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Metagenomic insights into the effect of oxytetracycline on microbial structures, functions and functional genes in sediment denitrification

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ABSTRACT

Denitrification is an indispensable pathway of nitrogen removal in aquatic ecosystems, and plays an important role in decreasing eutrophication induced by excessive reactive nitrogen pollution. Aquatic environments also suffer from antibiotic pollution due to runoff from farms and sewage systems. The aim of this study was to investigate the effect of oxytetracycline stress on denitrifying functional genes, the microbial community and metabolic pathways in sediments using high-throughput sequencing and metagenomic analysis. The oxytetracycline was observed to significantly inhibit the abundance of *nirK* and *nosZ* genes ($P < 0.001$). KEGG pathway annotation indicated that oxytetracycline treatment decreased the abundance of nitrate reductase, nitrite reductase and N_2O reductase. Functional annotations revealed that oxytetracycline exposure decreased the abundance of the protein metabolism subsystem in the bacterial community. Metagenomic sequencing demonstrated that the abundance of *Proteobacteria* and *Firmicutes* increased with oxytetracycline exposure while the *Actinobacteria* decreased. In sediments, *Pseudomonas* and *Bradyrhizobium* were major contributors to denitrification and oxytetracycline exposure resulted in a decreased abundance of *Bradyrhizobium*. These results indicated that oxytetracycline residues influences the denitrifier community and may heighten occurrence of reactive nitrogen in aquatic ecosystems.

1. Introduction

In the past few years, the production of reactive nitrogen (mainly nitrates) has increased dramatically due to human activities (Diaz and Rosenberg, 2008; Seitzinger, 2008). A mass of this reactive nitrogen poured into the river areas (Galloway et al., 2008; Yin et al., 2014), and ultimately lead to a series of environmental issues in these aquatic ecosystems (Deegan et al., 2012; Paerl et al., 2014). Therefore, the elimination of these nitrates from aquatic ecosystem has aroused people's attention (Yin et al., 2016; Yin et al., 2017). Denitrification is an indispensable step in nitrogen cycle and a major dissimilatory pathway of nitrates removal from river ecosystems (Beaulieu et al., 2011; Kool et al., 2011). Denitrification is therefore a target for bioremediation of environmental nitrogen pollution (Morse et al., 2012; Wu et al., 2017). However, other unrelated anthropogenic forms of pollution such as antibiotic residues can hinder denitrification through modifications of natural bacterial populations. (Conley et al., 2009; Yin et al., 2017).

Nowadays, antibiotics are widely used for disease prevention and treatment, animal growth promotion and improving animal product quality, due to the huge demand for human and animal clinics, aquaculture and farming (Looft et al., 2012; Yang et al., 2011; Zhou et al., 2013). It is estimated that 80–90% of these antibiotics and their metabolites are directly discharge into aquatic environments including rivers via urine and feces (Martinez, 2008; Zhang et al., 2013). There was a large amount of reports indicated a widespread distribution of antibiotics in river ecosystems (Chen and Zhou, 2014; Kummerer, 2009). Antibiotics residues in aquatic environments may disrupt the microbial community structure and thereby disturb the associated biological processes due to the antibacterial properties (Novo et al., 2013; Yin et al., 2016). Thus, the residual antibiotics will also inhibit the denitrification process and disturb its balance leading reactive nitrogen accumulation in aquatic ecosystems (Yin et al., 2016, 2017). There have been several studies investigated the effects of antibiotic residues on the denitrification process in river sediments (Yin et al., 2016, 2017). These studies primarily focused on changes in the genes of denitrifying bacteria and denitrification metabolites such as nitrous oxide, nitrate and nitrite. However, there was a lack of focus on bacterial communities and metabolic pathways of denitrification.

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Therefore, a systematic study concerning the effects of antibiotics on denitrifying bacterial flora and its metabolic pathways is necessary to further clarify the mechanism of antibiotics on denitrification (Miao et al., 2015a; Wu et al., 2017).

In China, the usage amount of antibiotics has accounted for more than 20% of the world usage (Zhao et al., 2015). The most widely detected antibiotics residues in aquatic environments are tetracyclines, sulfonamides, macrolides and fluoroquinolones (Zhang et al., 2015b; Zhao et al., 2015). Tetracyclines could inhibit the protein synthesis and have been widely detected in river environments at relatively high concentrations (approximately 20–500 ng L^{-1})(Chen and Zhou, 2014; Shi et al., 2014; Yan et al., 2013). However, systemic effects of tetracyclines on denitrifying microorganisms in the sediments are lacking. Of the several common tetracyclines, oxytetracycline (OTC) was one of the most used antibiotics and its environmental behavior has attracted increasing attention (Oliveira et al., 2013).

In this study, OTC was selected as a representative antibiotic to investigate the potential influence of antibiotic residues on denitrification. We coupled this focus with metagenomic sequencing methods because they are extremely accurate and the sequencing depth can easily encompass complex bacterial communities and metabolic pathways (Miao et al., 2015b; Ye et al., 2012; Zhang et al., 2012). We investigated the effects of OTC stress on denitrifying functional genes, microbial communities and metabolic pathways in sediments.

2. Materials and methods

2.1. experimental design and sample collection

Surface (0–10 cm) sediment was collected from the campus pond located in South China Agricultural University in Guangdong province, China. The sediment had a pH of 6.34, moisture content of 49.71%, organic matter content of 5.17%, total nitrogen content of 0.22% and a total phosphorus content of 0.083%. Sediment samples were pre-incubated to remove as much as possible ambient antibiotics biologically. Briefly, the sediment was placed into a Plexiglas aquarium filled with artificial water possessing nutrient levels similar to the sediment in suit and was incubated at 20 °C for 2 months (Hou et al., 2015; Yin et al., 2016). After preincubation, the sediments were prepared for antibiotic-added incubation experiments. Sediment (4 kg) and 20 kg of nutrient-balanced water were mixed and placed into Plexiglas aquariums using three replicates for each test group. OTC was added to water samples to 1 μg L^{-1} as appropriate before mixing. The aquariums were incubated in the dark at 20 °C and nutrient-balanced water was added to aquariums twice a week to make up for evaporative losses. The sediment samples were collected on days 1, 4, 16 and 30 after the start of the experiment.

2.2. DNA extraction and PCR amplification

Sediment samples were freeze-dried using an FD-1E-50 Freeze-Dryer (Bilon, Shanghai, China). Total genomic DNA was extracted from 0.25 g dried sediment with a PowerSoil DNA Isolation Kit (Mobio, USA) following guidance from the manufacturer. The concentrations and purity of the DNA were determined by UV spectroscopy (NanoDrop, USA) and qualified DNA extracts were stored at − 80 °C for the future analyses.

Four denitrifying genes including nitrite reductase genes *nirS* and *nirK*, nitrous oxide reductase gene *nosZ* and nitrate reductase gene *narG* were amplified by PCR. PCR amplifications were performed in a 25 μL volume reaction and included 2.5 μL of dNTPs (2.5 mM, TaKaRa, Dalian, China), 2 μL of 10 × Buffer (Mg^{2+} plus, TaKaRa), 0.125 μL of Taq DNA polymerase (5 U uL^{-1}, TaKaRa), 0.5 μL of each forward and reverse primers (10 mM) and 1 μL of genomic DNA. Primers were synthesized by Sangon (Shanghai, China) and are listed in Table S1 along with PCR reaction conditions. Amplicons were verified using electrophoresis on 1.0% agarose gels.

2.3. Real-time quantitative PCR

Gene expression levels were estimated using standard curves and real-time quantitative PCR. The PCR products were purified by using the DNA Fragment Purification Kit (TaKaRa, Dalian, China) and cloned into the PMD19-T Vector (TaKaRa, Dalian, China) using the procedures supplied by the manufacturer. Then ligated products were introduced into *Escherichia coli* DH5α utilizing the EASY-T1 Simple Cloning Kit (Tiangen Biotech Co. Ltd., China). Plasmids carrying target gene inserts were extracted from *E. coli* DH5α utilizing the E.Z.N.A Plasmid Miniprep Protocol (Omega, Norcross, GA, USA). Dilutions of the appropriate target gene products in plasmids were used to construct the standard curves. Plasmid concentrations were measured by spectrometry (NanoDrop Lite, Thermo, USA). Standard reactions and triplicate samples were carried out using a LightCycler 96 instrument (Roche, Switzerland). Reaction volumes for real time quantitative PCR were 20 μL and consisted of 10 μL SYBR green Premix Ex Taq II (TaKaRa, Dalian, China), 1 μL template DNA, 0.8 μL of each primer (10 mM, Sangon) and 7.4 μL molecular biology-grade water. Reaction conditions and primers are listed in Table. S1. The data analysis used the LightCycler 96 software supplied with the instrument. Positive (plasmid DNA) and negative (nuclease-free water) controls were run in conjunction with each sample. The gene standard curves had an R^2 more than 0.998 and reaction efficiencies from 92% to 106%.

2.4. Sequencing and bioinformatics analysis

The Novegene Institute (Beijing, China) performed high-throughput sequencing and library construction using the Illumina Hiseq. 2500 platform. Approximately 10 Gb of data were generated for each sample. The raw metagenomic data were firstly trimmed using quality control pipeline (recommended by Novegene Institute) to removed reads with adapters (> 15 bp overlap) or containing three or more unknown nucleotides ('N'). The filtration strategy was conducted by using Galaxy (http://usegalaxy.org/) and the 'FASTQ Groomer' and 'Filter by quality' tools were used to transform quality formats, and remove low quality sequences. The 'FASTQ to FASTA' was utilized to generate a fasta file for subsequent analysis.

To explore denitrifying genes in sediments, the quality-filtered Illumina reads were aligned to local gene databases. The local nucleotide databases of denitrifying genes were created by downloading DNA sequences of *nirS* (*K*), *narG*, and *nosZ* genes from RDP FunGene (http://fungene.cme.msu.edu/). For identifying the bacterial hosts of the denitrifying genes, all assigned sequences were subjected to BLASTx against the NCBI-nr database with E-value cut off at 10^{-5}.

The quality-filtered Illumina reads of all 24 sediment samples were submitted to the Metagenomics RAST server (MG-RAST) (http://metagenomics.anl.gov/) for functional annotation and nitrogen metabolic pathway analysis. The MG-RAST ID numbers are shown in Supplemental materials. For functional annotation, the reads were annotated against the SEED database at levels 1–3 with the default settings (Luo et al., 2014). Similarly, for nitrogen metabolic pathway analysis, the reads were searched against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database and visualized with KEGG mapper (http://www.genome.jp/kegg/) (Yu and Zhang, 2012).

2.5. Data analysis and statistical analysis

Statistical analyses were analyzed by one-way ANOVA (Duncan's multiple range test for variable) using SPSS software (version 22.0). The SPSS software was also used to calculate the Pearson Indices (R) and Significant Correlation (P) of the correlations between microbial community and functional genes. The distribution of bacterial community (genus > 0.1% of total in each samples) was analyzed by principal