

# Dynamics of Microbial Communities in an Earthen Shrimp Pond during the Shrimp Growing Period

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Received: August 17, 2011

Accepted: October 30, 2011

Published: December 1, 2011

doi:10.5539/enrr.v1n1p171

URL: <http://dx.doi.org/10.5539/enrr.v1n1p171>

## Abstract

Correlations between the activities of microbial communities and water quality in an earthen pond were investigated monthly during the shrimp growing period. The TAN gradually decreased compared to the beginning time, thereby resulting in an overall decrease of 81.3%. In contrast, the overall nitrate concentration increased 46.3% over this same period. Microbial community analysis using PCR-DGGE showed that changes in community dynamics that occurred during the shrimp growing period might be correlated to water quality. Overall, there were 3 groups of microbial dynamics. The 1<sup>st</sup> was observed in all investigations, was microflora found in the shrimp culturing system, which included the following: *Nitrosomonas eutropha*, *Exiguobacterium SKRP 5*, and *Exiguobacteria sp. CNJ771*. The 2<sup>nd</sup> involved the replacement of bacteria from one type to another, such as from *Flavobacteriales bacterium* to *Aquiflexum balticum*, which are involved in shrimp shell degradation. The 3<sup>rd</sup> consisted of microbes observed at only one time point, such as *Synechococcus sp. Y0011* (W<sub>0</sub>), and *Stenotrophomonas maltophilia* (W<sub>3</sub>), or at several time points, such as *Pseudomonas lanceolata* (W<sub>0</sub> and W<sub>3</sub>)

and *Burkholderia* sp. WBF2 ( $W_1$  and  $W_2$ ). Although this third group of bacteria was not found at all time points tested, the group was important for balancing the shrimp culturing system, which is important for a successful shrimp cultivation.

**Keywords:** Microbial community, DGGE, 16S rRNA genes, Shrimp farming water

## 1. Introduction

In spite of many challenges, Thailand has retained global dominance in shrimp production for over a decade, providing a major source of income, foreign exchange generation, and livelihood opportunities (Pouliotte, Islam, Smit, & Islam, 2006). Thai shrimp farming essentially consists of small-scale, owner-managed and owner-operated practices (Kongkeo & Davy). The requirements for successful shrimp farming are the maintenance of water quality and of the balance between beneficial and pathogenic bacteria. Between 1994 and 1997, shrimp production in Thailand dropped by 40%, due to disease caused by bacterial pathogens and shrimp viruses (Moriarty, 1999); a high density of shrimp is conducive for the spread of pathogens. The solution lies in the field of microbial ecology, not in the field of pharmacology, i.e., in the development of new antibiotics or vaccines (Kesarcodi-Watson, Kaspar, Lategan, & Gibson, 2008). Using beneficial bacteria (probiotics) to displace pathogenic bacteria by competitive processes is a more efficient remedy than administering antibiotics. Also, many commercial shrimp ponds were operating with daily water exchange rates of 10–15% to maintain water quality. Water exchange rates were identified as an important factor contributing to several disease in shrimp growing areas. Therefore, water quality control and microbial community balance are necessary for successful shrimp cultivations (Sandifer & Hopkins, 1996).

Data regarding the abundance and phylogenetic relationship of microorganisms based on 16S rRNA gene-targeting techniques, such as denaturing gradient gel electrophoresis (DGGE), are suitable cultivation-independent tools for the analysis of complex microbial communities (Amann, Ludwig, & Schleifer, 1995). In order to gain insight into the correlation between microbial communities and water quality, microbial communities and their dynamics and activities in shrimp farming water were analyzed by comparative sequence analysis of the 16S rRNA genes, which were amplified from total genomic DNA derived from the microbial community.

## 2. Materials and Methods

### 2.1 Shrimp farming water samples

Shrimp farming water samples were collected from a semi-intensive shrimp culture pond of white shrimps (*Penaeus vannamei*) located in Thung-Kru district, Bangkok, Thailand. A total of 4 water samples were studied in this report.  $W_0$  represented water samples taken before shrimp were released into the earthen pond.  $W_1$ ,  $W_2$  and  $W_3$  represented water samples taken one, two and three months, respectively, after the shrimp were released. Sample collection, stabilization, and transportation to the laboratory as well as sample storage were done according to the Standard Methods of Strickland and Parsons (Strickland & Parsons, 1972).

### 2.2 Analysis of water quality

Water samples were taken from the shrimp pond at 3 different depths: 10, 50 and 80 cm. Levels of total ammonia nitrogen (TAN, including  $\text{NH}_3^+$  and  $\text{NH}_4^+$ ), nitrate, nitrite, phosphate, chlorophyll *a* and temperature and salinity were determined as previously described (Strickland & Parsons, 1972).

### 2.3 Total Genomic DNA Extraction

For each time point and water depth, seven liters of shrimp water were successively filtered through 0.45- and 0.2- $\mu\text{m}$  filters. The filtrates were resuspended in 10 ml of 1X phosphate buffer solution (PBS) and frozen at  $-20^\circ\text{C}$ . Total genomic DNA was extracted using a method modified from Zhou et al. (Zhou, Bruns, & Tiedje, 1996). For each sample, a 5-ml suspension (mixture of PBS and filtrate) was centrifuged at 14,000 rpm for 5 min. Cell pellets were collected and resuspended in 300  $\mu\text{l}$  of lysozyme solution containing 10 mg/ml lysozyme, 0.15 M NaCl, and 0.1 M  $\text{Na}_2\text{EDTA}$ , pH 8.0. The resuspended samples were incubated at  $37^\circ\text{C}$  for 1 h and mixed by inversion every 15 min. After cooling on ice, 300  $\mu\text{l}$  of SDS buffer (0.5 M Tris-HCl, 0.1 M NaCl, pH 8.0, 4% sodium dodecyl sulfate) was added to each of the samples. The samples were incubated for 10 min and were placed at  $55^\circ\text{C}$  for 10 min. Next, the genomic DNA was extracted and purified from the lysates by three sequential phenol-chloroform extractions followed by precipitation with isopropanol (Sambrook, Fritsch, & Maniatis, 1989). The DNA pellets were washed with 70% ethanol resuspended in sterile TE buffer (50 mM Tris-HCl, 1 mM  $\text{Na}_2\text{EDTA}$ , pH 8.0) and stored at  $-20^\circ\text{C}$ .



the final sample period ( $W_3$ ); this period has the highest nitrate concentration, indicating the presence of an increasing phytoplankton mass due to accumulation of nitrate in the pond, which stimulates their growth by nitrate utilization (Dortch, 1990).

### 3.2 Microbial community composition in the shrimp cultivation pond

In the shrimp culture pond, the major source of nitrogen waste come from protein in artificial feed pellets. In fact, only 20-30 % of the nitrogen found in feed is converted into shrimp biomass (Kutako, Powtongsook, & Menasveta, 2009), while the rest is accumulated in high organic-content sediment at the bottom of the pond (Kutako, et al., 2009). In nature, ammonia is subsequently converted into nitrite and nitrate by the nitrification process, and the nitrate is thereafter eliminated from the pond by the denitrification process in the sediment. The nitrification and denitrification processes are linked with specific groups of bacteria naturally found in ponds. Generally,  $\beta$ - and  $\gamma$ -proteobacteria are found in a higher percentage of bacterial communities from polluted waters (Rubin & Leff, 2007), whereas cyanobacteria are used as feed for increasing the growth rate and size of shrimp (Focken, Groth, Coloso, & Becker, 1998) or for nutrient removal in aquaculture systems (Chaowanapreecha, Wantawin, & Ruengjitchatchawalya, 2007). In this study, a DGGE profile of the microbial community over the course of a single shrimp growing period of 3 months is shown (Fig. 1). The majority of the 14 bacteria found (Table 2) were identified as  $\beta$ -proteobacteria (8 out of 14 or 57.14%), which consists of several different groups of bacteria involved in degradation, nitrogen fixation and ammonia oxidation (Rubin & Leff, 2007). These bacteria included *Nitrosomonas*, *Flavobacterium*, *Exiguobacterium*, *Burkholderia* and *Nitrospira*. The remaining bacteria identified in this study belonged to the following groups:  $\alpha$ -proteobacteria (1 out of 14 or 7.14%),  $\gamma$ -proteobacteria (2 out of 14 or 14.28%), cyanobacteria (2 out of 14 or 14.28%) and Cytophaga-Flavobacterium-Bacteroides (CFB; 1 out of 14 or 7.14%). Changes in the microbial community's composition occurred over time as demonstrated by changes in the dominant bands observed from the DGGE profile and the affiliated sequences retrieved following DNA extraction. The observed microbial community in the water before the addition of shrimp ( $W_0$ ) consisted of 8 bands (Fig. 1). Five of these bands (a1, a3, a5, a7 and a12) were identified as members of the  $\beta$ -proteobacteria group, which represented the majority of bacteria identified in this study. Band a1 was identified as a member of *Nitrosomonas* sp. This nitrifying bacterium is microflora, found in shrimp grow-out ponds (Ghosh, Sasmal, & Abraham), that remove ammonia from the water; it is also found in various other environments (Kowalchuk & Stephen, 2001). Ammonia oxidizing bacteria are classified into 5 genera: *Nitrosomonas*, *Nitrosovibrio*, *Nitrosococcus*, *Nitrosolobus* and *Nitrospira*, while nitrite oxidizing bacteria are classified into three genera: *Nitrobacter*, *Nitrococcus* and *Nitrospira* (Altschul, et al.). *Nitrosomonas* and *Nitrobacter* are commonly used commercially in aquaculture as bioremediators (Altschul, et al.). Band a3 was closely related to *Flavobacterium*, which is a cellulose digesting bacterium involved in shrimp shell degradation (W. C. Chen, Tseng, Hsieh, Wang, & Wang). Bands a5, a7 and a12 were identified as *Exiguobacterium* spp. These bacteria have been found in a variety of environments (Vishnivetskaya, Kathariou, & Tiedje, 2009), including alkaline (Gopalsamy, Mody, Datta, & Jha, 2008), low temperature (Ponder, Thomashow, & Tiedje, 2008), or in aquaculture systems (Lopez-Cortes, Schumann, Pukall, & Stackebrandt, 2006). In an aquaculture system, these bacteria have the potential to improve the survival rate and development of *Artemia* sp. (Fardeau, Combet-blanc, & Ollivier, 2008), which is used as feed in shrimp nurseries. Moreover, these bacteria, much like *Bacillus* sp. (Kim, et al., 2005), can produce polypeptide antibiotics, such as bacitracin, gramicidin S, polymyxin, and tyrotricin, which are active against a wide range of bacteria (Drablos, Nicholson, & Ronning, 1999; Morikawa, Ito, & Imanaka, 1992; Perez, Suarez, & Castro, 1993) that might be harmful to shrimp. Two of the bands (a6 and a11) were identified as members of cyanobacteria. The presence of cyanobacteria was already suggested by the increase in chlorophyll *a* content in the shrimp water that was observed during the shrimp growing period. Increased levels of chlorophyll *a*, as shown in Table 1, were due to the growth of phytoplankton from nitrogen uptake or nitrogen-fixing in the shrimp pond (Sprober, Shafik, Prosing, Kovocs, & Herodek, 2003). Additionally, one band, a9, was closely related to *Pseudomonas* sp., a member of  $\gamma$ -proteobacteria. Based on traditional culturing studies, the *Pseudomonadaceae* family of bacteria is usually considered to be a predominant bacterial population in mariculture environments (Deng, et al., 2009).

Based on the DGGE profile, the microbial community at the end of the first month of cultivation ( $W_1$ ) consisted of 8 bands (Fig. 1). Six of the bands (b1, b3, b4, b5, b7 and b12) were identified as members of the  $\beta$ -proteobacteria group, which is the most prevalent group in this study. Of these, the 4 bands (b1, b3, b5, and b7) had the same identity as the a1, a3, a5, and a7 bands, respectively, from the sample taken at  $W_0$ . The remaining three bands, namely, b4, b9 and b12, represent new members detected in the  $W_1$  sample. Band b9 was identified as *Nitrobacter winogradskyi*, a member of the  $\alpha$ -proteobacteria group. These nitrite-oxidizing bacteria can oxidize nitrite to nitrate (Teske, et al., 1994) as suggested by the observed increase in nitrate concentration

between  $W_0$  and  $W_1$ . Band b12 was closely related to *Burkholderia*, which has been used for such agricultural purposes as biodegradation, including microcystin degradation (Kemes, et al., 2008), biocontrol (Parke & Gurian-Sherman, 2001) and serving as a plant-growth-promoting rhizobacteria (Compant, et al., 2005). However, bands a9, a11 and a12, which were present at  $W_0$ , had faded out by the time the  $W_1$  sample was taken.

The microbial community at the end of the second month ( $W_2$ ) consisted of 8 bands. Six of them (c1, c3, c4, c5, c8, and c13), i.e., the majority of the bands, were identified as members of the  $\beta$ -proteobacteria group. These 6 bands were similar, respectively, to bands b1, b3, b4, b5, b7 and b12 from the  $W_1$  time point. Band c6 was identified as the same member of the cyanobacteria as band b6 in  $W_1$ . Band c10, identified as *Nitrobacter winogradskyi*, shared the same identity as band b9.

The microbial community at the end of the third month ( $W_3$ ) consisted of 9 bands. Five of them were identified as members of the  $\beta$ -proteobacteria: d1 (or a1, b1, c1), d3 (or a5, b5, c5), d4 (or a7, b7, c8), d10 and d11 (or a12). Band d1, d3 and d4 were previously found in all the other cultivation periods, while a new band, d10, was identified as *Nitrosospira* sp. This bacterium is a low-salinity nitrifying bacterium (Bernhard, Donn, Giblin, & Stahl, 2005). During the course of this study, the salinity was gradually decreased from 10 to 2 psu to prevent shrimp disease (Limsuwan, et al., 2002). Band d11, in  $W_3$ , had a similar sequence to that of band a12 in  $W_0$ , suggesting the reappearance of this bacterium. The  $\beta$ -proteobacteria represented by bands c3 and c4, which were found in  $W_2$ , were not detected in  $W_3$ . Band c3 (or a3, b3), which was identified as closely related to the shrimp shell-degrading bacterium *Flavobacterium* (H. C. Chen & Hsu, 1997) was not found in  $W_3$ . Band d12 was closely related to *Aquiflexum balticum*, a member of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group of bacteria, which is considered to be important for the degradation of complex polysaccharides in aquatic environments (Brettar, Christen, & Hofle, 2004); these polysaccharides have also been found in shrimp shells (Mathur & Narang, 1990). A role in polysaccharide degradation might explain the observation that the shrimp shell degradation bacterium *Flavobacterium* is replaced by *Aquiflexum balticum* during the course of this study. Moreover, *Aquiflexum balticum* thrives at the optimum salinity and temperature of 1.5 psu and 30 °C, respectively, which was similar to the conditions in the shrimp pond at the time the  $W_3$  sample was taken (Brettar, et al., 2004). Band d6 (or b9, c10) was a  $\alpha$ -proteobacteria that is closely related to *Nitrobacter winogradskyi*, which was also found in  $W_1$  and  $W_2$ . Therefore, nitrite concentrations, as shown in Table 1, were stabilized by this bacterium. Additional members of the  $\gamma$ -proteobacteria (d8) and *Cytophaga-Flavobacterium-Bacteroides* (d12) groups were first detected in  $W_3$ . Band d8 was closely related to *Stenotrophomonas maltophilia*, which has the potential to degrade proteins (Garcia, et al., 2002) and might be the nitrogen source of ammonia-oxidizing bacteria for the production of ammonium (Broderick, 1978).

Overall, there were 3 groups of microbial dynamics observed in this study. The first dynamic consisted of the microbes that were found at all the time points investigated: *Nitrosomonas eutropha*, *Exiguobacterium SKRP 5*, and *Exiguobacteria* sp. CNJ771. The ammonia oxidizing bacteria, *Nitrosomonas eutropha*, has been reported as typical of the flora in earthen shrimp ponds, due to the excess ammonia that is found in these ponds. The sources of excess ammonia had been from overfeeding, shrimp feces or the sediment for nitrite production (Lied & Braaten, 1984). Moreover, this bacterium also correlated with *Nitrobacter winogradskyi*, a nitrite oxidizing bacteria which can oxidize nitrite to nitrate (Lees & Simpson, 1957) that was keeping nitrite concentrations at less than 0.05 mg/l, while nitrate concentrations increased over the growth period. The other ammonia-oxidizing bacteria, *Nitrosospira* sp., which was only found in  $W_3$  sample, might be working with *Nitrosomonas eutropha* to control the ammonia concentration in the shrimp pond system. *Exiguobacterium spp.*, another type of microflora found in aquacultures, was also found in *Artemia* cysts (C. s. Orozco-Medina, Maeda-Martínez, & López-Cortés, 2002) and gut rumen (C. Orozco-Medina, Lopez-Cortes, & Maeda-Martinez, 2009), salmon intestinal systems (Ring, Sperstad, Kraugerud, & Krogdahl, 2008) and shrimp farming sediment (Boonapatcharoen, Techkarnjanaruk, Wanichpongpan, & Ruenglerpanyakul, 2551). Therefore, it was expected that *Exiguobacterium spp.* would also be found during this investigation. Moreover, *Exiguobacterium undae* (found in  $W_2$  and  $W_3$ ) and *Exiguobacterium* sp. (found in  $W_0$  and  $W_4$ ) were also associated with the activity of *Exiguobacterium SKRP 5* and *Exiguobacteria* sp. CNJ771 during the shrimp growing period. The second group of microbial dynamics involved the replacement of *Flavobacteriales bacterium* with *Aquiflexum balticum*, both of which are bacteria involved in shrimp shell degradation. In the first two months ( $W_0$  to  $W_2$ ), *Flavobacteriales bacterium* was responsible for the degradation of shrimp shells. However, as the salinity gradually decreased to 2 psu (to control pathogens in the shrimp pond) and the temperature rose to 28.6 °C, the environmental conditions became optimal for *Aquiflexum balticum*; this finding might account for the observed replacement of *Flavobacteriales bacterium* with *Aquiflexum balticum*. The third group of microbial dynamics involved bacteria that were observed only at one time point, such as *Synechococcus* sp. Y0011 ( $W_0$ ), *Stenotrophomonas*

*maltophilia* ( $W_3$ ), or at several time points, such as *Pseudomonas lanceolata* ( $W_0$  and  $W_3$ ) and *Burkholderia* sp. WBF2 ( $W_1$  and  $W_2$ ). As previously mentioned, although this third group of bacteria was not found at all of the time points investigated, they have an important role in balancing the shrimp culturing system, which is needed for a successful shrimp cultivation.

#### 4. Conclusions

The importance of the microbial community composition for water quality and shrimp production suggests the need for management strategies that promote beneficial processes, while controlling adverse processes. The interrelationships between various functional groups within the microbial community are not completely understood, and relationships between microbes, system inputs, water quality, and shrimp health are complex. In this study, we investigated changes in water quality and microbial community composition and dynamics in shrimp farming water over the course of a single shrimp growing period. Moreover, we studied their correlations with one another. Overall, the microbial community during the shrimp growing period consisted of 14 species, including  $\alpha$ -proteobacteria (1 out of 14),  $\beta$ -proteobacteria (8 out of 14),  $\gamma$ -proteobacteria (2 out of 14), Cyanobacteria (2 out of 14), and CFB (1 out of 14). These species were grouped into 3 different types of microbial dynamics. The first group dynamic consisted of microbes that were found in all samples, which included *Nitrosomonas eutropha*, *Exiguobacterium SKRP 5*, and *Exiguobacteria* sp. CNJ771. The second group dynamic involved the replacement of *Flavobacteriales bacterium* with *Aquiflexum balticum*, both of which are associated with shrimp shell degradation. The third group dynamic involved bacteria that were observed only once, such as *Synechococcus* sp. Y0011 ( $W_0$ ) and *Stenotrophomonas maltophilia* ( $W_2$ ), or several times, such as *Pseudomonas lanceolata* ( $W_0$  and  $W_3$ ) and *Burkholderia* sp. WBF2 ( $W_1$  and  $W_2$ ). Changes in the composition of the microbial community were significantly linked to temperature, salinity and nutrient (ammonia, nitrite and nitrate) concentrations. Future studies aimed at understanding the role of each micro-organism within the shrimp pond community will provide greater guidance not only for the development of more successful shrimp culturing systems but also for the application of these developments for sustainable environmental protections.

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Table 1. Physical and chemical properties of water column samples obtained during the shrimp growing period

| Parameter                                      | Water samples  |                |                |                |
|--|----------------|----------------|----------------|----------------|
|  | W <sub>0</sub> | W <sub>1</sub> | W <sub>2</sub> | W <sub>3</sub> |
| Physical property                              |                |                |                |                |
| - pH   | 7.8±0.03       | 7.9±0.08       | 8.0±0.07       | 7.8±0.1        |
| -Temperature (°C)                              | 22.4±0.3       | 18.2±0.5       | 27.3±0.6       | 28.6±0.2       |
| -Salinity (PSU)                                | 10             | 7              | 5              | 2              |
| Chemical property                              |                |                |                |                |
| - Chlorophyll <i>a</i> (µg L <sup>-1</sup> )   | 42.67±5.2      | 71.00±4.3      | 164.63±11.4    | 187.16±15.1    |
| - Total Ammonia Nitrogen (mg L <sup>-1</sup> ) | 0.262±0.014    | 0.209±0.033    | 0.023±0.003    | 0.049±0.013    |
| -Nitrite nitrogen (mg L <sup>-1</sup> )        | < 0.05         | < 0.05         | < 0.05         | < 0.05         |
| -Nitrate nitrogen (mg L <sup>-1</sup> )        | 2.723±0.059    | 3.165±0.009    | 3.605±0.070    | 3.977±0.061    |

(90-day) (Means±SD, N=3)

Table 2. The 16 S rRNA gene sequences of the microbial community in shrimp pond water with high similarity by Blast searches

| Band           | Bacteria   | % Identity | Group                                    | Accession Number |
|----------------|--|------------|--|------------------|
| a1, b1, c1, d1 | <i>Nitrosomonas eutropha</i>                                     | 99         | β-proteobacteria                         | HQ433572         |
| a3, b3, c3     | <i>Uncultured Flavobacteriales bacterium</i><br>clone LiUU-11-73 | 84         | β-proteobacteria                         | HQ433562         |
| a5, b5, c5, d3 | <i>Exiguobacterium SKRP 5</i>                                    | 99         | β-proteobacteria                         | HQ433563         |
| a6, b6, c6     | <i>Synechococcus sp.</i> RS9915                                  | 99         | Cyanobacteria and<br>Chloroplast         | HQ433564         |
| a7, b7, c8, d4 | <i>Exiguobacteria sp.</i> CNJ771                                 | 99         | β-proteobacteria                         | HQ433553         |
| a 9, d5        | <i>Pseudomonas lanceolata</i>                                    | 99         | γ -proteobacteria                        | HQ433567         |
| a11            | <i>Synechococcus sp.</i> Y0011                                   | 92         | Cyanobacteria and<br>Chloroplast         | HQ433561         |
| a12, d11       | <i>Exiguobacterium sp.</i>                                       | 97         | β-proteobacteria                         | HQ433569         |
| b4, c4         | <i>Exiguobacterium undae</i>                                     | 98         | β-proteobacteria                         | HQ433557         |
| b9, c10, d6    | <i>Nitrobacter winogradskyi</i>                                  | 99         | α-proteobacteria                         | HQ433573         |
| b12, c13       | <i>Burkholderia sp.</i> WBF2                                     | 99         | β-proteobacteria                         | HQ433556         |
| d8             | <i>Stenotrophomonas maltophilia</i>                              | 99         | γ-proteobacteria                         | HQ433555         |
| d10            | <i>Nitrospira sp.</i>  | 93         | β-proteobacteria                         | HQ433552         |
| d12            | <i>Aquiflexum balticum</i>                                       | 92         | Cytophaga–Flavobacter<br>ium–Bacteroides | HQ433566         |

Remark: a = microbial community in W<sub>0</sub> (water samples taken before shrimp were released into the earthen pond)

b, c and d = microbial community in W<sub>1</sub>, W<sub>2</sub> and W<sub>3</sub> (water samples taken one, two and three months after shrimp were released, respectively)

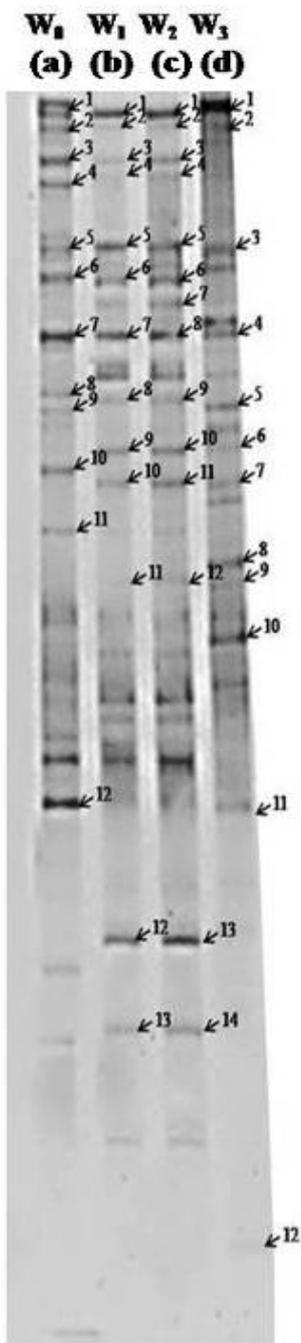


Figure 1. DGGE profile of bacterial amplicons amplified from the water: W<sub>0</sub> (Lane a), microbial community before the release of shrimp into the earthen pond; W<sub>1</sub> (Lane b), W<sub>2</sub> (Lane c) and W<sub>3</sub> (Lane d), depict the microbial community at time points 1, 2 and 3 months, respectively, during the shrimp growing period