Ecological Indicators 38 (2014) 218–224

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Bacterioplankton assemblages as biological indicators of shrimp health status

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ARTICLE INFO

Article history:
Received 27 May 2013
Received in revised form 30 October 2013
Accepted 2 November 2013

Keywords:
Bacterioplankton
Community assemblages
Indicator orders
Shrimp diseases

ABSTRACT

The close correlation between the incidence of shrimp disease and the surrounding microbial community is well established. However, it remains uncertain whether particular bacterial assemblages are indicative of shrimp health status (healthy or diseased). To test this hypothesis, we used 454 pyrosequencing to compare the bacterioplankton composition of ponds with healthy shrimp populations (PHS) with that of ponds with diseased shrimp populations (PDS). The results showed that the bacterial communities in PDS were markedly distinct ($P < 0.05$) from those of PHS but that the diversity was unchanged. This sudden shift in the bacterioplankton communities was accompanied by severe mass mortality of the shrimp. The differentiation of the bacterial communities was primarily shaped by the total phosphate and by the chemical oxygen demand. In particular, we identified 11 indicator orders (in which 6 for healthy status and 5 for diseased status) that differentiated PHS from PDS. In a given pond, especially, the sum of the relative abundance of the disease indicator phylotypes and that of the healthy indicator phylotypes could be used to estimate the health status of the pond. This result suggests that the bacterioplankton composition determines, at least in part, the health status of the shrimp. Overall, this study provides direct evidence that the composition of the bacterioplankton community can serve as a biological indicator to evaluate the occurrence of shrimp diseases.

1. Introduction

Economic incentives have spurred the development of intensive shrimp farming in coastal regions. To sustain high production, the feed supply generally exceeds the required level in shrimp ponds, and antibiotics are routinely applied to prevent disease even if pathogens are not evident (Cabello, 2006). These practices cause a gradual deterioration of water quality and the emergence of highly virulent pathogens (Holmström et al., 2003; Broughton and Walker, 2009; Zhou et al., 2012), particularly in the mid- to late crop periods (Sugiura et al., 2006; Ma et al., 2013). The deteriorating water quality causes pervasive and severe shrimp diseases (Defoirdt et al., 2011; Ferreira et al., 2011) that have become a major threat to aquaculture. For this reason, there is an urgent need to establish environmentally friendly and reliable technologies to control pathogenic bacteria.

Recently, a number of studies have indicated that bacterioplankton play a direct role in numerous shrimp diseases (Lucas et al., 2010; Defoirdt et al., 2011). This finding raises the possibility of the alternative control of shrimp diseases by managing the bacterial community. One successful example is the application of probiotics to displace specific pathogenic bacteria by competitive processes. Nevertheless, most, if not all, of these probiotics generally focus on vibriosis diseases (Moriarty, 1998; Vinod et al., 2006; Defoirdt et al., 2011); this restriction is unwarranted because new pathogenic bacteria, such as *Salmonella enterica* (Cabello, 2006), *Streptococcus* sp. (Hasson et al., 2009) and *Spiroplasma mirum* (Liang et al., 2011), have been regularly isolated from shrimp ponds. As a result, probiotics applications that target single types of pathogenic bacteria are no longer effective in treating shrimp diseases in certain cases. In addition, there is ample evidence that disease emergence is the result of a disturbance of the complex interactions between shrimp and bacterioplankton communities (Lucas et al., 2010; Defoirdt et al., 2011). Therefore, the strategies for disease control should

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http://dx.doi.org/10.1016/j.ecolind.2013.11.002

focus on the level of the entire community rather than on a single pathogen or a few pathogens (Wanjugi and Harwood, 2013). To achieve this goal, we first need to obtain the organisms, termed deleterious species, that are closely correlated with the occurrence of disease. Conversely, desirable alternative species or probiotics could be applied to displace deleterious common bacteria.

During the aquaculture production cycle, shrimp are in continuous and direct contact with the ambient bacterioplankton communities. Given that the microflora holds the key positions in ecological processes and food web dynamics (Yu et al., 2009; Xiong et al., 2012a; Ponsard et al., 2013) and certain bacteria are potential shrimp pathogens (Cabello, 2006; Hasson et al., 2009; Liang et al., 2011; Zhou et al., 2012), we hypothesize that sudden shifts in the bacterial community potentially lead to shrimp disease outbreaks and vice versa. Thus, the shrimp health status of ponds could be distinguishable and indicated by the bacterioplankton composition. This hypothesis has not been tested in aquaculture ponds. To do so, we collected water samples from culture ponds with healthy and diseased shrimp populations to identify indicative assemblages closely associated with shrimp health status and the factors that drive variations in these indicative species. This basic information will be essential for manipulating geochemical factors and the bacterioplankton community to control the occurrence of disease.

2. Materials and methods

2.1. Experimental design and water sample collection

The shrimp farm ponds investigated in this study are located in Zhanqi, Ningbo, eastern China (29°32′ N, 121°31′ E). These 30 ponds are approximately the same size (2000 m^2) and identically managed in terms of sea water, daily water exchange rate (5%), water depth (1.5 m), shrimp stocking density (360,000 ind/pond), feed type and schedule. The ponds are located in greenhouses to maintain stable temperatures during the cool season. Bottom aeration is applied to maintain suitable dissolved oxygen levels. Shrimp (*Litopenaeus vannamei*) juveniles were inoculated on 25 March 2012. A disease outbreak caused massive mortality of shrimp in 6 ponds on 27 May, 63 days after inoculation. The shrimp showed black patches on their shells, stopped eating and almost all died within 3 days. We collected water samples from the six ponds with diseased shrimp populations (PDS) and additional samples from six ponds with healthy shrimp populations (PHS) as a reference at a depth of 50 cm below the water surface to compare the bacterioplankton composition of PDS with that of PHS. Samples were transported to the lab within 4 h and kept at 4 °C.

Water temperature and pH were recorded with appropriate sensors at a depth of 50 cm. The concentrations of total organic carbon (TOC), total nitrogen (TN), total phosphate (TP), NO_3^--N and PO_4^{3+}-P and the chemical oxygen demand (COD) were analyzed according to standard methods (APHA, 1989). For the measurement of chlorophyll a (Chl a), a water sample was filtered through a 0.45 μm polycarbonate membrane (diameter 45 mm; Millipore, Boston, MA, USA), then extracted in 80% DMF (N,N-dimethyl formamide) for 24 h at 4 °C. The concentration of Chl a in the supernatant was determined using a spectrophotometer (UV-1601, Shimadzu, Japan) at wavelengths of 647 and 665 nm according to the equation $\text{Chl a} = 12.70_{A665} - 2.79_{A647}$ (Inskeep and Bloom, 1984).

2.2. DNA extraction

On the sampling day, approximately 1 L of water samples for DNA extraction were pre-filtered through nylon mesh (100 μm pore size). The samples were subsequently filtered through a 0.2 μm polycarbonate membrane (Millipore) to collect microbial biomass. The community DNA was extracted using a Power Soil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The DNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored at −80 °C until amplification.

2.3. Bacterial 16S rDNA amplification using the Roche 454 pyrosequencing adapter

An aliquot (50 ng) of DNA from each sample was used as the template for amplification. The V1–V3 hypervariable regions of bacterial 16S rRNAs (*Escherichia coli* positions 27F–519R) were amplified using the primer set 27F: AGAGTTTGATCMTGGCTCAG with the Roche 454 'A' pyrosequencing adapter and a unique 10 bp barcode sequence and the primer 519R: GWATTACCGCGGCKGCTG with the Roche 454 'B' sequencing adapter at the 5′-end of each primer. This region furnished nearly the same resolution as that of the nearly full length sequence (Kim et al., 2011). Each sample was amplified in triplicate with a unique barcode primer (in a 50 μL reaction system) under the following conditions: 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products for each sample were combined and purified with a PCR fragment purification kit (TaKaRa Biotech, Japan).

An equimolar amount of PCR products (assuming that the same size of amplicons had a similar molar mass) for each sample were combined in a single tube and run on a Roche FLX 454 pyrosequencing machine (Roche Diagnostics Corporation, Branford, CT, USA), producing reads from the forward direction 27F with the barcode.

2.4. Processing of pyrosequencing data

Sequencing reads were quality filtered and chimera checked using the Quantitative Insights Into Microbial Ecology (QIIME v1.5.0) workflow (Caporaso et al., 2010a), with minor modifications as described previously (Xiong et al., 2012b). Briefly, the bacterial reads whose length was outside the bounds of 200 and 450 bp and cases in which the homopolymer run exceeded 6 were removed by PyroNoise (Quince et al., 2009). Sequences with the same barcode were then assigned to the same sample (Caporaso et al., 2010a). Bacterial phylotypes were identified using uclust (Edgar, 2010) and assigned to operational taxonomic units (OTUs, 97% similarity). Representative sequences from each phylotype were aligned using PyNAST (Caporaso et al., 2010b), and the most abundant sequence in the OTU was selected as the representative sequence. The taxonomic identity of each phylotype was determined using the Greengenes database (DeSantis et al., 2006). To avoid sample size-based artifacts, we used a randomly selected subset of 4500 sequences (corresponding to the smallest sequencing effort for any of the samples) per sample to calculate the diversities and distances between samples.

2.5. Statistical analysis

We used an unpaired *t*-test to evaluate the differences in water variables and bacterial abundances between PHS and PDS. Principal coordinates analysis (PCoA) and cluster analysis were performed to evaluate the overall differences in microbial community structure based on Bray–Curtis distances. Dissimilarity tests (e.g., MRPP, ANOSIM and Adonis) were then employed to test the significance of the differences found between the bacterial community compositions (Anderson, 2001; Clarke, 1993). To make the various environmental factors comparable, the raw environmental data were standardized with the equation SNDE = (x – mean of the raw data)/standard deviation of the raw data, where SNDE represents