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The Coupling of Carbon, Nitrogen and Sulphur Transformational Processes in River Sediments Based on Correlationship among the Functional Genes

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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 *q*PCR. The correlation among functional genes from 135 river sediment samples was calculated. We supposed that the correlationship among functional genes could be used as a reference index speculating the coupled systems of C-N-S in this reasearch, 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

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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 Garciagil, 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

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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

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The Huaihe River is located in the eastern China, watershed area of approximately 270,000 km^2, 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 ℃ 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 situated 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℃ 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℃ 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_2O_{volume}=1g: 5ml).

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The organic matter (OM) was determined by the loss of ignition (LOI) in a muffle furnace at 550±5 ℃ for 6 h. The total nitrogen (TN) content was measured using the Kjeldahl method. Concentrations of NH_4^+-N, NO_3^--N and NO_2^--N and 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 5uL 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 ddH_2O and 1uL 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 interaction 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). Network graph was employed to investigate the key functional genes and nutrient elements of affecting the coupling transformation of C, N and S.

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 sections investigated, 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 secondaty 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

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164 In 13 functional genes investigated in this study, the abundance of *dsrB* and *pmoA*1 genes was
165 relative higher, and that of *hzo* and *aprA* genes lower (Table 2).

166 For N-cycling genes, the abundance of *amoA*-AOB was substantially lower as compared to
167 *amoA*-AOA. Comparing to *nirK* and *nosZ*, *nirS* displayed higher abundance. In the functional genes
168 related to C-cycle, the *mcrA* abundance exhibited the highest coefficient of variance. Table 2 also
169 demonstrated that in contrast to *pmoA* and *pmoA*2 genes, type II methanotrophs possessing the *pmoA*1
170 gene were predominant. In two genes involving in sulfate reduction, the abundance of *dsrB* gene was
171 significantly more than that of *aprA* gene in sediments.

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

175 Table 3 displayed the correlation coefficent among 13 functional genes involving in C-, N- and
176 S-cycle in sediments. In the functional genes involving in N-cycle, abundances of *cmo*, *hzo*,
177 *amoA*-AOB, *nirS*, *nosZ* genes were correlated between each other. Meanwhile, abundances of
178 methanotrophic (*pmoA*, *pmoA*1, *pmoA*2), *mcrA* genes were correlated between each other in C-cycle.
179 With regard to S-functional genes, no direct relationships between *dsrB* and *aprA* were found.

180 Concerning the correlations among N-, C- and S-functional genes, the methanotrophic (*pmoA*,
181 *pmoA*1 and *pmoA*2) 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*, *pmoA*1and *pmoA*2).

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

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200 the abundance of *hzo* gene and *mcrA* gene, predicting that methanogenesis and anammox could work
201 together, which also proved that anammox coupled to methanogenesis (Fig 3).

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

$$CH_3OH + NO_3^- \rightarrow N_2 + CO_2 + OH^- + H_2O$$

$$CH_3OH \rightarrow H_2O + CO_2 + CH_4$$

211 Du et al. (2017) confirmed that it existed in reactor that a novel partial-denitrification combied
212 with anammox process, since the nitrite for anammox could be acquired from partial-denitrification
213 process. In our study, the abundance of *hzo* gene showed positive correlations with the denitrifying
214 genes (*nirK*, *nirS* and *nosZ*), suggesting that denitrification might cooperate with anammox. Bai et al.
215 (2013) proposed that an integrated process was developed by an anaerobic reactor, in which

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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*, *pmoA*1 and *pmoA*2) 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_2O→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

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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:

$$3CH_4 + 8NO_2^- + 8H_+ \rightarrow 3CO_2 + 4N_2 + 10H_2O$$

In our study, methanotrophic genes (*pmoA*, *pmoA*1 and *pmoA*2) 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:

$$CH_4 + NH_4 + +O_2 \rightarrow CH_3OH + NO_2^- + H_2O$$

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

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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 trithionate and thiosulfate through three consecutive pairs of electron transfer ($3SO_3^{2-} \rightarrow S_3O_6^{2-} \rightarrow S_2O_3^{2-} \rightarrow S^{2-}$). (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 NH_2OH, N_2H_4 and HNO_2, NO and N_2O, 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”

268 losses of nitrogen and sulfate (Fdz-Polanco et al., 2001).

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

$$3SO_4^{2-} + 4NH_4^+ \rightarrow 3HS^- + 4NO_2^- + 4H_2O + 5H^+$$

270 Coupled with the process of anammox, a summary equation of SRAO was displayed:

$$3SO_4^{2-} + 8NH_4^+ \rightarrow 3HS^- + 4N_2 + 12H_2O + 5H^+$$

271 In addition to SO_4^{2-}, NO_2^- is the most favourable electron acceptor (Rikmann et al., 2012). The
272 possible half-reactions for SRAO, as suggested by Yang et al. (2009), would be as follows:

$$4NH_4^+ + 8H_2O \rightarrow 4NO_2^- + 32H^+ + 24e^-$$

$$3SO_4^{2-} + 24H^+ + 24e^- \rightarrow 3S^{2-} + 12H_2O$$

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

277 Previous studies had confirmed the existence of microaerophilic sulfate and nitrate co-reduction
278 system under laboratory conditions (Bowles et al., 2012;Brunet and Garciagil, 1996). The abundance
279 of denitrifying genes (*nirS*, *nirK* and *nosZ*) had a positive correlation with *aprA* gene, which also
280 inferred the co-reduction system based on the assumption of this research (Fig 4). Additionally,
281 several sulfur-reduced compounds (H_2S, FeS and $S_2O_3^{2-}$) could act as electron donors for dissimilatory

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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*, *pmoA*1 and *pmoA*2) were positively correlated with *aprA* gene, which can also infer the occurrence of sulfate-dependent aerobic methane oxidation process, thereby futher 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

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CH_4 dynamics from wetlands, the effect of nutrients on CH_4 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, NH_4^+-N, NO_3^-, TN and OP had significantly positive correlation with the abundances of methanogenic (*mcrA*) gene (Fig 6). And, the stepwise regression presented a following regression: $\log mcrA = 6.359 + 0.006 * NH_4^+ - N + 0.5 * TN - 0.001 * TP + 0.325 * pH$ (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*, *pmoA*1 and *pmoA*2) and *cmo* gene were positively influenced by OM, NH_4^+-N, NO_3^-, 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 NH_4^+ concentrations (Lammel et al., 2015;Stempfhuber et al., 2014). From Fig 6, it could be seen that both of OM and NH_4^+-N contributed to the increase of the abundance of AOB and the correlation coefficent between *amoA*-AOB and OM and between *amoA*-AOB and NH_4^+-N was (r=0.424，p<0.01) and (r=0.459，p<0.01), respectively.

The *hzo* gene involving in the anaerobic ammonia oxidation (anammox, $NH_4^+ + NO_3^- \rightarrow H_2O + N_2$) 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 *hzo* gene was mainly related to the contents of OM, NH_4^+, NO_3^-, TN in this study (Fig 6).

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In this study, all of the denitrifying genes (*nirK*, *nirS* and *nosZ*) was positively correlated with OM, NH_4^+-N, NO_3^--N, TN and OP (Fig 6), which implied that the lower content of nitrogen in sediments was disadvantageous for denitrification in river sediments.

The *aprA* gene and *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 *aprA* gene was positively correlated with OM, NH_4^+-N, NO_3^--N, TN, and OP, but no direct correlations between the *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 *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 *amoA*-AOA and *dsrB* played a secondary role in the coupling transformation of C, N and S, while *aprA, mcrA and hzo* closely participate in the coupling processes (Fig 6). There was a positive correlation between the abundance of *dsrB* gene and *amoA*-AOA gene, but *dsrB* gene was not related to *amoA*-AOB gene. It indicated that *amoA*-AOA gene had an important

effect on the coupling process of ammonia oxidation and sulfite reduction. Similarly, in ammonia oxidation genes (*amoA*-AOA and *amoA*-AOB), *aprA* gene only had a positive correlation with. *amoA*-AOB gene, which suggested that *amoA*-AOB gene played a key role in the coupling process of ammonia oxidation and sulfate reduction. Network graph displayed that *aprA* gene played a more important role than *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 *nirS* gene exhibited a greater weight than the *nirK* gene, indicating that *nirS*-encoding bacteria may take precedence over *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 *nirS*- and *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

354 a minor role in the coupling transformation of C, N and S, while S-functional gene (*aprA*),
355 C-functional gene (*mcrA*), N-functional gene (*hzo*) were the key functional genes that participate in
356 the coupled processes in the elemental biogeochemical cycle. Despite the fact that this hypothesis still
357 has to be verified experimentally it is safe to conclude that C and N might play an important
358 modulating role in the coupling of carbon, nitrogen and sulphur. Transcription and protein group can
359 be carried out to further verify if the processes exactly occurred.

360

361 **Author contributions**

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

366

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371 **Compliance with ethical standards**

372 The work has not been published previously and not under consideration for publication
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575 **Table 1.** The chemical properties of sediment samples

Indices	pH	OM g kg^{-1}	NH_4^+-N mg kg^{-1}	NO_3^--N mg kg^{-1}	NO_2^--N mg kg^{-1}	TN g kg^{-1}	IP mg kg^{-1}	OP mg kg^{-1}	TP mg kg^{-1}	C/N ratio
Mean	7.78	38.45	62.40	22.04	0.24	0.87	470.93	85.82	674.52	79.12
Median	7.80	34.66	44.21	12.62	0.15	0.69	448.72	73.94	644.56	52.17
Minimum	6.08	10.31	2.87	0.10	0.01	0.01	92.93	2.16	152.65	21.17
Maximum	8.83	173.09	304.46	157.48	1.40	4.77	1631.96	509.17	2108.46	1184.45
CV(%)*	5.44	56.75	86.91	124.30	94.93	85.17	39.96	65.88	39.05	145.03

576 Notes: CV—coefficient of variance.

577

578 **Table 2.** The abundance of functional genes (copies ·g^{-1} dw soil) related to C, N, S cycles

Functional genes	Mean	CV%	Minimum	Maximum
nirK	1.27×10^{8}	128.94	2.17×10^{6}	9.00×10^{8}
nirS	1.55×10^{9}	163.00	6.63×10^{6}	1.54×10^{10}
nosZ	1.44×10^{8}	193.50	3.30×10^{5}	1.73×10^{9}
hzo	1.28×10^{6}	126.67	3.33×10^{4}	1.13×10^{7}
amoA-AOA	7.76×10^{7}	317.86	1.16×10^{6}	2.43×10^{9}
amoA-AOB	1.25×10^{7}	67.38	2.32×10^{6}	6.5×10^{7}
mcrA	7.76×10^{7}	315.34	4.31×10^{7}	2.15×10^{11}
pmoA	1.32×10^{9}	248.39	3.38×10^{6}	2.58×10^{10}
*pmoA*1	1.82×10^{10}	210.38	9.88×10^{6}	2.08×10^{11}
*pmoA*2	5.06×10^{8}	225.95	5.29×10^{6}	6.18×10^{9}
cmo	1.18×10^{8}	91.29	4.15×10^{6}	7.45×10^{8}
dsrB	7.82×10^{9}	146.30	1.92×10^{8}	5.80×10^{10}
aprA	6.62×10^{6}	205.35	8.62×10^{3}	1.09×10^{8}

579 Notes: CV—coefficient of variance. Denitrification, including *nirS* and *nirK* for nitrite reductase, and *nosZ* for
580 nitrous oxide reductase; Anammox, including *hzo* for hydrazine oxidoreductase; Nitrification, including *amoA*
581 encoding bacterial and archaeal ammonia monooxygenase; Methanogenesis, including *mcrA* for the methyl
582 coenzyme M reductase; Aerobic methane oxidation, including *pmoA* encoding the alpha-subunit of pMMO, in which
583 *pmoA* gene from conventional type I methanotrophs, conventional type II methanotrophs and type II methanotrophs

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584 possessing the *pmoA*2 gene. Anaerobic nitrite-dependent methane oxidation, including *cmo* gene for M. oxyfera
585 specific primers; Sulfur reduction, including *dsrB* for dissimilatory sulfite reductase and *aprA* for
586 adenosine-5'-phosphosulfate (APS) reductase.

587 **Table 3.** The correlation coefficent among the abundance of 13 functional genes (n=135)

Items	*hzo*	*cmo*	*AOA*	*AOB*	*nirK*	*nirS*	*nosZ*	*mcrA*	*pmoA*	*pmoA*1	*pmoA2*	*dsrB*
cmo	0.763**											
AOA	0.042	-0.04										
AOB	0.492**	0.575**	0.361**									
nirK	0.294**	0.462**	-0.161	0.159								
nirS	0.366**	0.617**	-0.188*	0.253**	0.810**							
nosZ	0.251**	0.534**	-0.069	0.394**	0.483**	0.550**						
mcrA	0.515**	0.677**	0.210*	0.501**	0.259**	0.357**	0.444**					
pmoA	0.503**	0.510**	0.142	0.308**	0.135	0.260**	0.316**	0.594**				
*pmoA*1	0.566**	0.788**	-0.107	0.503**	0.414**	0.586**	0.540**	0.481**	0.402**			
*pmoA*2	0.565**	0.766**	-0.138	0.373**	0.429**	0.599**	0.476**	0.525**	0.457**	0.874**		
dsrB	0.247**	0.021	0.294**	0.151	-0.088	-0.121	-0.14	0.123	0.102	-0.078	-0.051	
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

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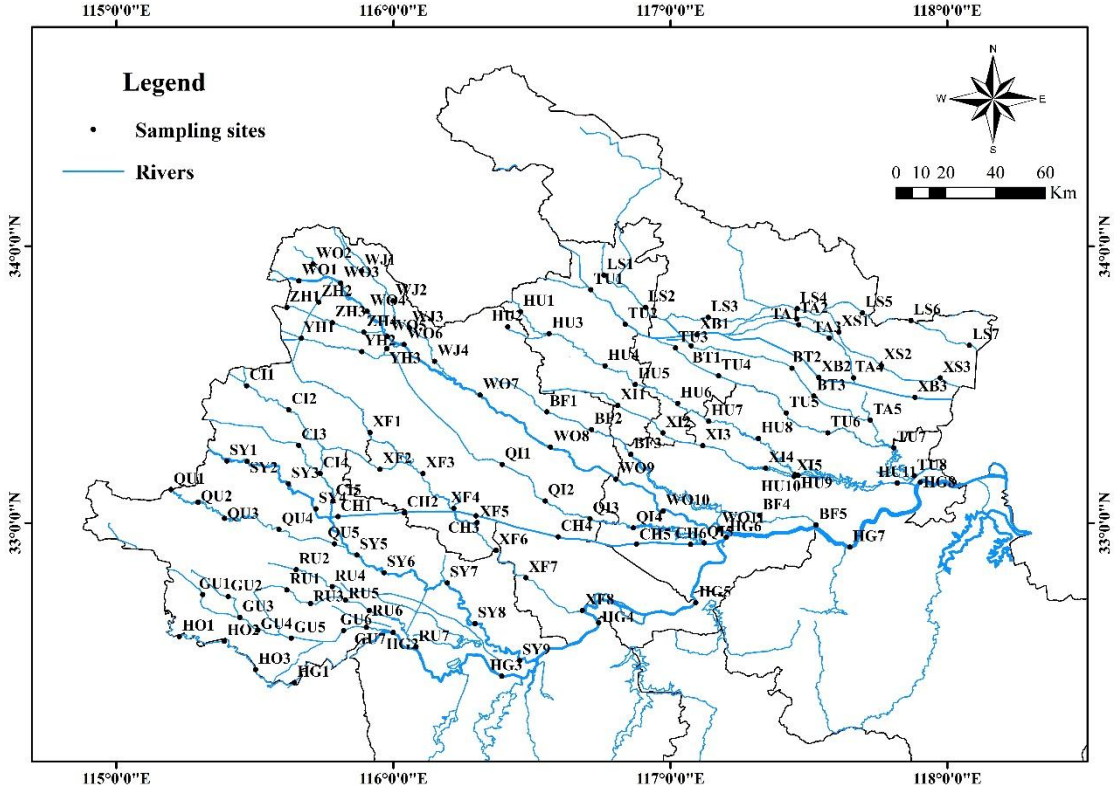

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590 **Fig.1.** Sketch map of sampling sites of rivers in northern Anhui province

591 Z-Zhaohe River, Y-Youhe River, XS-Xinhe River, X-Xiehe River, XF-Xifeihe River, WJ-Wujiahe
592 River, W-Guohe River, T-Tuohe River, TA-Tanghe River, S-Shayinghe River, R-Runhe River,
593 Q-Quanhe River, QI-Qianhe River, L-Suihe River, H-Huihe River, HG-Huaigan river, HO-Honghe
594 River, G-Guhe River, CH-Cihuai River, C-Cihe River, XB-Bianhe River, BT-Beituohe River,
595 BF-Beihe River.

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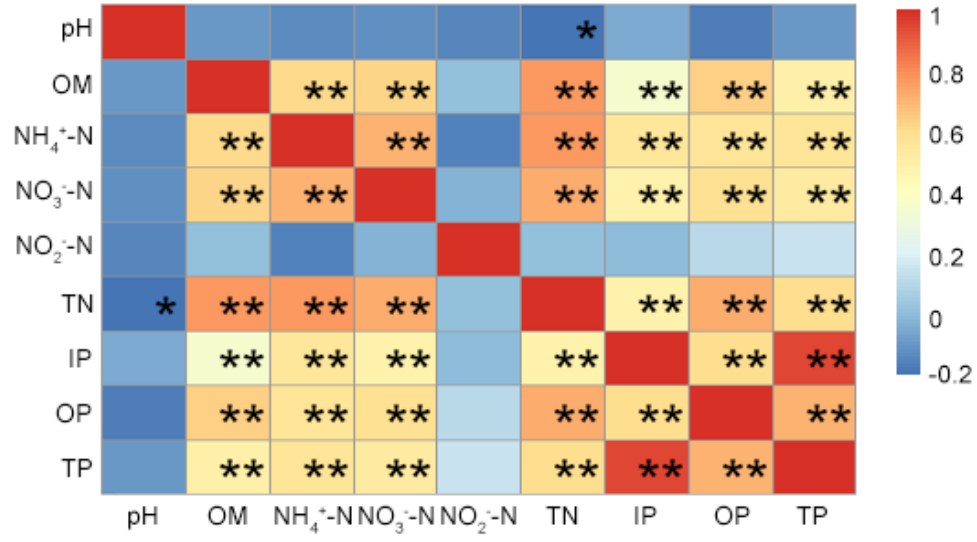
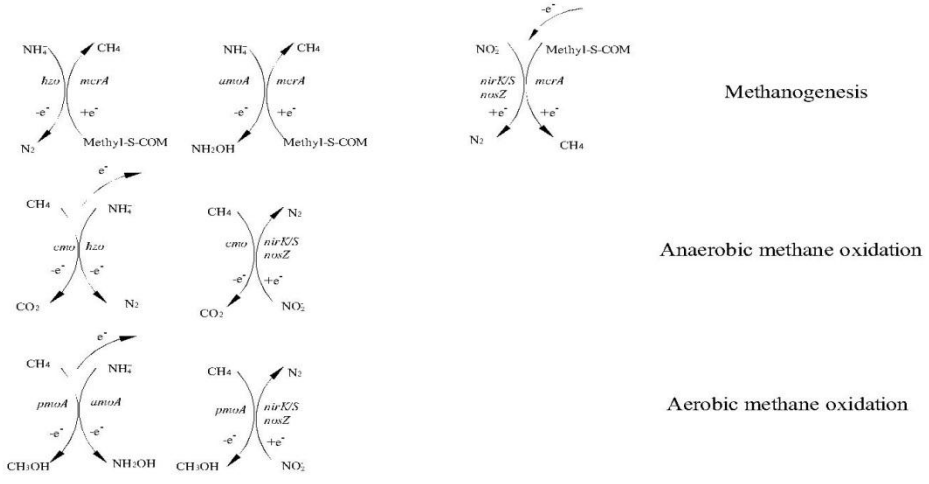


596

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



598
599 **Fig.3.** Coupling of methane / nitrogen cycles in river sediments
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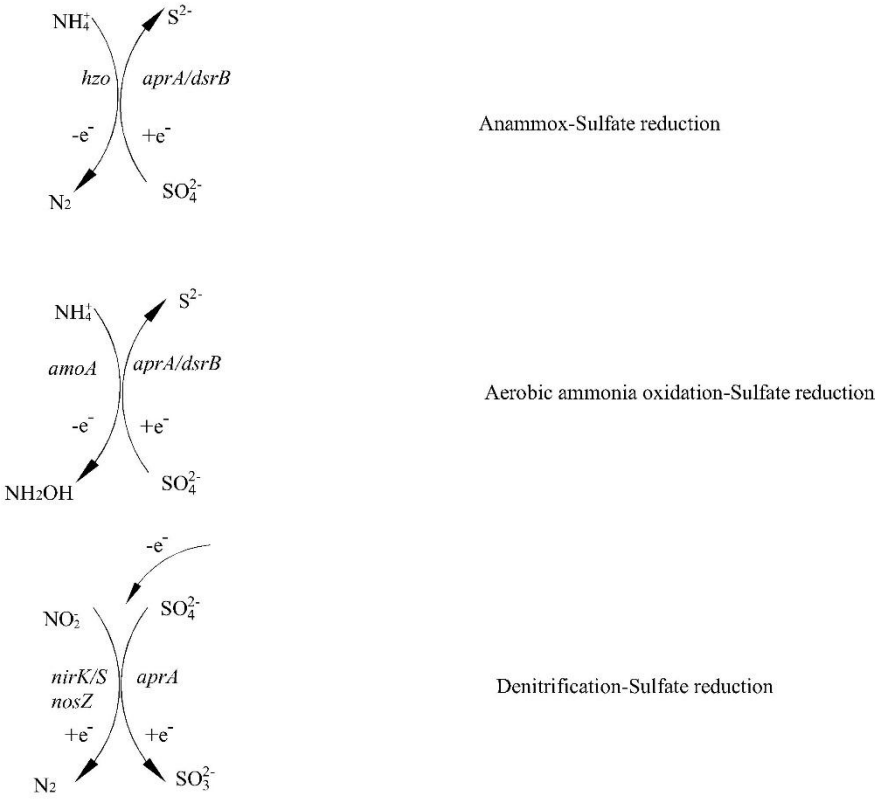

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605

606 **Fig.4.** Coupling of nitrogen / sulphur cycles in river sediments

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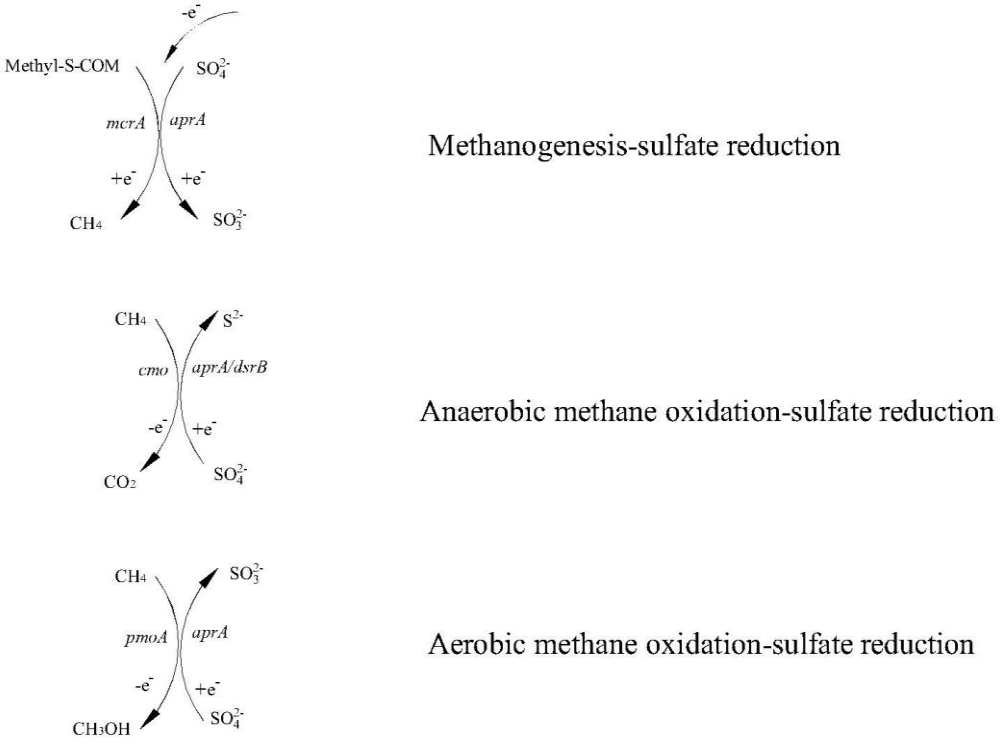

607

608 **Fig.5.** Coupling of methane / sulphur cycles in river sediments

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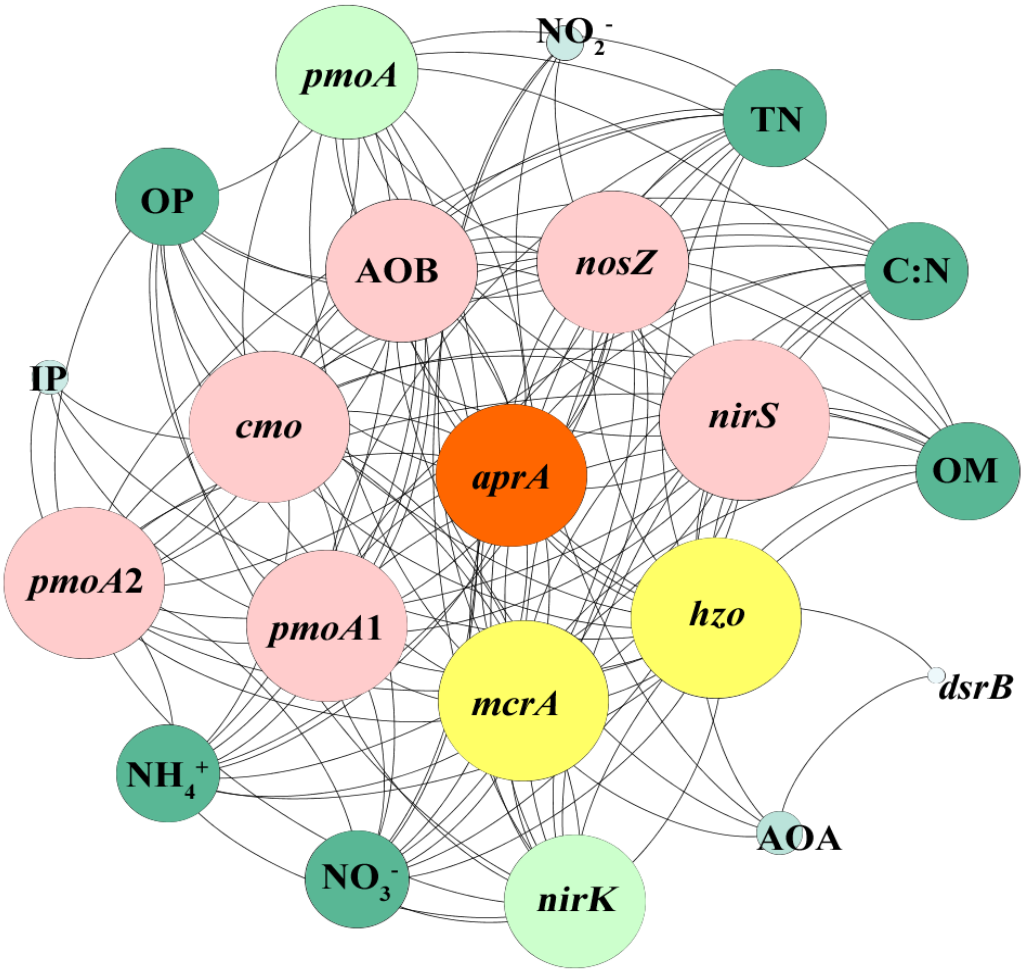

Fig.6. Relationships between different chemical properties and functional genes