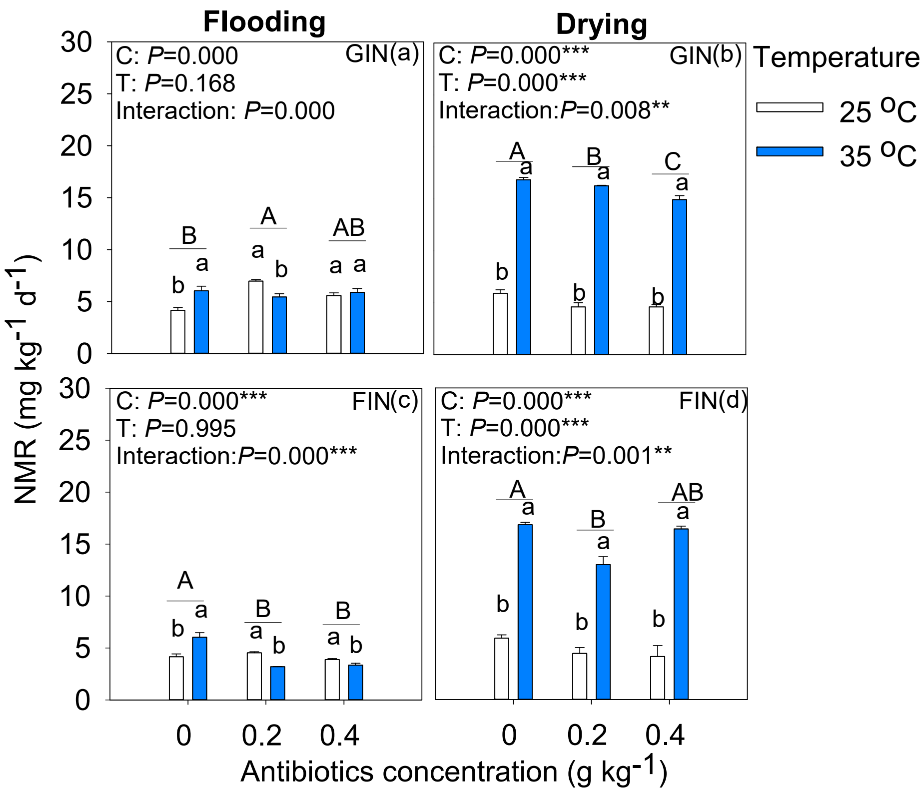


Figure 5. Net N-mineralization (NMR) with the antibiotics content of 0, 0.2 and 0.4 g kg^{-1} in the flooding and drying conditions at 25 °C and 35 °C. Different uppercase letters indicate significant differences among antibiotics concentrations of 0, 0.2 and 0.4 g kg^{-1}; Different lowercase letters represent significant differences between 25 °C and 35 °C at the same antibiotic concentration ($p < 0.05$). Different numbers of asterisks represent a significant difference (** $p < 0.01$, *** $p < 0.001$).

3.3. TDN Input Amount and Multiple Exceeding Standard

Hydrological condition, temperature, and their interaction can significantly influence TDN amount and MES under both GIN and FIN addition ($p = 0.000$, Table 2). Moreover, the interaction of antibiotics concentration and temperature can significantly influence TDN amount and MES under FIN addition ($p < 0.05$, Table 2). The amounts of TDN input via net nitrogen mineralization without antibiotics addition from the WLF zone of Pengxi River are 6883.8 (flooding, 25 °C), 9987.3 (flooding, 35 °C), 9781.6 (drying, 25 °C), and 27,866.5 (drying, 35 °C) t $year^{-1}$ (Table 2), which respectively are 21.0, 29.8, 30.4 and 84.8 times the annual permissible discharge amount of pollutants calculated from a municipal wastewater treatment plant with a peak flow of 60,000 m^3/d according to Class I (A) of the Wastewater Discharge Standard (GB18918-2002) in China. The corresponding multiple exceeding standard (MES) got to 21.3, 16.5, 21.7, 74.2 on average after FIN addition, while reach 31.6, 28.5, 23.2, 78.6 on average after GIN addition. Thus, the nutrient exports from the WLF zone via net N mineralization, especially with or without antibiotics pollution under global warming are considerable, which is critical for eutrophication control in the TGR tributaries. In addition, the amounts of TDN input via net nitrogen mineralization is much larger than the amount of TDN release of 49.3 t $year^{-1}$ by the leaf decay of *Cynodon dactylon* (L.) Pers in the same region [24]. It has been found that spring algal bloom was accompanied by a nonpoint source with nutrients loads of 10,613 t $year^{-1}$ on average for TDN from 2005 to 2008 in Pengxi River within Yunyang County section [35].

Our results showed that the amount of TDN exports originated from net N mineralization of WLF-zone soil without antibiotics was account for about 64.9% (flooding, 25 °C), 92.2% (flooding, 35 °C), 94.1% (drying, 25 °C) and 262.6% (drying, 35 °C) of that loads from the nonpoint source, respectively. However, the corresponding proportion changed into 65.8%, 51.0%, 67.0%, 229.5% after FIN addition, and 97.8%, 88.3%, 71.6%, 243.2% after GIN addition on average. To summary, the WLF zone is an important source of nutrients in the Three Gorges Reservoir [36], the load of nutrient exports from the net N mineralization of WLF-zone soil with or without antibiotics could not be neglected, and control of net N mineralization under drying is especially needed for alleviating water eutrophication.

Table 2. TDN inputs amount and multiple exceeding standard (MES). ANOVA *p*-values of concentration, hydrological regime and temperature on TDN inputs amount and MES were presented by the linear mixed model.

Concentration (g kg^{-1})	Hydrological Regime	Temperature (°C)	Replicate	Antibiotics Types			
				GIN		FIN	
				TDN Amount (t/a)	MES	TDN Amount (t/a)	MES
0	Flooding	25	3	6883.8	21.0	6883.8	21.0
0.2	Flooding	25	3	11,531.1	35.1	7523.6	22.9
0.4	Flooding	25	3	9228.9	28.1	6441.0	19.6
0	Flooding	35	3	9987.3	30.4	9987.3	30.4
0.2	Flooding	35	3	9007.8	27.4	5290.6	16.1
0.4	Flooding	35	3	9732.5	29.6	5542.3	16.9
0	Drying	25	3	9781.6	29.8	9781.6	29.8
0.2	Drying	25	3	7593.1	23.1	7346.9	22.4
0.4	Drying	25	3	7608.0	23.2	6878.6	20.9
0	Drying	35	3	27,866.5	84.8	27,866.5	84.8
0.2	Drying	35	3	26,912.1	81.9	21,516.0	65.5
0.4	Drying	35	3	24,717.8	75.2	27,191.5	82.8
ANOVA *p*-values							
Concentration				0.050	0.050	0.286	0.286
Hydrological condition				0.000	0.000	0.000	0.000
Temperature				0.000	0.000	0.000	0.000
Concentration × Hydrological condition				0.090	0.090	0.072	0.072
Concentration × Temperature				0.894	0.894	0.018	0.018
Hydrological condition × Temperature				0.000	0.000	0.000	0.000
Concentration × Hydrological condition × Temperature				0.093	0.093	0.110	0.110

4. Conclusions

The amounts of TDN exports via net nitrogen mineralization without antibiotics from the WLF zone of Pengxi River are 6883.8 (flooding, 25 °C), 9987.3 (flooding, 35 °C), 9781.6 (drying, 25 °C), and 27,866.5 (drying, 35 °C) t year^{-1}, which is 21.0, 29.8, 30.4 and 84.8 times of the permissible discharge amount of pollutants in China according to (GB18918-2002). Moreover, the amount of net N nitrogen mineralization is also dependent on the types of antibiotics and hydrological conditions. The assessment in the present research of the TDN input via net nitrogen mineralization under antibiotics addition and warming will provide valuable information regarding the contribution of this process to nitrogen budgets in the WLF zone to eutrophication in the Three Gorges Reservoir. However, an integrated model combined with the hydraulic gradients, hydrogeological parameters and meteorological conditions needs to be developed in future research due to the real complicated field environment. The nitrogen application strategy and nitrogen loss control technology need further development in the WLF zone. The control of N mineralization in the WLF zone is one of the effective strategies, especially during the dry period of the WLF zone, for alleviating water eutrophication, but further study is needed.

Author Contributions: J.L., D.L. and Q.N. designed the experiment; C.Y. and Y.D. performed the experiments; Y.D., X.Y., C.X. and J.L. analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

WLF	water level fluctuation;
TDN	total dissolved inorganic nitrogen;
TGR	Three Gorges Reservoir;
GIN	Griseofulvin;
FIN	Fosfomycin;
N	nitrogen;
NMR	net nitrogen mineralization rate;
NAR	net ammonification rate;
NNR	net-nitrification rates;
NH_4-N	ammonium nitrogen;
NO_3-N	nitrate nitrogen;
WHC	maximum soil water holding capacity;
MES	Exceeding standard multiple.

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