The Coupling of Carbon, Nitrogen and Sulphur Transformational Processes in River Sediments

Based on Correlationship among the Functional Genes

Mingzhu Zhang¹, Yang Li¹, Qingye Sun¹, Piaoxue Chen¹, Xuhao Wei¹

¹School of Resources and Environmental Engineering, Anhui University, Hefei, Anhui Province, 230601, China

Correspondence to: Qingye Sun (sunqingye@ahu.edu.cn)

Abstract: Microorganisms in sediments play an important role in C-, N- and S-cycles by regulating forms and contents of these elements. The coupled system or synergistic reaction among three elemental cycles can effectively alleviate the pollution of C, N, and S in sediments. However, ecological processes coupling C-, N- and S-cycles in sediments are still poorly understood. In order to understand the ecological processes mediated by microorganisms living in river sediments, a total of 135 sediment samples were collected from Huaihe River and its branches located in the Northern of Anhui Province, the abundance of functional marker genes (mcrA, pmoA, cmo, amoA, hzo, nirK, nirS, nosZ, dsrB, aprA), involving in C-, N- and S-transformation, were determined by qPCR. The correlation among functional genes from 135 river sediment samples was calculated. We supposed that the correlation among functional genes could be used as a reference index speculating the coupled systems of C-N-S in this research, then the distinct coupling relation of C-N-S was revealed, and probable genetic mechanisms were also expounded based on the hypothesis. The study found that amoA-AOA and dsrB possibly played a secondary role, while S-functional gene (aprA), C-functional
gene \((mcrA)\) and N-functional gene \((hzo)\) were the key functional genes that participate in the coupled processes in the elemental biogeochemical cycle. The results also demonstrated that C, N might have combined effects on the coupling of carbon, nitrogen and sulphur transformation.

**Keywords:** river sediment, coupled systems, C, N, and S cycles, functional genes

1 Introduction

Rivers play a substantial part in elemental biogeochemical processes (Aufdenkampe et al., 2011), which can regulate the carbon (C), nitrogen (N) and sulphur (S) cycles and act as a good indicator of environmental changes (Crump et al., 2009; Williamson et al., 2008). However, the nutrient elements (such as carbon, nitrogen and sulphur) originating from domestic sewage, farm drainage, industrial effluent, etc. flow into the river, and deposit into the sediments (Cheng et al., 2014; Liu et al., 2014; Fonti et al., 2015), which lead to the deterioration of river ecosystems.

Studies demonstrated that microorganisms in the artificial environments could couple the transformation processes of different elements by inter-specific cooperation or coordination of inter-gene from the same species (Zhi and Ji, 2014). In coupling with methane-nitrogen cycle, anammox-methanogenesis (Bai et al., 2013), nitrite-driven anaerobic methane oxidation (Ettwig et al., 2010), aerobic methane oxidation-denitrification (AME-D) (Knittel and Boetius, 2008; Modin et al., 2008; Modin et al., 2007) and denitrification-methanogenesis (Kodera et al., 2017; Wang et al., 2017)
have been confirmed. For the coupling of S and N cycles, Fdz-Polanco et al. (2001) firstly approved the sulfate-reducing anaerobic ammonium oxidation (SRAO) process to explain “abnormal” losses of nitrogen and sulfate. And subsequently several laboratory studies were conducted for purpose of speculate the pathway of SRAO (Rikmann et al., 2012; Zhang et al., 2009; Schrum et al., 2009). The occurrence of microaerophilic sulfate and nitrate co-reduction system has been previously reported (Bowles et al., 2012; Brunet and Garcìagil, 1996). For the coupling of C and S cycles, the pathway of sulfate-dependent anaerobic methane oxidation had been discovered, which was common completed by anaerobic methanotrophic archaea and sulfate-reducing bacteria (M et al., 2003).

Recently, the coupling cycle between different elements in natural or constructed wetlands, such as methane oxidation coupled to nitrogen fixation (Larmola et al., 2014), methane oxidation coupled to ammonium oxidation (Zhu et al., 2010), methane oxidation coupled to denitrification (Zhu et al., 2016; Long et al., 2016; Long et al., 2017; Luo et al., 2017; Zhang et al., 2018), methane oxidation coupled to sulfate reduction (Xu et al., 2014; Weber et al., 2017; Emil et al., 2016), etc., received extensive attention. The coupling cycle between different elements was mainly driven by functional groups from bacteria and/or archaea living in sediments. The enzymes coded by functional gene(s) in functional groups catalyze each reaction step in the biogeochemical cycle of elements. At presently, the functional genes have been regarded as appropriate indicators for the related biogeochemical processes in the C and N cycles (Petersen et al., 2012; Rocca et al., 2014). The development of
molecular biological technique greatly facilitate the quantitation of functional genes in environmental samples (Lammel et al., 2015; Petersen et al., 2012). Many studies have used the abundance of functional groups or functional genes involving in elemental cycle to explore the elemental metabolic pathways in different ecosystems (Bru et al., 2011; Xie et al., 2014; Smith et al., 2015).

Studies have shown that the microbial functional groups that complete a biogeochemical reaction may come from different microbial groups, and the same type of bacteria or archaea may also participate in different steps of the biogeochemical cycle. Therefore, compared with the microbial functional group, the correlationship among the functional genes can not only better reveal the coupling relationship of elemental metabolic processes in environmental media (especially for some natural ecosystems or more complex environmental media, such as sediments), but also predict some undetected coupling reactions. The main aims of this study were: (1) to analyze the correlation among the different functional genes related to some known coupled metabolic processes in sediments, and (2) to predict the possible coupling systems in sediments based on the correlation among the functional genes; and (3) to illustrate the key functional genes that participate in certain specific metabolic processes or steps in the elemental biogeochemical cycle.

2 Materials and Methods

2.1 Site description
The Huaihe River is located in the eastern China, watershed area of approximately 270,000 km², involving 5 Provinces (Henan, Anhui, Shandong, Jiangsu and Hubei) and 165 million population, situated in a transition zone of northern-southern climates in China (Meng et al., 2014; He et al., 2015) and belongs to monsoon climate from north subtropical to south warm temperature, and from humid to semihumid-semiarid. The average annual precipitation and the annual evaporation in the basin are some 883mm and 900-1500mm, respectively. The rainfall of flood season (June to September) usually amounts to 70% of the annual value. The average annual temperature ranges 13.2-15.7°C and frost free period is about 200-240 day. In the basin, a complex interaction of meteorological and hydrological processes frequently trigger and exacerbate flood and drought events (Wang et al., 2014; Zhang et al., 2015). Water resources per capita and per unit area in Huaihe River basin is less than one-fifth of the Chinese average. And more than 50% of the water resources are over-exploited (Jiang, 2011). In this basin, agricultural cultivation and livestock have a long history. Textile, household appliances, steel, cement and fertilizer, as the major industries, mainly distribute along the main stream and branches of Huaihe River, which are running through the main economic areas in the middle-eastern of China (Tian et al., 2013). In recent decades, a large number of nutrient from farm drainage, domestic sewage, industrial effluent, etc., had entered into the main stream and branches and deposited in the river sediment.

2.2 Sample collection and pretreatment
In this study, the main stream and the leftward branches located in the Anhui Province were chosen to do as the investigated area. The length of main stream of Huaihe River in Anhui Province is more than 400km and its leftward branches in Anhui Province mainly include Honghe river, Guhe River, Runhe River, Shayinghe River, Xifeihe River, Cihuai River, Qianhe River, Guohe River, Beifeihe River, Xiehe-Huihe River, Tuohe River, Bianhe River, Suihe River, etc. All branches investigated are located in Wanbei plain, which is a part of North China Plain. A total of 135 sections from main stream and its branches were chosen to collect the sediment samples. Before field sampling, all of sampling sections were set by the remote sensing map (Fig 1).

In each sampling section, 5 subsamples of surface sediment (depth: 0-10cm) were collected by Pedersen sampler and then mixed into a sample. The sediment sample was immediately loaded into a sterile self-sealing bag and then stored in the incubator with 4°C in the field. After returning to laboratory, each sample was divided into two parts, one was used to analyze the chemical properties and another was directly extracted DNA for the molecular biological test. The samples using to analyze chemical properties were desiccated by the method of vacuum freeze drying and then screened. After screening, the samples were loaded into the self-sealing bag and then stored in the refrigerating cabinet with -20°C until the chemical analysis was carried out.

2.3 Chemical analysis of sediment samples

The pH was assessed by the Mettler Toledo FE20 pH meter (sediment_{mass} : H_{2}O_{volume}=1g : 5ml).
The organic matter (OM) was determined by the loss of ignition (LOI) in a muffle furnace at 550±5°C for 6 h. The total nitrogen (TN) content was measured using the Kjeldahl method. Concentrations of NH₄⁺-N, NO₃⁻-N and NO₂⁻-N in sediment samples were determined using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). SMT (standard measurement and test) (Ruban et al., 2001) method is used to measure the total phosphorus (TP) inorganic phosphorus (IP) and organic phosphorus (OP) in the sediment.

2.4 DNA extraction

Total DNA in sediment samples were extracted by using the PowerSoil® DNA isolation kit (Mo Bio Carlsbad USA) in accordance with the manufacturer's instructions. Each extracted genomic DNA was preserved at −20°C until use.

2.5 Real-time fluorescent quantitative PCR

Quantitative analyses of functional genes, including amoA of AOA, amoA of AOB, hzo, nirK, nirS, nosZ, mcrA, pmoA, dsrB and aprA, were performed. The information on the primers selected for amplification are listed in supporting information (Table S1). Real-time PCRs were implemented on a Stepone real-time PCR system (Applied Biosystems USA). Each PCR mixture (10 uL) was composed of 5 uL of Bestar® SYBR qPCR Master Mix Ex TaqTM II (2×), 0.25 uL of each primer (concentration of 10 uM), 0.2 uL of ROX reference dye (50×), 3.3 uL of ddH₂O and 1 uL of template DNA (Bestar Biosystem, German). After generating PCR fragments of the respective functional genes using M13
PCR from clones, standard curves for real-time PCR were prepared based on a serial dilution of known copies of PCR fragments. The $R^2$ value of each standard curve was above 0.99.

**2.6 Data analysis**

To further investigate the interactions among the environmental parameters, pearson correlation analysis was applied to determine the significant correlations among the chemical properties. Correlation analysis was calculated to evaluate ecological associations among different functional marker genes involving in C-, N- and S-transformation using SPSS Statistics 20 (IBM, USA). NetworkSpearman graphanalysis was employed to investigate the key functional genes and nutrient elements of affecting the coupling transformation of C, N and S, the p-values in the correlation were adjusted statistically significant (PFDR<0.05). Network analysis was carried out by Gephi software according to the relationships between sediment parameters and functional genes..C, N and S cycles and coupled pathways were carried out following Auto CAD software.

Stepwise regression models between functional genes and chemical parameters were established by using SPSS Statistics 20 (IBM, USA). In stepwise regression analysis, environmental parameters, (i.e. pH, OM, NH$_4^+$-N, NO$_3^-$-N, NO$_2^-$-N, TN, IP, OP and TP) were used as candidate variables to integrate with functional genes related to C, N and S cycles.

**3 Results**
3.1 Chemical properties of river sediments

Table 1 presented the main chemical properties of 135 sediment samples. The pH values of river sediments were alkaline (with a mean of 7.78) and exhibited a lower coefficient of variance (CV) in all of chemical properties detected. TN displayed a higher CV among the different sampling sections rather than OM and TP. In 135 investigated sections, the content of inorganic nitrogen in sediments displayed a following order: NH$_4^+$-N > NO$_3^-$-N > NO$_2^-$-N, and NO$_3^-$-N contents among different sections showed the highest CV in inorganic nitrogen. IP content with a lower CV is higher than OP content in sediments. In five sections (i.e., sections C1, Q2, T3, TA1 and G6) with higher OM, NH$_4^+$-N, TN and TP, there were three sections (C1, TA1 and G6) locating in the farmland area. The first branch of the Huaihe River generally exhibited a lower content of nutrients rather than the secondary branches, especially OM, NH$_4^+$-N, NO$_3^-$-N and TN contents in sediments. Data analysis presented that OM (29.50±13.98 g·kg$^{-1}$), NH$_4^+$-N (34.92±34.33 mg·kg$^{-1}$), NO$_3^-$-N (7.01±6.85 mg·kg$^{-1}$) and TN (0.41±0.34 g·kg$^{-1}$) in the sediments of Guohe River (a first branch of the Huaihe River) were significantly lower than those in the sediments of its secondary branches (OM: 43.54±21.68 g·kg$^{-1}$; NH$_4^+$-N: 73.45±58.09 mg·kg$^{-1}$; NO$_3^-$-N: 35.35±20.01 mg·kg$^{-1}$ and TN: 0.85±0.66 g·kg$^{-1}$, $p<0.05$). The similar characteristics were found in the Shayinghe River (a first branch of Huaihe River) with the secondary branches.

Data analysis indicated that there was a significantly positive correlation among the different
chemical properties except for the pH and NO$_2^-$-N (Fig 2). The higher positive correlation between OM and TN in sediments indicated that both had the same source.

**3.2 Quantities of functional genes related to C, N and S cycles in river sediments**

In 13 functional genes investigated in this study, the abundance of *dsrB* and *pmoA1* genes was relative higher, and that of *hzo* and *aprA* genes lower (Table 2).

For N-cycling genes, the abundance of *amoA*-AOB was substantially lower as compared to *amoA*-AOA. Comparing to *nirK* and *nosZ*, *nirS* displayed higher abundance. In the functional genes related to C-cycle, the *mcrA* abundance exhibited the highest coefficient of variance. Table 2 also demonstrated that in contrast to *pmoA* and *pmoA2* genes, type II methanotrophs possessing the *pmoA1* gene were predominant. In two genes involving in sulfate reduction, the abundance of *dsrB* gene was significantly more than that of *aprA* gene in sediments.

All of the functional genes investigated in this study displayed higher CV (67.38%-317.86%), indicating a significant difference in abundance of detected N-, C- and S-cycling genes among 135 river sections.

Table 3 displayed the correlation coefficient among 13 functional genes involving in C-, N- and S-cycle in sediments. In the functional genes involving in N-cycle, abundances of *cmo*, *hzo*, *amoA*-AOB, *nirS*, *nosZ* genes were correlated with each other. Meanwhile, abundances of methanotrophic (*pmoA*, *pmoA1*, *pmoA2*), *mcrA* genes were correlated between each other in C-cycle.
With regard to S-functional genes, no direct relationships between *dsrB* and *aprA* were found.

Concerning the correlations among N-, C- and S-functional genes, the methanotrophic (*pmoA, pmoA1* and *pmoA2*) genes were correlated with the abundance of *nosZ*. The abundance of *mcrA* gene had a positive correlation with denitrifying genes (*nirK, nirS* and *nosZ*) and *hzo* genes. It was noted that the *dsrB* and *aprA* gene abundance were positively correlated with *hzo* gene abundance. Interestingly, positive correlation was also found between the abundance of *aprA* gene and C-functional genes (*mcrA, pmoA, pmoA1* and *pmoA2*).

4 Discussions

The cycles of carbon, nitrogen and sulphur in environment are made up of a series of chemical reactions (Parey et al., 2011; Lammel et al., 2015). For the sediment containing a large amount of organic matter and being in the state of reduction, the oxidation-reduction reaction should be the most important chemical reaction (Vincent et al., 2017). The substance of the oxidation-reduction reaction is the gain or loss of electrons or the offset of share electron pair. In river sediment, some elements get electrons to be reduced, while other elements lose electrons to be oxidized in the oxidation-reduction reaction. The enzymes from microorganisms, as catalyzer, can accelerate the oxidation-reduction reactions in sediment (Kandeler et al., 2006; Rocca et al., 2014; Parey et al., 2011). Although sediment is an important place of elemental cycles, ecological processes regulating methane, nitrogen and sulfur cycles are poorly understood.
4.1 Coupling of methane / nitrogen cycles in river sediments

Bai et al. (2013) revealed that the methanogenesis could coexist with anammox in a single anaerobic reactor. Based on the hypothesis of this research, there was a positive correlation between the abundance of hzo gene and mcrA gene, predicting that methanogenesis and anammox could work together, which also proved that anammox coupled to methanogenesis (Fig 3).

Studies showed that coupling the nitrate reduction and anaerobic digestion to form a bioreactor, in which denitrification and methanogenesis process can be carried out simultaneously. The coupled process could handle the high-strength carbon- and nitrate-containing wastewater, which had received extensive attention recently (Chen et al., 2009; Sun et al., 2015; Kodera et al., 2017). Based on our hypothesis, the abundance of mcrA gene was positively correlated with denitrifying genes (nirK, nirS and nosZ) in this study, which can also speculate that simultaneous denitrification and methanogenesis (SDM) process might occurred (Fig. 3). The simultaneous removal of carbon and nitrogen in the anaerobic environment through methanogenesis and denitrification was proved to be achievable (Chen et al., 2009).

\[
\text{CH}_3\text{OH} + \text{NO}_3^- \rightarrow \text{N}_2 + \text{CO}_2 + \text{OH}^- + \text{H}_2\text{O}
\]

\[
\text{CH}_3\text{OH} \rightarrow \text{H}_2\text{O} + \text{CO}_2 + \text{CH}_4
\]

Du et al. (2017) confirmed that it existed in reactor that a novel partial-denitrification combied with anammox process, since the nitrite for anammox could be acquired from partial-denitrification.
process. In our study, the abundance of hzo gene showed positive correlations with the denitrifying genes (nirK, nirS and nosZ), suggesting that denitrification might cooperate with anammox. Bai et al. (2013) proposed that an integrated process was developed by an anaerobic reactor, in which methanogenesis, denitrification and anammox were coupled, with methanogenesis first, then denitrification and anammox simultaneously. Accordingly, the whole abundance of mcrA gene was the highest compared with denitrifying genes (nirK, nirS and nosZ) and hzo gene in this study. Therefore, we postulated the plausible stoichiometric equations, which were deciped in table S2.

Methane oxidation coupled to denitrification consisted of nitrite-driven anaerobic methane oxidation (Ettwig et al., 2010) and aerobic methane oxidation coupling to denitrification (Zhu et al., 2016). This research exhibited that methanotrophic (pmoA, pmoA1 and pmoA2) genes and cmo gene were positively correlated with denitrifying genes (nirS and nosZ), which inferred the existence of aerobic methane oxidation coupled to denitrification (AME-D) process and anaerobic nitrite-dependent methane oxidation process in river sediments as is hypothesized (Fig 3). According to the speculation of the electron transfer pathway, since aerobic/anaerobic methane oxidation both are the processes of releasing electrons, while the released electrons are accepted by denitrification processes (NO$_2^-$→NO and N$_2$O→N$_2$). To date, the aerobic methane oxidation coupled to denitrification (AME-D) mechanism still remains obscure, and relevant studies have been carried out to propose different explanations of AME-D progress (Stein and Klotz, 2011); (Modin et al., 2007).
Zhu et al. (2016) summarized the potential energy reactions included in AME-D process. Under anaerobic conditions, NO$_3^-$ and NO$_2^-$ played a crucial role in supplying electron acceptors in denitrification processes (Zhu et al., 2016), a tentative inference about AME-D progress on this result is depicted in table S2. Ettwig et al. (2010) confirmed the existence of nitrite-driven anaerobic methane oxidation and explained the source of O$_2$ and the production of N$_2$. Dedicated stable isotope studies showed that this organism could make its own molecular oxygen from nitrite via nitric oxide. The produced oxygen was mainly used to oxidize methane in an anaerobic environment according to the expected stoichiometry:

\[ 3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}_+ \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O} \]

In our study, methanotrophic genes (pmoA, pmoA1 and pmoA2) were positively correlated with amoA-AOB, which can predict the coupled system of aerobic methane oxidation-aerobic ammonia oxidation based on the correlationship between the functional genes related to C, N cycles (Fig 3). Some investigators had confirmed that aerobic methanotrophs could oxidize ammonium through pMMO, since methane monooxygenase (pMMO) and ammonia monooxygenase (AMO) may be evolutionarily related (Holmes et al., 1995; Klotz and Norton, 1998). The coupled system might be:

\[ \text{CH}_4 + \text{NH}_4^+ + + \text{O}_2 \rightarrow \text{CH}_3\text{OH} + \text{NO}_2^- + \text{H}_2\text{O} \]

Recent study had confirmed the co-occurrence of nitrite-dependent anaerobic ammonium and methane oxidation processes in subtropical acidic forest soils (Meng et al., 2016). Anammox and
nitrite-dependent anaerobic methane oxidation (n-damo) which linked the microbial nitrogen and carbon cycles are two new processes of recent discoveries (Zhu et al., 2010; Meng et al., 2016). In this research, the abundance of cmo gene had a positive correlation with hzo, which also predicted the coupled system of nitrite-dependent anaerobic ammonium and methane oxidation processes on the basis of our hypothesis (Fig 3).

4.2 Coupling of nitrogen / sulphur cycles in river sediments

Sulfate-reducing ammonia oxidation (SRAO) could simultaneously remove ammonium and sulfate in one anaerobic reactor, and several published works verified this process could occurred both in laboratory-scale bioreactors or nature (Fdz-Polanco et al., 2001; Rikmann et al., 2012). Our results found that the abundance of hzo gene had a positive correlation with dsrB and aprA gene, indicating the occurrence of sulfate-reducing ammonia oxidation (SRAO) process, which further support our hypothesis (Fig. 4).

The pathway of sulfites reduced to hydrogen sulfide may be: (1) transforming thithionate and thiosulfate through three consecutive pairs of electron transfer \(3\text{SO}_3^{2-} \rightarrow \text{S}_3\text{O}_6^{2-} \rightarrow \text{S}_2\text{O}_3^{2-} \rightarrow \text{S}^{2-}\). (2) losing six electrons directly, and not forming above intermediates, which is called the coordinate 6 electron reaction (Parey et al., 2011). In addition, the process of anammox was responsible for anaerobic nitrogen removal (Rikmann et al., 2012). At present, the transformation of intermediate involved in anammox still remains ambiguous and it is reported that the intermediate contained
NH₂OH, N₂H₄ and HNO₂, NO and N₂O, etc. Up to now, many investigations have been focused on the feasible metabolic pathway and reaction equations of the synchronously ammonia and sulfate removal. Sulfate-reducing ammonium oxidation (SRAO) process was first proposed to explain “abnormal” losses of nitrogen and sulfate (Fdz-Polanco et al., 2001).

Possibility of SRAO was noted by Strous et al. (2002), Zhang et al. (2009), Schrum et al. (2009)

\[ 3\text{SO}_4^{2−} + 4\text{NH}_4^+ → 3\text{HS}^− + 4\text{NO}_2^- + 4\text{H}_2\text{O} + 5\text{H}^+ \]

Coupled with the process of anammox, summary possible equations of SRAO was noted by Strous et al. (2002), Zhang et al. (2009), Schrum et al. (2009)

\[ 3\text{SO}_4^{2−} + 8\text{NH}_4^+ → 3\text{HS}^− + 4\text{N}_2 + 12\text{H}_2\text{O} + 5\text{H}^+ \]

In addition to SO₄²⁻, NO₂⁻ is the most favourable electron acceptor (Rikmann et al., 2012). The possible half-reactions for SRAO, as suggested by Yang et al. (2009), would be as follows:

\[ 4\text{NH}_4^+ + 8\text{H}_2\text{O} → 4\text{NO}_2^- + 32\text{H}^+ + 24\text{e}^- \]

\[ 3\text{SO}_4^{2−} + 24\text{H}^+ + 24\text{e}^- → 3\text{S}^{2−} + 12\text{H}_2\text{O} \]

Previous research did not clearly indicate the existence of aerobic ammonia oxidation-sulfate reduction process. In this research, the abundance of *amoA*-AOA gene was positively correlated with *dsrB* gene, we can speculate the coupled system of aerobic ammonia-sulfate reduction according to our hypothesis, which might occur through horizontal gene transfer (Fig. 4).

Previous studies had confirmed the existence of microaerophilic sulfate and nitrate co-reduction.
system under laboratory conditions (Bowles et al., 2012; Brunet and Garciagil, 1996). The abundance of denitrifying genes (*nirS*, *nirK* and *nosZ*) had a positive correlation with *aprA* gene, which also inferred the co-reduction system based on the assumption of this research (Fig 4). Additionally, several sulfur-reduced compounds (H$_2$S, FeS and S$_2$O$_3^{2-}$) could act as electron donors for dissimilatory nitrate reduction (Brunet and Garciagil, 1996).

4.3 Coupling of methane / sulphur cycles in river sediments

There were two methane-oxidizing mechanisms of aerobic and anaerobic/aerobic oxidation in sediment. For the coupling of C and S, the pathway of sulfate-dependent anaerobic methane oxidation had also been discovered (M et al., 2003; Xu et al., 2014). In this study, the positive correlation between *cmo* gene and *aprA* gene could speculate the coupling relation of anaerobic methane oxidation-sulfate reduction. Similarly, the abundance of methanotrophic genes (*pmoA*, *pmoA1* and *pmoA2*) were positively correlated with *aprA* gene, which can also infer the occurrence of sulfate-dependent aerobic methane oxidation process, thereby further supporting the hypothesis (Fig. 5).

The coexistence of methanogenesis and sulfate reduction has been shown before (Maltby et al., 2018). In this research, the positive correlation between *aprA* gene and *mcrA* gene could also deduce the presence of methanogenesis within the sulfate reduction zone, which further verified the
hypothesis that the correlationship among functional genes could be used to predict the coupled systems (Fig 5).

**4.4 Linking the abundance of functional genes and environmental parameters**

In the methane cycle, the *mcrA* gene (methylcoenzyme M reductase) is exclusively linked to methanogens. Although previous studies have been performed to identify the main factors controlling CH₄ dynamics from wetlands, the effect of nutrients on CH₄ dynamics is poorly understood. Previously studies found that organic matter, nitrogen and phosphorus was the important regulating factors in the process of methanogenesis (Yang, 1998). In our study, correlation analysis indicated that the content of OM, NH₄⁺-N, NO₃⁻, TN and OP had significantly positive correlation with the abundances of methanogenic (*mcrA*) gene (Fig 6). And, the stepwise regression presented a following regression: \( \log mcrA = 6.359 + 0.006 \times \text{NH}_4^+ - N + 0.5 \times \text{TN} - 0.001 \times \text{TP} + 0.325 \times \text{pH} \) \((R^2 = 0.49, P<0.001)\), which indicated that N had a greater effect on *mcrA* than C and P. The abundance of methanotrophic genes (*pmoA, pmoA1* and *pmoA2*) and *cmo* gene were positively influenced by OM, NH₄⁺-N, NO₃⁻, TN (Fig. 6), suggesting that C and N co-limitation of the methanotrophs.

In the process of ammonia oxidation, studies indicated that the *amoA*-AOB was generally more sensitive to higher OM and NH₄⁺ concentrations (Lammel et al., 2015; Stempfhuber et al., 2014). From Fig 6, it could be seen that both of OM and NH₄⁺-N contributed to the increase of the abundance of AOB and the correlation coefficient between *amoA*-AOB and OM and between *amoA*-AOB and

\[ \text{corr coeff} = 0.38 \]
The $\text{NH}_4^+$-$\text{N}$ was $(r=0.424, \ p<0.01)$ and $(r=0.459, \ p<0.01)$, respectively.

The $\text{hzo}$ gene involving in the anaerobic ammonia oxidation (anammox, $\text{NH}_4^+\text{NO}_3^-\rightarrow\text{H}_2\text{O}+\text{N}_2$) process (Schmid et al., 2010) mainly mediated by anammox bacteria and was shaped by various environmental factors in natural habitats (Bai et al., 2015). The abundance of $\text{hzo}$ gene was mainly related to the contents of OM, $\text{NH}_4^+$, $\text{NO}_3^-$, TN in this study (Fig 6).

In this study, all of the denitrifying genes ($\text{nirK}$, $\text{nirS}$ and $\text{nosZ}$) was positively correlated with OM, $\text{NH}_4^+$-$\text{N}$, $\text{NO}_3^-$-$\text{N}$, TN and OP (Fig 6), which implied that the lower content of nitrogen in sediments was disadvantageous for denitrification in river sediments.

The $\text{aprA}$ gene and $\text{dsrB}$ gene could serve as marker genes for sulfate reduction energy metabolism (Bae et al., 2015; Meyer and Kuever, 2007). We found that the abundance of $\text{aprA}$ gene was positively correlated with OM, $\text{NH}_4^+$-$\text{N}$, $\text{NO}_3^-$-$\text{N}$, TN, and OP, but no direct correlations between the $\text{dsrB}$ copy numbers and any nutrient characteristics of the Huaihe river sediment were detected. This result is different from study of (Bae et al., 2015), who presented that there was a positive correlation between $\text{dsrB}$ gene and TP concentrations.

Integrating the gene abundance data with environmental parameters provided a comprehensive overview of these interactions related to nitrogen, methane and sulphur cycle, which showed that among the nutrient characteristics of Huaihe River sediment, organic matter and nitrogen nutrients had comprehensive and complicate impact on the coupling transformational processes of C, N and S in
river sediment (Fig 6).

Network graph also showed that \textit{amoA}-AOA and \textit{dsrB} played a secondary role in the coupling transformation of C, N and S, while \textit{aprA}, \textit{mcrA} and \textit{hzo} closely participate in the coupling processes (Fig 6). There was a positive correlation between the abundance of \textit{dsrB} gene and \textit{amoA}-AOA gene, but \textit{dsrB} gene was not related to \textit{amoA}-AOB gene. It indicated that \textit{amoA}-AOA gene had an important effect on the coupling process of ammonia oxidation and sulfite reduction. Similarly, in ammonia oxidation genes (\textit{amoA}-AOA and \textit{amoA}-AOB), \textit{aprA} gene only had a positive correlation with \textit{amoA}-AOB gene, which suggested that \textit{amoA}-AOB gene played a key role in the coupling process of ammonia oxidation and sulfate reduction. Network graph displayed that \textit{aprA} gene played a more important role than \textit{dsrB} gene in the coupling of N-S and C-S, indicating that the process of sulfite reduction might occur toughly.

In addition, network graph showed that the \textit{nirS} gene exhibited a greater weight than the \textit{nirK} gene, indicating that \textit{nirS}-encoding bacteria may take precedence over \textit{nirK}-encoding bacteria in river sediments investigated in the coupling processes of N-C and N-S. Enwall et al. (2010) held that different habitat and nutrient content resulted in the differences in abundance of the \textit{nirS}- and \textit{nirK}-type denitrifiers. Kim et al. (2011) also suggested that both types of denitrifiers apparently occupy different ecological niches.
5 Conclusions

Appropriate marker genes abundance can determine quantification of microbial functional groups. A direct relationship was established between the nutritional status and the distributions of functional genes. The C-N, C-S and N-S coupled systems might be inferred in this research based on the correlationship among functional genes. Compared with other genes, the amoA-AOA and dsrB played a minor role in the coupling transformation of C, N and S, while S-functional gene (aprA), C-functional gene (mcrA), N-functional gene (hzo) were the key functional genes that participate in the coupled processes in the elemental biogeochemical cycle. Despite the fact that this hypothesis still has to be verified experimentally it is safe to conclude that the abundance of functional genes involved in C, N and S cycles were mainly influenced by OM, NH$_4^+$-N, NO$_3^-$-N, and TN contents, indicating that organic matter and nitrogen nutrients might play an important modulating role in the coupling of carbon, nitrogen and sulphur. Despite the fact that this hypothesis still has to be verified experimentally it is safe to conclude that C and N might play an important modulating role in the coupling of carbon, nitrogen and sulphur. Transcription and protein group can be carried out to further verify if the processes exactly occurred.

Author contributions

MZZ, YL, and QYS proposed and organized the overall project. MZZ performed the majority of
the experiments. PXC and XHW gave assistance in sampling and the analyses of chemical properties. MZZ and QYS wrote the main manuscript text. YL contributed insightful discussions. All authors reviewed the manuscript.

**Funding**

Financial supports from the National Science and Technology Major Project (2012ZX07204-004).

**Compliance with ethical standards**

The work has not been published previously and not under consideration for publication elsewhere. This article does not contain any studies with human participants or animals performed by any of the authors.

**References**


Bae, H. S., Holmes, M. E., Chanton, J. P., Reddy, K. R., and Ogram, A.: Distribution, Activities, and
Interactions of Methanogens and Sulfate-Reducing Prokaryotes in the Florida Everglades, Applied & Environmental Microbiology, 81, 7431, 2015.


Chen, S., Sun, D., and Chung, J. S.: Simultaneous methanogenesis and denitrification of aniline wastewater by using anaerobic-aerobic biofilm system with recirculation, Journal of Hazardous...


He, Y., Ye, J., and Yang, X.: Analysis of the spatio-temporal patterns of dry and wet conditions in the Huai River Basin using the standardized precipitation index, Atmospheric Research, 166, 120-128, 2015.


corresponding process are commonly assumed yet rarely observed, Isme Journal, 9, 1693, 2014.


Stempfhuber, B., Welzl, G., Wubet, T., Schöning, I., Marhan, S., Buscot, F., Kandeler, E., and Schloter,


Table 1. The chemical properties of sediment samples

<table>
<thead>
<tr>
<th>Indices</th>
<th>pH</th>
<th>OM g·kg⁻¹</th>
<th>NH₄⁺-N mg·kg⁻¹</th>
<th>NO₃⁻-N mg·kg⁻¹</th>
<th>NO₂⁻-N mg·kg⁻¹</th>
<th>TN g·kg⁻¹</th>
<th>IP mg·kg⁻¹</th>
<th>OP mg·kg⁻¹</th>
<th>TP mg·kg⁻¹</th>
<th>C/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.78</td>
<td>38.45</td>
<td>62.40</td>
<td>22.04</td>
<td>0.24</td>
<td>0.87</td>
<td>470.93</td>
<td>85.82</td>
<td>674.52</td>
<td>79.12</td>
</tr>
<tr>
<td>Median</td>
<td>7.80</td>
<td>34.66</td>
<td>44.21</td>
<td>12.62</td>
<td>0.15</td>
<td>0.69</td>
<td>448.72</td>
<td>73.94</td>
<td>644.56</td>
<td>52.17</td>
</tr>
<tr>
<td>Minimum</td>
<td>6.08</td>
<td>10.31</td>
<td>2.87</td>
<td>0.10</td>
<td>0.01</td>
<td>0.01</td>
<td>92.93</td>
<td>2.16</td>
<td>152.65</td>
<td>21.17</td>
</tr>
<tr>
<td>Maximum</td>
<td>8.83</td>
<td>173.09</td>
<td>304.46</td>
<td>157.48</td>
<td>1.40</td>
<td>4.77</td>
<td>1631.96</td>
<td>509.17</td>
<td>2108.46</td>
<td>1184.45</td>
</tr>
<tr>
<td>CV(%)</td>
<td>5.44</td>
<td>56.75</td>
<td>86.91</td>
<td>124.30</td>
<td>94.93</td>
<td>85.17</td>
<td>39.96</td>
<td>65.88</td>
<td>39.05</td>
<td>145.03</td>
</tr>
</tbody>
</table>

Notes: CV—coefficient of variance.

Table 2. The abundance of functional genes (copies·g⁻¹ dw soil) related to C, N, S cycles

<table>
<thead>
<tr>
<th>Functional genes</th>
<th>Mean</th>
<th>CV%</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>nirK</td>
<td>1.27×10⁸</td>
<td>128.94</td>
<td>2.17×10⁶</td>
<td>9.00×10⁸</td>
</tr>
<tr>
<td>nirS</td>
<td>1.55×10⁹</td>
<td>163.00</td>
<td>6.63×10⁶</td>
<td>1.54×10¹⁰</td>
</tr>
<tr>
<td>nosZ</td>
<td>1.44×10⁸</td>
<td>193.50</td>
<td>3.30×10⁵</td>
<td>1.73×10⁹</td>
</tr>
<tr>
<td>hzo</td>
<td>1.28×10⁶</td>
<td>126.67</td>
<td>3.33×10⁴</td>
<td>1.13×10⁷</td>
</tr>
<tr>
<td>amoA-AOA</td>
<td>7.76×10⁷</td>
<td>317.86</td>
<td>1.16×10⁶</td>
<td>2.43×10⁹</td>
</tr>
<tr>
<td>Items</td>
<td>hzo</td>
<td>cmo</td>
<td>AOA</td>
<td>AOB</td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>cmo</td>
<td>0.763**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOA</td>
<td>0.042</td>
<td>-0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOB</td>
<td>0.492**</td>
<td>0.575**</td>
<td>0.361**</td>
<td></td>
</tr>
<tr>
<td>nirK</td>
<td>0.294**</td>
<td>0.462**</td>
<td>-0.161</td>
<td>0.159</td>
</tr>
<tr>
<td>nirS</td>
<td>0.366**</td>
<td>0.617**</td>
<td>-0.188*</td>
<td>0.253**</td>
</tr>
<tr>
<td>nosZ</td>
<td>0.251**</td>
<td>0.534**</td>
<td>-0.069</td>
<td>0.394**</td>
</tr>
<tr>
<td>mcrA</td>
<td>0.515**</td>
<td>0.677**</td>
<td>0.210*</td>
<td>0.501**</td>
</tr>
<tr>
<td>pmoA</td>
<td>0.503**</td>
<td>0.510**</td>
<td>0.142</td>
<td>0.308**</td>
</tr>
<tr>
<td>pmoA1</td>
<td>0.566**</td>
<td>0.788**</td>
<td>-0.107</td>
<td>0.503**</td>
</tr>
<tr>
<td>pmoA2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dsrB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: CV—coefficient of variance. Denitrification, including nirS and nirK for nitrite reductase, and nosZ for nitrous oxide reductase; Anammox, including hzo for hydrazine oxidoreductase; Nitrification, including amoA encoding bacterial and archaeal ammonia monooxygenase; Methanogenesis, including mcrA for the methyl coenzyme M reductase; Aerobic methane oxidation, including pmoA encoding the alpha-subunit of pMMO, in which pmoA gene from conventional type I methanotrophs, conventional type II methanotrophs and type II methanotrophs possessing the pmoA2 gene. Anaerobic nitrite-dependent methane oxidation, including cmo gene for M. oxyfera specific primers; Sulfur reduction, including dsrB for dissimilatory sulfite reductase and aprA for adenosine-5’-phosphosulfate (APS) reductase.

Table 3. The correlation coefficient among the abundance of 13 functional genes (n=135)
\textit{pmoA2} 0.565** 0.766**  -0.138  0.373**  0.429**  0.599**  0.476**  0.525**  0.457**  0.874**
\textit{dsrB} 0.247**  0.021  0.294**  0.151  -0.088  -0.121  -0.14  0.123  0.102  -0.078  -0.051
\textit{aprA} 0.324**  0.497**  -0.005  0.334**  0.373**  0.440**  0.342**  0.323**  0.246**  0.450**  0.408**  -0.103

\textbf{Fig.1.} Sketch map of sampling sites of rivers in northern Anhui province

**Fig. 2.** The correlation analysis among different chemical properties

**Fig. 3.** Coupling of methane / nitrogen cycles in river sediments
Fig. 4. Coupling of nitrogen / sulphur cycles in river sediments
Fig. 5. Coupling of methane / sulphur cycles in river sediments
Fig. 6. Relationships between different chemical properties and functional genes