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Variation of Functional Diversity of Microbial Communities in Water of Polyculture of *Portunus trituberculatus*, *Litopenaeus vannamei* and *Ruditapes philippinarum*

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Abstract

The functional diversity (FD) of the microbial communities found in pond waters was examined with the BIOLOG method for five different aquaculture groups: (1) monoculture of crab *Portunus trituberculatus*, (C); (2) polyculture of crab and white shrimp *Litopenaeus vannamei*, (CS); (3) polyculture of crab and short-necked clam *Ruditapes philippinarum,* (CB); (4) polyculture of crab, white shrimp, and short-necked clam (CSB); (5) polyculture of white shrimp and short-necked clam (SB). The results showed that the average well color development (AWCD) value which represents microbial metabolic activity was highest in the CSB group ($P < 0.05$). The McIntosh index, Shannon index, and Shannon evenness index, in the CSB group were significantly higher compared to the others ($P < 0.05$). These data indicated that both the microbial metabolic activity and functional diversity of the water microbial community in the CSB group were significantly higher than in the other communities ($P < 0.05$). The principal component analysis (PCA) results indicated that the carbon sources and the metabolic activity of the water microbial communities in the CSB group were different from the others. The redundancy analysis (RDA) results revealed that the FD of the microbial communities in all five groups was closely related to environmental factors. The critical environmental variables influencing the functional diversity of the water microbial communities were TOC, NO_2^--N and NH_4^+-N in July, and TP, NH_4^+-N and PO_4^{-3}–P in September. This study may provide a novel strategy for optimizing polyculture structure in swimming crab ponds.

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Introduction

Polyculture can improve biological diversity and environmental stability in an aquaculture pond, thereby increasing the utilization of input material, strengthening self-purifying of the pond water, improving economic efficiency, and reducing environmental pollution (Jena *et al*., 2008; Rahman *et al*., 2008). The intensity of aquaculture has increased, and the accumulation of organic matter such as residual feed, feces, and biological residues can lead to deterioration of the environment (Tacon *et al.*, 2003). It is well known that microbial communities have a close relationship with farmed aquatic organisms and that they play important roles in the material cycle and energy flow in aquaculture ecosystems, bioremediation of polluted environments (Zhou *et al.*, 2009), and bio-indicators (Paerl *et al.*, 2002). Therefore, the study of the functional diversity (FD) of the microbial community is of great importance for aquaculture ecosystems.

BIOLOG technology is one of the commonly used methods for investigating microbial FD. It characterizes the functional diversity of a microbial community based on the utilization of different carbon sources. It can be used to evaluate the structure and function of microbial communities in different environments. Many recent studies have addressed the FD of microbial communities in the soil (Schutter and Dick, 2001; Liu *et al*., 2012). However, little research has focused on the microbial communities in aquaculture systems. We used the BIOLOG technique and redundancy analysis (RDA) to assess the variation in the FD of microbial communities in pond water from polyculture containing swimming crab (*Portunus trituberculatus*), white shrimp (*Litopenaeus vannamei*), and short-necked clam (*Ruditapes philippinarum*). We also assessed the relationship between FD and environmental variables. The purpose of this study was to provide a foundation for establishing micro-ecology polyculture protocol for swimming crab ponds.

Materials and Methods

Pond and enclosures. The experimental pond, approximately 2 ha. in area with water depth ranging from 1.6–1.7 m, was located in Ganyu County, Jiangsu Province, China (34°58′N, 119°20′E). The culture pond was divided into equally sized enclosures (L × W × D = 5 × 5 × 2 m) lined with waterproof polyvinyl plastic (Tian *et al.,* 2001).

Animals and experimental design. Juvenile swimming crab, white shrimp, and short-necked clam were purchased from Ganyu Jiaxin Aquatic Food Co., Ltd (Ganyu, Jiangsu, China).

Five different culture groups were tested: (1) monoculture of crab *Portunus trituberculatus*, control (C); (2) polyculture of crab and white shrimp *Litopenaeus vannamei*, (CS); (3) polyculture of crab and short-necked clam *Ruditapes philippinarum,* (CB); (4) polyculture of crab, white shrimp, and short-necked clam (CSB); (5) polyculture of white shrimp and short-necked clam (SB).

There were three replicates of each group in 15 separate enclosures. Stocking information for the different experimental groups and the control are shown in Table 1.

Table 1. Stocking information for different groups

Groups	*Portunus trituberculatus*		*Litopenaeus vannamei*		*Ruditapes philippinarum*	
	Density individuals/m²	*Weight g/ind*	*Density ind/m²*	*Weight g/ind*	*Density ind/m²*	*Weight g/ind*
C	6		0		0	
CS	6		45		0	
CB	6	0.57±0.13	0	0.045±0.010	15	0.91±0.12
SB	0		45		15	
CSB	6		45		15	

The swimming crabs and short-necked clams were stocked on July 2, and the white shrimp were stocked on July 14. All of the animals were harvested on October 3. Blue clams *Aloidis laevis* were fed twice a day (06:00 and 18:00), and the feeding rate changed from 80.0% to 2.0% of body weight of crab as they grew (Zhou *et al.*, 2010). A commercial pellet feed manufactured by Lianyungang Chia Tai Feed Co., Ltd

(Lianyungang, Jiangsu, China), was fed to the shrimps twice a day (06:00 and 18:00), and the feeding rate was determined by the growth of the shrimps. Growth of crabs and shrimp were determined by sampling 15–20 individuals every 10 days. They were returned to their enclosure after weighing. No fertilization or any antibiotic was used during this study period. The aerators were used twice a day (05:00–06:00 and 14:00–15:00) on sunny days, and three times a day (05:00–06:00, 14:00–15:00 and 22:00–0:00) on cloudy or rainy days over the entire course of the experiment.

In July and September 2012, 2L water samples were collected in sterile vessels at the same time, from intermediate depth water (0.5 m) of each enclosure, to measure physical and chemical properties of the pond and to study the microbial communities.

Environmental properties. Physical and chemical properties of all the sampling sites were measured with the YSI 556 system (YSI Incorporated, USA) for in situ salinity and temperature. The concentrations of total nitrogen (TN), total phosphorus (TP), ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), and active phosphate (PO_4^{-3}–P) were determined with standard methods (GB/T 12763.4-2007). The chlorophyll *a* (Chl *a*) concentration was measured using the acetone extraction method (GB/T 12763.6-2007). Total organic carbon (TOC) was measured with a total organic carbon analyzer (TOC-5000A, Shimadzu, Inc., Japan).

Eco-plate analysis. In recent years, the BIOLOG technique has been used extensively to characterize the FD of microbial communities in different environments by evaluating the utilization of different carbon sources. In this experiment, eco-plates (Biolog Eco-plate TM, USA) were used to analyze the FD of microbial communities from each pond sample. An Eco-plate contains three replicate sets of 31 carbon substrates and a blank. Based on the microbial metabolic pathways for three major nutrients, the 31 carbon sources in the BIOLOG Eco-plates were divided into six different types: polymers (n=4), carbohydrates (n=10), carboxylic acids (n=7), amino acids (n=6), phenolic compounds (n=2), and amines (n=2) (Choi and Dobbs, 1999).

An aliquot from each sample was loaded onto an Eco-plate (150 μL/well) to determine the metabolic potential of the microbial communities in the water sample. The plates were incubated at 25°C, and color development in each well was recorded as optical density (OD) at 590 and 750 nm with a plate reader at 24 h-intervals for 168 h.

The microbial activity in each microplate was expressed as the average well color development (AWCD), which reflected the overall ability of the microbial communities to metabolize the available carbon sources (Choi and Dobbs, 1999). AWCD was determined using the following equation:

$$AWCD = \sum[(C_i\text{-}R)_{590}-(C_i\text{-}R)_{750}]/n$$

where C_i is the optical density value from each well, R is the optical density value from the water blank well, and n is the number of substrates (Garland and Mills, 1991).

Microbial community diversity indices are comprehensive indicators that reflect species richness and evenness. The trends in these indices can account for the dynamic features of a microbial community. The Shannon index and Shannon evenness index are based on the concept of evenness or equitability (i.e., the extent to which each species is represented among a sample), and they are used to evaluate both richness and evenness of community. The Simpson index is the probability that two individuals drawn at random from a community will belong to the same species, and it is used to evaluate the dominance of specific species (Magurran, 1988). The McIntosh index is used to evaluate evenness of community based on the hyperspace of species (Atlas 1984).

An optical density reading at 72 h was used for statistical analysis. The metabolic FD of the microbial community from each water sample was measured using the Shannon index, Simpson index, McIntosh index, and Shannon evenness index.

Based on previously published work on soil microbial communities (Schutter and Dick, 2001), the optical densities of the wells in the plates were used to calculate the FD of the water microbial community with the following equations.

The Shannon index was calculated using the equation:

$$H= -\sum P_i \ln P_i, \quad P_i = n_i/N$$

where n_i is the optical density of each well and N is the sum of the optical densities of all the wells.

The Simpson index was calculated using the equation:

$D=1-\sum(P_i)^2$, $P_i = n_i/N$

where P_i is the proportion of the optical density of each well to the sum of optical density of all wells.

The McIntosh index was calculated based on the equation:

$$U = \sqrt{\left(\sum n_i^2\right)}$$

where n_i is the optical density of each well.

The Shannon evenness index was calculated using the equation:

$S-E=H/lnS$,

where H is the Shannon index and S is the number of wells that had the color change.

SPSS17.0 was used to perform analysis of variance (one-way ANOVA), multiple comparisons test (Duncan), and principal components analysis (PCA) based on the optical density readings at 72 h.

A multivariate ordination method was used to analyze the relationship between microbial FD and environmental variables (CANOCO 4.5 software). To test whether weighted-averaging techniques or linear methods were appropriate, a detrended correspondence analysis (DCA) was performed. The longest gradients that resulted from the DCA were 0.112 (July) and 0.142 (September) for the analysis based on Eco-plates. Accordingly, redundancy analysis (RDA) was performed (Braak and Smilauer, 2002) to study the relationship between microbial communities and the environmental factors. All the environmental factors were tested to determine whether they correlated with the FD of the water microbial communities using the Monte Carlo permutation test ($P < 0.05$).

Results

Dynamics of the AWCD of the water microbial community. The AWCD value was determined at intervals of 24 h and increased throughout the incubation period. The dynamic varying traces of the AWCD values are shown in Fig. 1.

Fig. 1 Variation of average well color development (AWCD) in all carbon sources utilized by water microbial communities in different groups
C: monoculture of crab; CS: 6 crab, 45 shrimp/m²; CB: 6 crab, 15 clam/m²; SB: 45 shrimp, 15 clam/m²; CSB: 6 crab, 45 shrimp, 15 clam/m².

The amount of carbon sources used by the water microbial communities increased gradually as incubation time progressed in all the different groups. The AWCD value of the CSB group was significantly higher compared to the other groups in July ($P < 0.05$). In September, the CSB and SB had the highest AWCD compared to the other groups ($P < 0.05$). The AWCD value in CB was the lowest during the experiment. These results indicated that metabolic activity of the water microbial community in the CSB group was greater compared to the other groups, except for SB ($P < 0.05$).

Dynamic features of different types of carbon source. The utilization of various carbon sources in the Biolog-ECO plates by the water microbial communities from the different groups were further analyzed (Fig. 2).
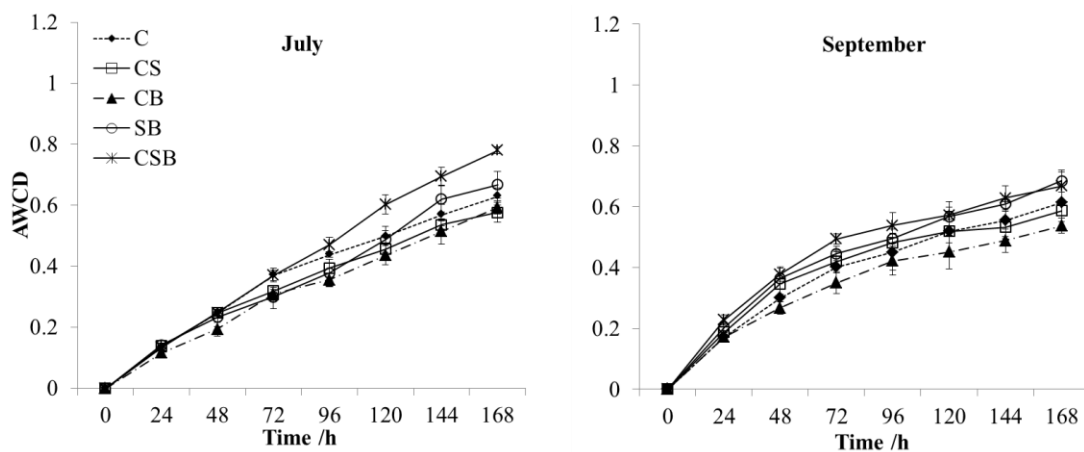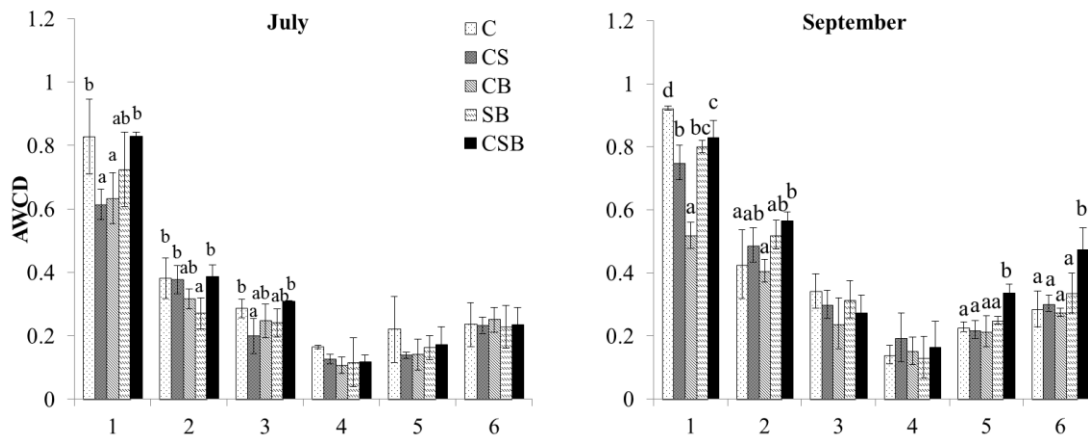

Fig.2 Variation of average well color development (AWCD) in different types of carbon sources utilized by water microbial communities in different groups
Note: Different letters indicate significant differences at P< 0.05.1: polymers, 2: carbohydrates, 3: carboxylic acids, 4: phenolic acids, 5: amines, 6: amino acids
C: monoculture of crab; CS: 6 crab, 45 shrimp/m²; CB: 6 crab, 15 clam/m²; SB: 45 shrimp, 15 clam/m²; CSB: 6 crab, 45 shrimp, 15 clam/m².

The water microbial communities utilized polymers to a greater extent as compared to the other five carbon sources during the experiment. In July, there were no significant differences in the utilization of phenolic acids, amines, and amino acids by the water microbial communities in any of the groups ($P > 0.05$). However, the water microbial communities in the C and CSB groups used significantly higher amounts ($P < 0.05$) of polymers (0.83 ± 0.12, 0.83 ± 0.01), carbohydrates (0.38 ± 0.06, 0.39 ± 0.04), and carboxylic acids (0.29 ± 0.03, 0.31 ± 0.00) than the communities in other groups.

In September, there were no significant differences in the utilization of carboxylic acids and phenolic acids by the water microbial communities in any of the groups ($P > 0.05$). However, the utilization of carbohydrates (0.57 ± 0.03), amines (0.34 ± 0.03), and amino acids (0.47 ± 0.07) by the water microbial communities in the CSB group were significantly higher compared to the others ($P < 0.05$). Generally, the carbon sources used by the water microbial communities were selective, but the selectivity changed with time.

Principal component analysis. PCA with a correlation matrix was used to evaluate the carbon source metabolization of the microbial community in a certain environment. The first two principal components (PC1 and PC2) were used to describe the information from carbon sources (Staley *et al*., 2012; Yan *et al*., 2013).

Table 2 shows the correlations between the different types of carbon sources used by the water microbial communities in the polyculture groups. In July, significant correlations were observed between utilization of the following: polymers and phenolic acids ($P < 0.05$); carboxylic acids and amines ($P < 0.01$); carbohydrates and phenolic acids ($P < 0.05$); and phenolic acids and amines ($P < 0.05$). In September, significant correlations were observed between utilization of the following: polymers and carboxylic acids ($P < 0.05$); carbohydrates and amines and amino acids ($P < 0.01$); and amines and amino acids ($P < 0.01$).

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Table 2 Correlation Matrix of different types of carbon sources utilized by water microbial communities in different groups

Time	Carbon type	1	2	3	4	5	6
July	1	1.000					
	2	0.131	1.000				
	3	0.702**	0.228	1.000			
	4	0.543*	0.447*	0.225	1.000		
	5	0.747**	-0.056	0.275	0.453*	1.000	
	6	-0.005	-0.231	-0.313	-0.234	0.322	1.000
September	1	1.000					
	2	0.348	1.000				
	3	0.491*	0.194	1.000			
	4	0.097	0.178	-0.132	1.000		
	5	0.348	0.611**	0.137	-0.033	1.000	
	6	0.308	0.715**	-0.202	0.198	0.795**	1.000

*Note: * correlation is significant at the 0.05 level, ** correlation is significant at the 0.01 level*
1: polymers, 2: carbohydrates, 3: carboxylic acids, 4: phenolic acids, 5: amines, 6: amino acids
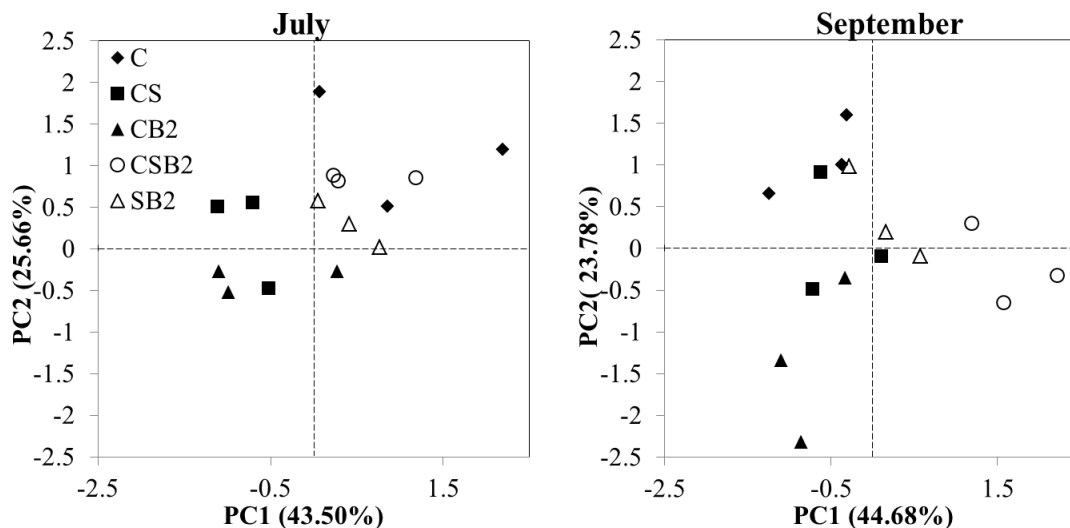
Fig. 3 Principal components analysis (PCA) of variation of carbon utilization profiles of water microbial communities in different groups
Note: C: monoculture of crab; CS: 6 crab, 45 shrimp/m²; CB: 6 crab, 15 clam/m²; SB: 45 shrimp, 15 clam/m²; CSB: 6 crab, 45 shrimp, 15 clam/m².

PC1 and PC2 are plotted against each other in Fig. 3 for illustration. In July, the variance analysis showed that component score coefficients of PC1 and PC2 were both significantly different in different groups ($P < 0.05$). PC1 and PC2 explained 43.50% and 25.66% of the data variance, respectively. There were significant differences between the C, CS and CB groups in PC1 and significant difference between the C and the CS, CB and SB groups in PC2 ($P < 0.05$). In September, the variance analysis showed that component score coefficients of PC1 and PC2 were both significantly different in different groups ($P < 0.05$). PC1 and PC2 explained 44.68% and 23.78% of the data variance, respectively. There were significant differences between CSB and the other groups in PC1 and significant differences between the CB and the C, CS and SB groups in PC2 ($P < 0.05$).

The carbon sources that were significantly correlated with PC1 and PC2 ($r^2 > 0.60$) in July and September are shown in Table 3.

Table 3. The main carbon sources significantly correlated with PC1 and PC2 in PCA of water microbial communities in different groups ($r^2 > 0.60$)

Carbon type	July		September	
	PC1	PC2	PC1	PC2
Polymers	0.927			
Carbohydrates		-0.604	0.855	
Carboxylic acids	0.716			0.901
Phenolic compounds	0.744			
Amines	0.716	0.609	0.862	
Amino acids		0.820	0.877	

Note: data was from Component Matrix of PCA.

Carbon sources such as polymers, carboxylic acids, phenolic compounds, and amino acids were intensively utilized by the water microbial communities in July, while carbohydrates, amino acids and carboxylic acids were more intensively used by the water microbial communities in September. Interestingly, the water microbial communities intensively metabolized amines both in July and September.

Changes in water microbial community diversity. Table 4 shows trends of the McIntosh index, Simpson index, Shannon index, and Shannon evenness index for the water microbial communities. In July, the McIntosh index of C and CSB were significantly higher compared to the others ($P < 0.05$). The Simpson index and Shannon index for SB were significantly lower compared to the others ($P < 0.05$). There were no significant differences in the Shannon evenness index between the groups ($P > 0.05$). In September, all of the diversity indices, except the Simpson index, were significantly higher in CSB compared to the others ($P < 0.05$).

Table 4. Variation of carbon utilization diversity indices of water microbial communities in different groups

Time	Water	McIntosh index	Simpson index	Shannon index	Shannon evenness
July	C	2.891 ± 0.113^b	0.937 ± 0.001^{bc}	3.075 ± 0.012^{bc}	0.907 ± 0.002^a
	CS	2.416 ± 0.112^a	0.941 ± 0.004^{bc}	3.094 ± 0.048^c	0.907 ± 0.016^a
	CB	2.310 ± 0.183^a	0.942 ± 0.002^c	3.105 ± 0.020^c	0.916 ± 0.004^a
	SB	2.452 ± 0.303^a	0.930 ± 0.004^a	2.993 ± 0.028^a	0.910 ± 0.028^a
	CSB	2.918 ± 0.118^b	0.936 ± 0.003^{ab}	3.042 ± 0.008^{ab}	0.898 ± 0.015^a
September	C	3.275 ± 0.305^b	0.930 ± 0.011^a	3.011 ± 0.090^a	0.879 ± 0.022^a
	CS	3.193 ± 0.245^b	0.942 ± 0.002^{ab}	3.097 ± 0.022^{ab}	0.905 ± 0.007^b
	CB	2.736 ± 0.134^a	0.936 ± 0.008^{ab}	3.071 ± 0.047^{ab}	0.909 ± 0.005^b
	SB	3.283 ± 0.106^b	0.943 ± 0.003^b	3.103 ± 0.020^{ab}	0.909 ± 0.002^b
	CSB	3.615 ± 0.059^c	0.947 ± 0.008^b	3.144 ± 0.008^c	0.921 ± 0.002^c

Note: Different letters in the same column indicate significant differences at P< 0.05.
C: monoculture of crab; CS: 6 crab, 45 shrimp/m^2; CB: 6 crab, 15 clam/m^2; SB: 45 shrimp, 15 clam/m^2; CSB: 6 crab, 45 shrimp, 15 clam/m^2

Environmental characteristics. Table 5 shows the variation between the different polyculture groups with respect to the water-related environmental factors. There was no difference in temperature and pH value among all the different groups throughout this study. In July, the TOC, TN, TP, and PO_4^{-3}–P for the CB group were significantly higher compared to the others ($P < 0.05$). The NH_4^+-N, NO_2^--N, and NO_3^--N values for the CSB group were significantly lower compared to the others ($P < 0.05$). C and SB had significantly lower Chl *a* than the other groups ($P < 0.05$). In September, the TOC and TP in the SB group were the highest compared to all the other groups ($P < 0.05$). TN and NH_4^+-N for CS group were higher compared to any of the other groups ($P < 0.05$). In contrast, NO_2^--N was significantly lower in the CS and SB than in the other groups ($P < 0.05$). The levels of NO_3^--N in C and CB, PO_4^{-3}–P in CS and CSB, and Chl *a* in SB were significantly higher than those for the other groups ($P < 0.05$).

		TOC /(mg/l)	TN /(mg/l)	TP /(µg/l)	NH$_4^+$-N /(µg/l)	NO$_2^-$-N /(µg/l)	NO$_3^-$-N /(µg/l)	PO$_4^{3-}$-P /(µg/l)	Chl$a$ /(µg/l)	T /(℃)	pH
July	C	8.04±1.11^{ab}	1.88±0.00^a	56.89±2.54^b	45.89±2.54^b	14.45±0.78^b	54.68±3.61^c	5.84±0.40^a	8.30±1.11^a	28.69±1.83	8.17±0.14
	CS	11.03±0.35^{bc}	1.76±0.13^a	46.85±2.12^a	67.40±4.02^c	15.89±1.08^b	48.76±1.39^b	6.33±0.34^a	10.49±1.02^b	28.69±1.83	8.23±0.15
	CB	15.11±4.51^c	2.17±0.14^b	90.36±4.36^d	46.66±8.66^b	14.45±1.70^b	43.44±2.44^b	9.74±1.64^b	11.43±1.37^b	28.69±1.83	8.11±0.08
	SB	13.76±1.31^c	1.83±0.02^a	58.57±3.57^{bc}	42.77±2.23^b	13.96±1.96^b	48.00±3.00^b	6.33±0.33^a	7.91±0.53^a	28.69±1.83	8.20±0.16
	CSB	6.68±0.55^a	1.93±0.06^a	63.59±2.59^c	25.28±4.28^a	9.73±0.83^a	25.37±4.37^a	7.30±0.40^a	10.05±0.27^b	28.69±1.83	8.14±0.12
Sept.	C	9.37±1.26^a	2.71±0.05^a	85.34±6.34^b	100.85±10.85^a	518.33±18.33^b	1068.38±61.62^c	15.10±1.10^a	7.15±1.72^a	23.10±1.31	8.12±0.11
	CS	10.11±0.37^a	3.87±0.10^e	88.69±9.69^b	367.86±17.86^d	391.01±11.01^a	655.85±25.85^a	37.01±2.01^c	6.44±0.55^a	23.10±1.31	8.13±0.14
	CB	8.19±0.66^a	3.20±0.08^c	58.57±7.57^a	109.92±0.08^a	535.67±14.13^b	1088.73±88.73^c	18.99±1.99^{ab}	13.07±1.28^b	23.10±1.31	8.27±0.29
	SB	13.01±3.10^b	3.01±0.01^b	155.62±25.62^d	194.95±14.95^b	435.70±25.70^a	673.78±33.78^a	23.37±2.37^b	19.52±2.11^c	23.10±1.31	8.20±0.17
	CSB	8.55±0.68^a	3.66±0.11^d	123.83±13.83^c	296.83±26.83^c	556.04±46.04^b	855.36±24.64^b	36.64±5.64^c	6.29±0.26^a	23.10±1.31	8.23±0.25

Table 5. Variation of water environmental factors in different groups
Note: Different letters in the same column indicate significant differences at P< 0.05.
C: monoculture of crab; CS: 6 crab, 45 shrimp/m^2; CB: 6 crab, 15 clam/m^2; SB: 45 shrimp, 15 clam/m^2; CSB: 6 crab, 45 shrimp, 15 clam/m^2.
TOC: total organic carbon; TN: total nitrogen; TP: total phosphorus; NH$_4^+$-N: ammonia nitrogen; NO$_2^-$-N: nitrite nitrogen; NO$_3^-$-N: nitrate nitrogen; PO$_4^{-3}$-P: active phosphate; Chl a: the chlorophyll a; T: temperature.

Redundancy analysis. RDA enables the identification of the environmental variables that could explain variation in the carbon metabolism of microbial communities (van Dobben *et al.*, 2001).

The eigenvalue associated with each axis and the carbon metabolic function-environment correlations are indicators of the extent to which the environmental variables explain the species responses (González *et al*. 2003). Table 6 shows the results obtained for the first two axes of the RDA in July and September. The RDA indicated that the first two axes explained 94.8% of the total variance in July and 96.0% of the total variance in September.

Table 6. Statistical summary of RDA for water microbial communities

	July		September	
	1	*2*	*1*	*2*
Eigenvalues	0.841	0.107	0.726	0.234
Cumulative percentage variance of species-environment relation	84.1	94.8	72.6	96.0

Table 7 shows the canonical coefficients of the environmental variables that were significantly correlated with the FD of the water microbial communities using the Monte Carlo permutation test (*P* < 0.05).

Table 7. Canonical coefficients of environmental variables with the first two axes of RDA for water microbial communities

Environmental variables	**July**		September	
	1	*2*	*1*	*2*
NH$_4^+$-N	-0.6339	-0.5188	-0.4211	-0.4032
NO$_2^-$-N	-0.6364	-0.2359		
TN			-0.0800	-0.6458
PO$_4^{3-}$-P	-0.4314	0.0585	-0.4157	-0.5772
TP			-0.7243	-0.1622
Chl *a*	-0.4088	-0.5792		
TOC	-0.9156	0.3318	-0.2885	0.3137

Note: NH$_4^+$-N: ammonia nitrogen; NO$_2^-$-N: nitrite nitrogen; NO$_3^-$N: nitrate nitrogen; TN: total nitrogen; PO$_4^{3-}$-P: active phosphate; TP: total phosphorus; Chl a: the chlorophyll a; TOC: total organic carbon.

In July, all of the environmental variables were negatively correlated with the first axis; and the ranking was TOC > NO$_2^-$-N > NH$_4^+$-N > PO$_4^{-3}$-P > Chl *a*. The second axis was negatively correlated with all of the environmental variables except PO$_4^{-3}$-P and TOC;

the ranking was Chl a > NH_4^+-N > TOC > NO_2^--N > PO_4^{-3}-P. Thus, in July, the most correlated environmental variable was TOC, followed by NO_2^--N and NH_4^+-N. These variables also had the closest association with the Shannon evenness index (positive), McIntosh index (negative), polymers (negative), carboxylic acids (negative), and amines (negative) (Fig. 4).
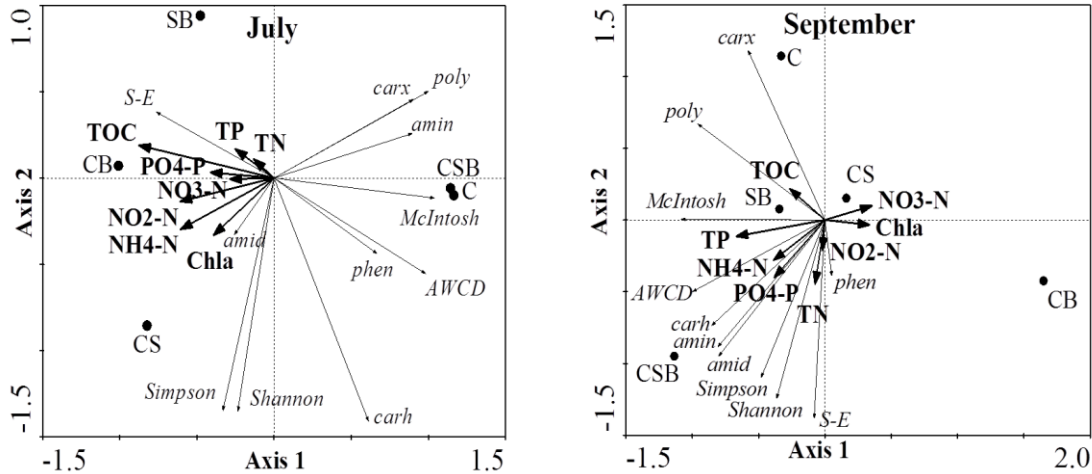

Fig. 4 Redundancy analysis (RDA) of variation of carbon metabolic function of water microbial communities in different groups with environmental variables.
Note: poly: polymers, carh: carbohydrates, carx: carboxylic acids, phen: phenolic acids,
 amin: amines, amid: amino acids
C: monoculture of crab; CS: 6 crab, 45 shrimp/m²; CB: 6 crab, 15 clam/m²; SB: 45 shrimp, 15 clam/m²; CSB: 6 crab, 45 shrimp, 15 clam/m².
TOC: total organic carbon; TN: total nitrogen; TP: total phosphorus; NH_4-N: ammonia nitrogen; NO_2-N: nitrite nitrogen; NO_3-N: nitrate nitrogen; PO_4-P: active phosphate; Chl a: the chlorophyll a.

In September, the first axis was negatively correlated with all of the environmental variables, and the ranking was TP > NH_4^+-N > PO_4^{-3}-P > TOC > TN. The second axis was also negatively correlated with the environmental variables, excluding the TOC, and the ranking was TN > PO_4^{-3}-P > NH_4^+-N > TOC > TP. Thus, in September, the most correlated environmental variable was TP, followed by NH_4^+-N and PO_4^{-3}-P. These variables also had the closest association with AWCD (positive), carbohydrates (positive), Shannon evenness index (positive), amines (positive), amino acid (positive), and McIntosh index (positive) (Fig. 4).

Discussion

In this study, the carbon sources utilized, and metabolic activity of the microbial communities in the tri-species polyculture containing crab, shrimp, and clam (CSB) were significantly higher than those in the other groups ($P < 0.05$, Fig.1). There were no significant distribution differences observed across the groups in the PC axes during the initial period in July. However, in September, significant differences were detected along the distribution of the PC1 axis in the CSB group compared to those in the other groups. The PCA results indicated that components of the microbial communities from different groups were constantly changing. Finally, the carbon sources utilized and metabolic activity of the water microbial communities of CSB were obviously different from those of the other groups. The carbon sources utilized and the metabolic activity of the water microbial communities from a polyculture system of *Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix,* and *Cyprinus carpio* were significantly higher than those in other systems (Tian *et al*., 2012). The results of the current study could be explained by optimal structure and function of the microbial community in the group with crabs, shrimps, and clams. This could indicate better energy flow and material cycle in the water, leading to the enrichment and diversity of microbial communities.

In the present study, in July, the McIntosh index of the CSB group was significantly higher than that of the other groups ($P < 0.05$), while the Simpson index and the Shannon index were lower than those of the others ($P < 0.05$). However, there were no significant differences in the Shannon evenness index for the groups. In contrast, all the diversity indices, except the Simpson index for the CSB group, were significantly higher ($P < 0.05$) than those for the other groups in September (Table 3). This was consistent with previous findings by Zhang, who found that the FD of soil microbes and the number of vegetation species were not entirely positively correlated (Zhang *et al*., 2013). Similar findings were reached by others (Rodríguez-Loinaz *et al*., 2008). One possible explanation is that different organisms may have different effects on the microbial community. Additionally, the carbon sources in the BIOLOG plates do not accurately represent the substrates present in ecosystems (Gomez *et al*., 2006), and thus provided a wide set of compounds that allowed estimation of relatively potential metabolic versatility (Gomez *et al*., 2004). Both of these factors could have led to changes in the microbial community diversity indices in this study.

As a reflection of environmental factors, the diversity of the microbial community was closely related to the physicochemical properties of the environment, and they influenced each other (Gomez *et al*., 2006). The RDA analysis showed that there were obvious distribution differences between July and September in the different groups, especially in the CSB group. In July, both the crab monoculture (C) and the CSB groups were clustered together along the positive trend of the first axis. In contrast, the other groups were distributed in a negative trend along the first axis. When the culture ended in September, the crab monoculture was distributed in a positive trend on the second axis. However, the CSB group was distributed in a negative trend on the second axis and no longer clustered with the C group. The other groups were distributed between the C and CSB groups on the RDA axes (Fig.4). This suggested that the composition and metabolism of the microbial community in the CSB group were mostly different from those in the crab monoculture. It also demonstrated that the water microbial community has a unique model of carbon utilization in the tri-species polyculture, with the highest capacity for carbon utilization.

The RDA analysis showed that the critical environmental variables influencing the functional diversity of the water microbial communities were TOC, NO_2^--N, and NH_4^+-N in July, and TP, NH_4^+-N and PO_4^{-3}–P in September. It indicated that the importance of influential variables varied over time during the experiment. Among them, nitrogen was the primary factor affecting microbial community metabolism throughout the experiment. TOC was a significant environmental variable for the FD of the microbial community in the early stage, however, it was replaced by phosphorus in September in the later period. Other studies showed similar findings (Li *et al*., 2014). Nitrogen has direct effects not only on the composition and distribution of microbial community (Bouvy *et al.*, 2011), but also the "consumers" of microorganism (flagellates and ciliates), and thus affect microbial community metabolism indirectly as well (Nakano *et al.*, 1998). The ammonium state of nitrogen, NH_4^+-N was the critical factor affecting microbial community metabolism both in July and September. The possible explanation is that microorganisms prefer utilizing NH_4^+-N which constituted 55%-99% of the total nitrogen uptake by microorganisms (Jorgensen *et al.*, 1999). Organic carbon could affect the growth and enzyme activities of microorganism, and therefore also microbial community metabolism (Findlay *et al*., 2003; Grossart 2010). TOC was the other factor affecting microbial community metabolism in July. It might be attributed to lack of organic carbon caused by reduced feed given in the earlier stage of experiment. TP and PO_4^{-3}–P were factors affecting microbial community metabolism (Nakano *et al.*, 1998; Bouvy *et al.*, 2011). They replaced TOC and were more important to water microbial communities in September. The amount of phosphorus in water may be insufficient due to its low solubility and mobility (Yuan *et al*., 2011) compared to carbon derived from the accumulation of organic matter such as residual feed, feces and biological residues.

Conclusions
In summary, the tri-species polyculture of swimming crab, white shrimp, and short-necked clam not only enhanced carbon utilization capacity of water microbes, but also improved metabolic activity and functional diversity of microbial community. The environmental variables significantly correlated with the functional diversity of the water microbial communities were total organic carbon, NO_2^--N and NH_4^+-N in July, and total phosphorus, NH_4^+-N and PO_4^{-3}–P in September. The present study also indicated that BIOLOG technology was able to detect changes in the structure and function of microbial communities in water and provided a foundation for establishing micro-ecology protocol for swimming crab polyculture. Furthermore, by identifying environmental variables influencing the FD of the microbial community through RDA analysis, it is convenient and beneficial for aquaculturists to recognize, monitor, and control the microbial functional diversity according to different culture stages in ponds.

Acknowledgements

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