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Succession of bacterioplankton community in intensive shrimp (*Litopenaeus vannamei*) aquaculture systems

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ABSTRACT

Bacterioplankton play an integral role in aquaculture systems, but our understanding of the succession of bacterioplankton community along intensive shrimp cultivation is fragmentary. Here we used high-throughput sequencing technique to study the dynamics of bacterioplankton community during the process of intensive shrimp (*Litopenaeus vannamei*) cultivation, and link community changes to physicochemical and biological factors. Community composition varied dramatically over time, supporting the idea of significant unidirectional temporal change in bacterioplankton assemblages, and could be clustered into 4 seral stages by CLUSTER analysis. Different dominant taxa were identified in different seral stages. Furthermore, we used a RMT-based network method to reveal the interspecies interactions among different bacterioplankton and found that these interactions were also dynamic along bacterioplankton succession. Canonical correspondence analysis and Pearson correlations showed that ammonium, nitrite, dissolved oxygen, cryptophytes and green algae constrained community composition by affecting the relative abundance of susceptible species. Besides, *Candidatus* Aquiluna rubra was identified as the keystone and generalist of the entire network, suggestive of an important role in driving bacterioplankton succession. These findings expand our understanding of the underlying mechanisms of bacterioplankton succession in ponds with intensive rearing shrimp, and suggest that stabilizing environmental variables (e.g. increasing DO, minimizing nutrient pollution and regulating phytoplankton community) could be a useful management tool for promoting and maintaining healthy aquaculture environments.

1. Introduction

Unlike terrestrial domesticated animals, aquaculture species, like shrimps, live constantly and directly in the culture water environment, and the condition of culture environment crucially affect the health status of reared animals (Lemonnier et al., 2010; Ma et al., 2013). As an indispensable component of water environment, bacterioplankton play central roles in shrimp culturing systems. On the one hand, bacterioplankton contributes essentially to energy flowing and nutrient cycling, such as discomposing organic matter (Cotner and Biddanda, 2002), promoting biogeochemical cycling of carbon (C), nitrogen (N), phosphorus (P) and sulphur (S) (Gonzalez et al., 2000), and affecting many other ecological processes (Wei et al., 2009). On the other hand, as shrimps and bacterioplankton share one water environment, many studies have suggested the transfer of microorganisms from surrounding water into the shrimp's internal habitat (Giatsis et al., 2015). In other word, the composition of bacterioplankton community directly interacts with the structure of shrimp internal microbiota (De Schryver and Vadstein, 2014; Xiong et al., 2015b), and the shift would lead to dysbiosis in gut microbial structure (Berry et al., 2012; Xiong et al., 2017; Zhu et al., 2016a). In addition, the bacterioplankton community structure and diversity are flexible with the physical, chemical and biological properties in the ambient water, which can be used as an indicator mirroring environmental quality (Dai et al., 2017; Teeling et al., 2012; Xu et al., 2014; Yeo et al., 2013). During the aquaculture production cycle, water quality is temporal variable, which, in turn, led to the dynamics in bacterioplankton community. Many studies have reported the temporal variability of bacterioplankton community along shrimp cultivation, e.g., Xiong et al. (2014c) revealed the high turnover and predictability of bacterioplankton community during shrimp cultivation. Lucas et al. (2010) reported that vibriosis could change the temporal dynamics of bacterioplankton and phytoplankton

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communities. However, with the increasing accumulation of microbial communities sequence data, it is not enough to focus only on the alpha- or beta-diversity patterns of bacterioplankton community.

Biodiversity encompasses not just species but also interactions among species, such as mutualism and competition (Olesen et al., 2007). Within habitats, species and their interactions assemble into large, complex ecological networks (Faust and Raes, 2012; Montoya et al., 2006). Explaining and predicting the structures, dynamics, and underlying mechanisms of such interspecies interactions are essential parts of any study of biodiversity and its responses to perturbations (Zhou et al., 2011). In the past two decades, a growing number of researches on interspecies interactions have been reported, principally in plant and animal ecology (Allesina et al., 2008; Bastolla et al., 2009; Martin et al., 2017). However, the study of interactions among different microbial taxa has been restricted, chiefly due to the difficulties in directly observing and quantifying microbial interactions under natural conditions (Deng et al., 2016). Recently, as time series analyses of microbial communities become increasingly available, network analysis has been put forward to deduce potential interactions among different taxa by uncovering co-occurrence or antagonistic associations (Faust et al., 2015; Steele et al., 2011). Among several association network approaches, a random matrix theory (RMT)-based network method developed by Deng et al. (2012), molecular ecological network analysis (MEN), has been widely applied in soil (Wang et al., 2017a), sediment (Ligi et al., 2014), seawater (Dai et al., 2017), and wastewater (Wang et al., 2017b), owing to its advantage in computational simplicity and noise tolerance. The shrimp culturing system is a complex ecosystem in which various bacterioplankton interact with each other. Through such interactions, bacterioplankton community is capable of accomplishing ecological functions which could not be achieved by individuals (Wang et al., 2017b). It is expected that the interspecies interactions among bacterioplankton are temporal variable along shrimp cultivation. However, to the best of our knowledge, such hypothesis has not been fully explored.

In the present study, we used multi-statistical and molecular ecological network analysis methods (i) to investigate the successional process of bacterioplankton community along intensive shrimp cultivation, (ii) to identify the environmental factors that drive this succession, and (iii) to screen keystone species that shape the composition of bacterioplankton community. To this end, water samples from six shrimp (*Litopenaeus vannamei*) intensive rearing ponds were collected to depict the bacterioplankton community and water quality over a whole shrimp cultivation cycle. This basic information will be essential for understanding the microbial ecology in aquaculture ecosystem and manipulating water physic-chemical properties and bacterioplankton community to promote and sustain the health of aquaculture environments.

2. Materials and methods

2.1. Experimental site and sampling

The intensive rearing ponds investigated in this study were located in Zhanqi, Ningbo, eastern China (29°32′N, 121°31′E). There were 30 ponds on this shrimp-farm, and these ponds were approximately uniform in size (2000 m^2) and depth (1.5 m). The ponds were within greenhouses to maintain a relatively stable temperature during the cool season. Bottom aeration was applied to maintain a suitable level of dissolved oxygen. Congeneric larval shrimp (*L. vannamei*) were introduced into the ponds with stocking density of 360,000 ind./pond on 8 April 2016. The farmers had adjusted the water parameters before stocking to make sure that the rearing conditions were similar across the ponds. One week later, samples were taken at various time points separated by 6 to 10 days (over a span of 87 days, from 15 April to 10 July) from six selected ponds. The ponds were identically managed in terms of disinfectant seawater inputs (salinity 22‰), 5% daily water exchange, feed type and feed schedule. No biological agent was introduced during the cultivation activities. Cultivation was terminated on 10 July 2016, and the growth status of shrimps were showed in Table A.1. Totally, 78 samples (6 ponds × 13 time points) were collected.

2.2. Water sample collection and analysis

Water temperature (WT), pH, salinity (SAL) and dissolved oxygen (DO) were recorded *in suit* with a YSI 6000 multi-parameter probe (YSI Inc., Yellow Springs, USA) at a depth of 50 cm. The content of green algae (Green), diatoms/dinoflagellates (Dia-dino), cryptophytes (Crypto) and total chlorophyll *a* (Chla) were also measured *in suit* with a FluoroProbe fluorometer (FluoroProbe-III, bbe Moldaenke, Schwentinental, Germany). To minor the spatial variability within ponds, samples were taken from four representative points and mixed to form a composite biological replicate sample (3 L) representing a given pond. All water samples were stored in the dark at 4 °C and were returned to the laboratory for further processing.

The levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD) were analyzed following standard methods (AQSIQ, 2007). For the analysis of ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and orthophosphate (PO_4^{3-}), samples were filtered through glass fiber filters (GF/F, 25 mm, 0.7 μm) with a filtration system (Vacuum Pump XF5423050, Millipore, Darmstadt, Germany), and measured with an automated spectrophotometer (Smart-Chem 200 Discrete Analyzer, Westco Scientific Instruments, Brookfield, USA).

For the bacterioplankton community analysis, 1000 mL of each water sample was prefiltered through a 100 μm pore-size sieve and then sequentially filtered onto three 0.2 μm pore-sized polycarbonate membranes (47 mm diameter, Millipore, Boston, MA, USA) (one for subsequent analysis, two for backup) on the sampling day. As much water as possible was filtered onto each membrane. The membranes were immediately frozen at −80 °C until they were needed.

2.3. DNA extraction, PCR amplification and Illumina Miseq pyrosequencing

Microbial DNA was extracted directly from the membrane using a Power Soil® DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The DNA extracts were quantified by the radios of 260/280 nm, and 260/230 nm using a spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The V3-V4 regions of bacterial 16S rDNA gene was amplified (30 μL reaction volume; started from 95 °C for 3 min; followed by 28 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s; and finalized with a 10 min extension step at 72 °C) using primer set 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′), with overhang sequences as adaptors to link to the barcodes at the 5′ end for each primer. PCR was performed in triplicate for each sample, and products were purified using a PCR fragment purification kit (Takara, Japan) and checked using the Quant-iT PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, CA, USA). The purified products were combined into equimolar ratios for paired-end (PE) library preparation, and 300 bp PE sequencing on an Illumina Miseq platform (Illumina, San Diego, CA, USA).

2.4. Processing of pyrosequencing data

The paired reads were joined with FLASH using default setting (Magoc and Salzberg, 2011). Raw FASTQ files were processed using the Quantitative Insights Into Microbial Ecology (QIIME) Pipeline (Version 1.9.0 http://qiime.org/tutorials/tutorial.html) (Caporaso et al., 2010). The operational taxonomic units (OTUs) at 97% similarity level were clustered using USEARCH (version 7.1 http://drive5.com/uparse/). The sequences were quality filtered on the basis of quality score,