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Full Length Research Paper

Distribution of pathogen in the Bohai sea in spring and summer

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The aquicultural pathogen *Vibrio* spp. is popular and harmful to mariculture animals and even resulted in human enterogastrtis. However, little is known about the abundance and distribution of marine pathogen in Bohai Sea. In the present study, the distributions of the typical pathogens, including *Escherichia Coli*, *Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio fluvialis* and *Vibrio harviyi*, were investigated using protein micro array method from the Bohai Sea samples, which collected in spring and summer in 2005, respectively. The results showed that: (1) Temporally, the tested typical pathogens were more abundant in summer than in spring, as supported by the total pathogenic *Vibrio*s averaged 3.05×10^4/L in spring while 2.48×10^5/L in summer; (2) Spatially, in summer, pathogenic *Vibrio*s in Bohai Bay was 4.87, 10.52 and 7.15 times higher than that in Liaodong Bay, Laizhou Bay and Central Bohai Sea, respectively (p = 0.034, 0.013 and 0.012, respectively). (3) Total pathogenic *Vibrio*s in coastal area was 4.68 times higher than that in central area ($p = 0.0279 < 0.05$), showing a decline trend in abundance. (4) All the pathogenic *Vibrio*s varied between spring and summer, with greatest variance in *V. fluvialis*. Both *V. parahaemolyticus* and *V. harveyi* had no significant variances. Bohai Bay was heavily polluted and relatively not fit for mariculture. *V. fluvialis* dominated in Bohai Sea and was a possible major pathogen of vibriosis.

Key words: Bohai sea, protein micro array, pathogenic *Vibrio.*

INTRODUCTION

There are more than ten strains of aquicultural pathogen bacteria, which is popular and harmful to maricultural animals. It is familiar to us that the typical strains including *Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio fluvialis, Vibrio harviyi* are pathogens and even the killers on marine products animals, for example, *V. fluvialis* can lead to fish, shell toxemia and blood poisoning. Furthermore, pathogenic *Vibrio*s even resulted in human enterogastrtis. The pathogen stain *Escherichia coli*, firstly detected in 1885, makes headlines for its varied roles in food poisoning, drug manufacture and biological research. Thus, the aquicultural pathogens *Vibrio* spp. and *E. coli* were the potential dangers for aquicultural industry and the pollution of marine environments. For environmental monitoring, detecting the abundance of acquicultural pathogens *Vibrio* spp. and *E. coli* in the littoral water exhibits a great importance. Previously, only several publication have reported the presence of *Vibrio* spp along the Gulf Coast (Kelly, 1982), West Coast (Kaysner et al., 1987; Jiang, 2001), and East Coast of the United States (Oliver et al., 1991; 1983; Weidchart et al., 1992; Xu et al., 1982; Tamplin et al., 1982) and in coastal waters of Denmark (Hoi et al., 1998), Hong Kong (Chan et al., 1986) and Japan (Aono et al., 1997; Oonaka et al., 2002). Unfortunately, the investigations of the pathogens are still in fancy and this make theirs abundance and distribution unclear in diverse marine environments.

The Bohai Sea is the largest inner sea of China (117.7 - 122.1°E and 37.2 - 40.8°N), with a total area of 77,000 km^2 and an average depth of 18 m (the maximum water depth is about 70 m at the north of the Bohai strait) and is divided into four parts with typical features: the Liaodong

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Bay, the Bohai Bay, the Laizhou Bay and the Central Bohai Sea (Figure 1). The Bohai Sea is the important base of the Chinese aquicultural industries. So the investigation of the pathogens is urgent work for the development in a healthy of the local aquicultural industry and could also provide a better understanding of the marine environmental pathogens.

Protein micro array is a rapid, high-performance, high-throughput solution for proteomics research (Eisenstein, 2006; Hurst et al., 2009) and is widely used (Izutsu et al., 2008; Berger and Bulyk, 2009). The objective of the present study was to investigate the distributions of five typical pathogens, including *E. coli, V. parahaemolyticus, V. alginolyticus, V. fluvialis* and *V. harviyi* with the purposes of monitoring the quality of marine seawater and providing the environmental information for the local aquicultural industry in the Bohai Sea.

MATERIALS AND METHODS

Sampling area and sampling sites

Two cruises of "HaiJian 21" were carried out in the Bohai Sea in March - April and June - July, respectively. The 46 sample stations planned distribute in alongshore and central of Bohai including 4 sections (Figure 1). Water at depth of 1 m was collected by an auto sampling system. 2 L water was filtered through a positive charged membrane. The membranes were washed by 2 ml PBS buffer and the elution was used for analysis stored at 4℃.

Detection of pathogens by protein micro-array

***Buffers* and *reagents*.**

PBS (phosphate buffered saline, 137 mM NaCl, 10 mM phosphate, ph 7.4) was used for dilutions. PBS-T (0.1%v/v Tween-20) was used for washes and PBS-TB (0.1% v/v Tween-20, 5% BSA (bovine serum albumin)) for slide blocking. BSA was purchased from Yuanhengshengma biotechnology research institute, Beijing, China. All other chemicals were A.R. and made in China.

Protein micro array

Antibodies and antigens: Antibodies used in these studies include rabbit anti-*E. coli* pAb (polyclonal antibody), rabbit anti-*V. fluvialis* pAb, rabbit anti-*V. harviyi* pAb, rabbit anti-*V. alginolyticus* pAb and anti-*V. parahaemolyticus* pAb. Five strains of bacteria antigens and the corresponding rabbit polyclonal antibodies were obtained, which were supplied after the pretreatment of bacterial adsorption to prevent nonspecific absorption. Cy3 was purchased from Amersham to label antibodies.

Protein micro array was performed as previously reported (Rowe et al., 1999; Stokes et al., 2001; Rao et al., 2004; Delehanty and Ligler, 2002; Howell et al., 2003). To prevent nonspecific adsorption to regions between the antibodies arrays, Tween-20 were used as backfilling agents. The concentration of Tween-20 was 0.1% (v/v). The concentration of the original stock suspension of bacteria was 1.0 × 108 cfu/mL. Before incubation with the antibody array, the bacteria suspension was diluted in different proportion with PBS. To determine the magnitude of cross-reaction in the bacteria-antibody system used for this study, protein micro arrays printed with five strains of bacteria were exposed to five rabbit-antibodies respectively.

Image obtaining

A Leica DML microscope fluorescence microscope was used to verify the performance of the immunoassay before utilizing more precise techniques at magnifications 100/ 200/ 400 /1000 to investigate the emergence of detectable patterns on the substrates after exposure to bacteria. An attractive feature of this simple approach is that patterns of adsorbed bacteria can be seen directly both before and after the incubation with antibody solution.

All micro arrays were imaged using a scan array lite micro array scanner (Packard BioScience) equipped with a 532 nm laser with 10 μm pixel resolutions. The fluorescence intensity of the two-dimensional array of spots was determined using the scan array express micro array analysis system (version 2.1, Packard BioScience).

Safety consideration

Appropriate safety precautions were exercised when handling bacterial preparations. Cleaning and coping with the slides was performed in a chemical hood by personnel wearing acid-resistant gloves and appropriate personal protective gear. All suspensions containing analyses were handled in the same conditions and stored in 4℃ for not more than one week prior to use. All equipment, benchtops, etc., exposed to these suspensions were disinfected with a 20% bleach solution and were rinsed with distilled water. Analyte suspensions were also treated with bleach (20%) and were rinsed down the sink with excess water. Contaminated disposables (test tubes, pipet tips etc.) were placed in closed bags and later sterilized before discarded.

RESULTS

Bacteriological counts were obtained from a total of 20 sampling stations in spring cruise and from 44 sampling stations in summer cruise, respectively (Figures 2 and 3).

Seasonal variability

Temporally, the abundance of the five typical pathogens in summer was higher than that in spring. The total pathogenic *Vibrio*s averaged 3.05 × 10^4/L in spring and 2.48 × 10^5/L in summer, respectively. The difference in the total pathogenic *Vibrio*s between spring and summer is significant ($p < 0.05$). Occurrence of *E. coli* and *Vibrios* enumerated in 1 L water according to sampling sites was showed in Figures 2, 3 and Table 1. Generally, the abundances of all pathogenic *Vibrio*s were relatively low and close to detective limit. Stations of BH6, BH15, BH16 and BH37 had relatively higher abundance of pathogenic V*ibrio*s (5 × 10^4/L). No pathogenic *Vibrio*s could be detected at station BH10 and BH12 (Figure 2). As showed in Figure 3, the highest abundance of *V. fluvialis* was found at station BH2 (2.00 × 10^6/L). In summer, *Vibrios* of all stations averaged 3.02 × 10^4/L, 1.43 × 10^4/L,

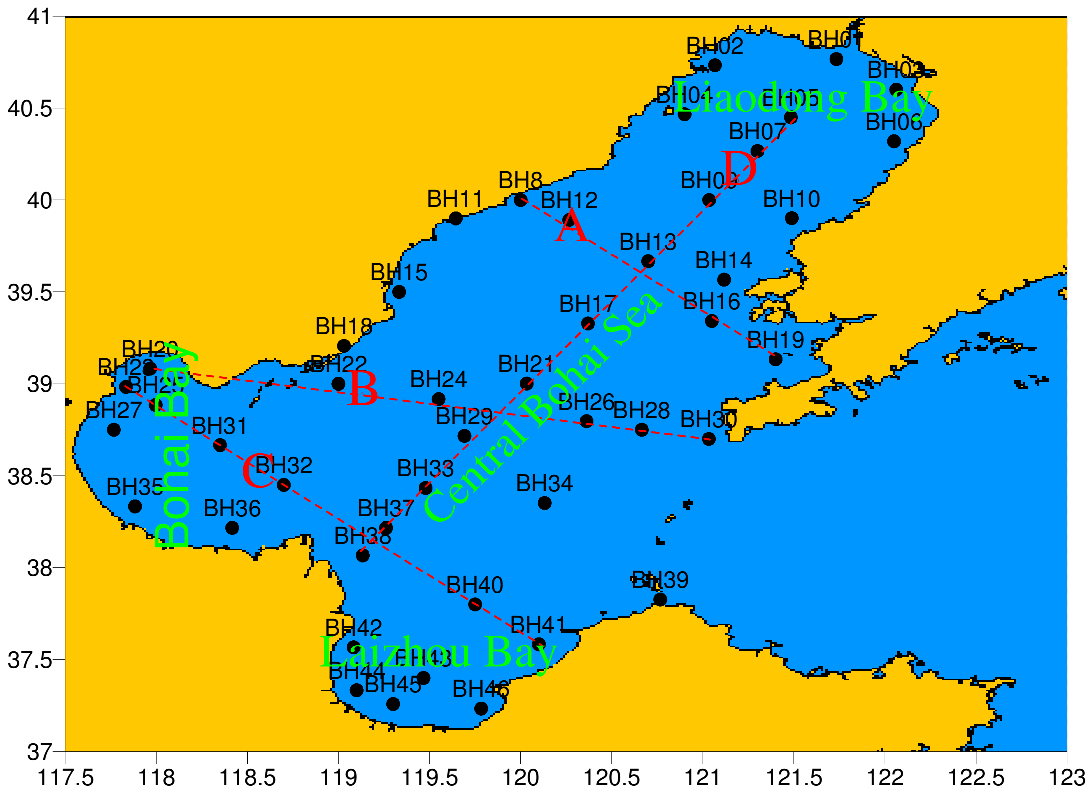

Figure 1. Sample sites in the Bohai sea.

3.45 × 10^4/L,1.43×10^5/L and 2.57×10^4/L, respective. Station BH23 had the highest abundance of pathogenic *Vibrio*s. No pathogenic *Vibrio*s could be detected at station BH16.

Distribution of pathogen in Bohai sea

In summer, spatially, pathogenic *Vibrio*s in Bohai Bay were 4.87, 10.52 and 7.15 times higher than in Liaodong Bay, Laizhou Bay and Central Bohai Sea, respectively ($p = 0.034$, 0.013 and 0.012, respectively). Total pathogenic *vibrio*s in coastal area was 4.68 times higher than that in central area ($p = 0.0279$), showing a decline trend in abundance. The difference among Liaodong Bay, Laizhou Bay and Central Bohai Sea is not significant.

Comparison of *vibrio*s between two cruises

All the pathogenic *vibrio*s varied in both spring and summer, with greatest variance in *V. fluvialis*. Both *V. parahaemolyticus* and *V. harveyi* had no significant variances. The average abundance of different *Vibrio*s showed great variations between spring and summer except for *V. parahaemolyticus* and *V. harviyi*. *V. fluvialis* had the greatest abundance among all the five pathogens (Figure 4).

Discussion

The highest concentration of *V. vulnificus* is 5.14 × 10^3CFU/100 ml in September in Mexico estuary (Lipp et al., 2001). Based on individual stations, the maximum concentrations at 66.7% of the stations were recorded in September (values ranged between 2.00 × 10^3 and 1.90 × 10^4 CFU/100 ml). In Southern California, the highest density of *V. cholerae* was found in San Diego Creek with a concentration of 4.25 × 10^5CFU/L (Jiang et al., 2001). In the present study, the highest concentration of *V. fluvialis* reached 2 × 10^6/L. Other pathogens can almost get to 10^5/L, as compared with other place, implying that serious pollution in Bohai Sea. The total pathogenic *Vibrio*s in coastal area were higher than that in central area, showing a decline trend in abundance (Figure 5). The distributions of pathogens were mostly determined by the distance to the shore and the estuary.

From our results, the pathogenic *Vibrio*s correspondingly showed great variation in season distribution and the total of five pathgonenic bacteria were more abundant in summer than that in spring. As previous report, the distributions of *Vibrio* spp. are related with biological and non-biological factors such as temperature and salinity (Ravel et al., 1994). In Bohai Sea, the surface temperature is 7.607 - 8.825℃ in spring while 24.18 - 27.24℃ in summer (Sun et al., 2004), this make temperature a most important factor to the distributions.

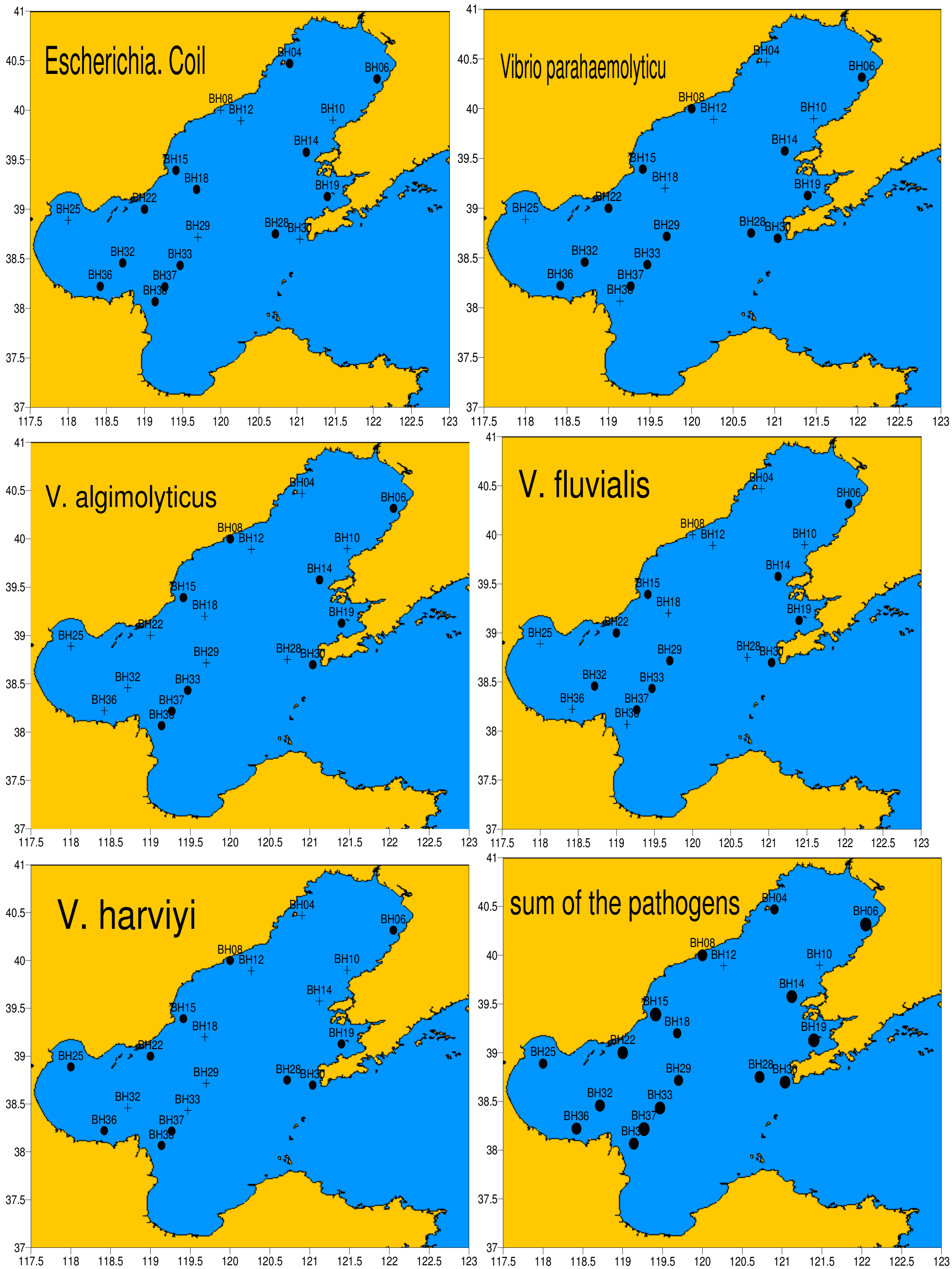

Figure 2. Distribution of pathogens and sum of the pathogens in spring cruise.

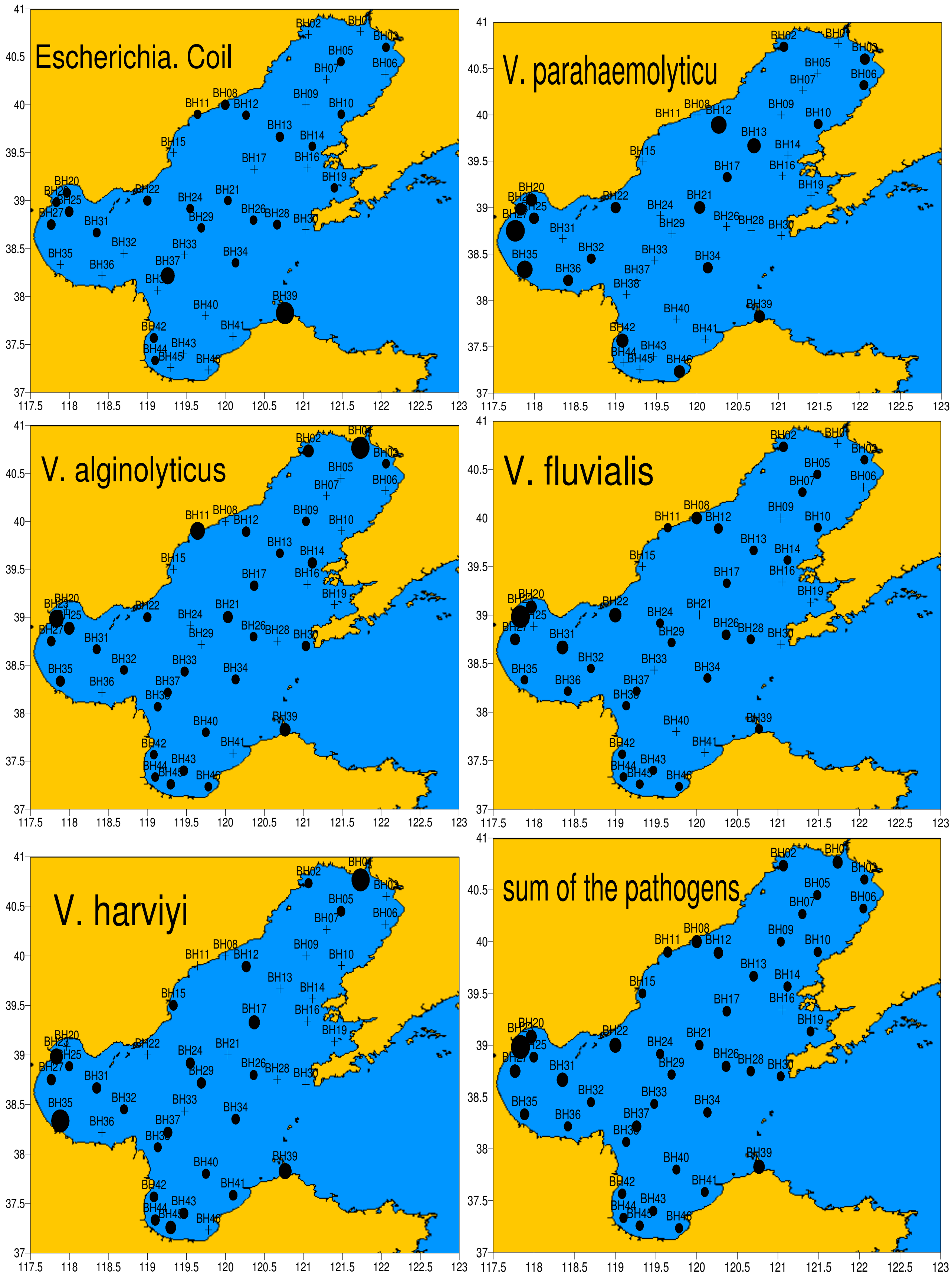

Figure 3. Distribution of pathogens and sum of the pathogens in summer cruise.

Table 1. Detected stations and percentage of pathogens in two cruises.

Pathogens	Spring cruise			Summer cruise		
	Detected stations	Percentage (%)	Mean of all stations	Detected stations	Percentage (%)	Mean of all stations
E. Coil	14	70	7.0×10^3/L	25	56.82	3.02×10^4/L
V. Parahaemolytucus	14	70	7.0×10^3/L	20	45.45	1.43×10^4/L
V. alginolyticus	10	50	5.0×10^3/L	30	68.18	3.45×10^4/L
V. fluvialis	11	55	5.5×10^3/L	32	72.73	1.43×10^5/L
V. harviyi	12	60	6.0×10^3/L	25	56.82	2.57×10^4/L

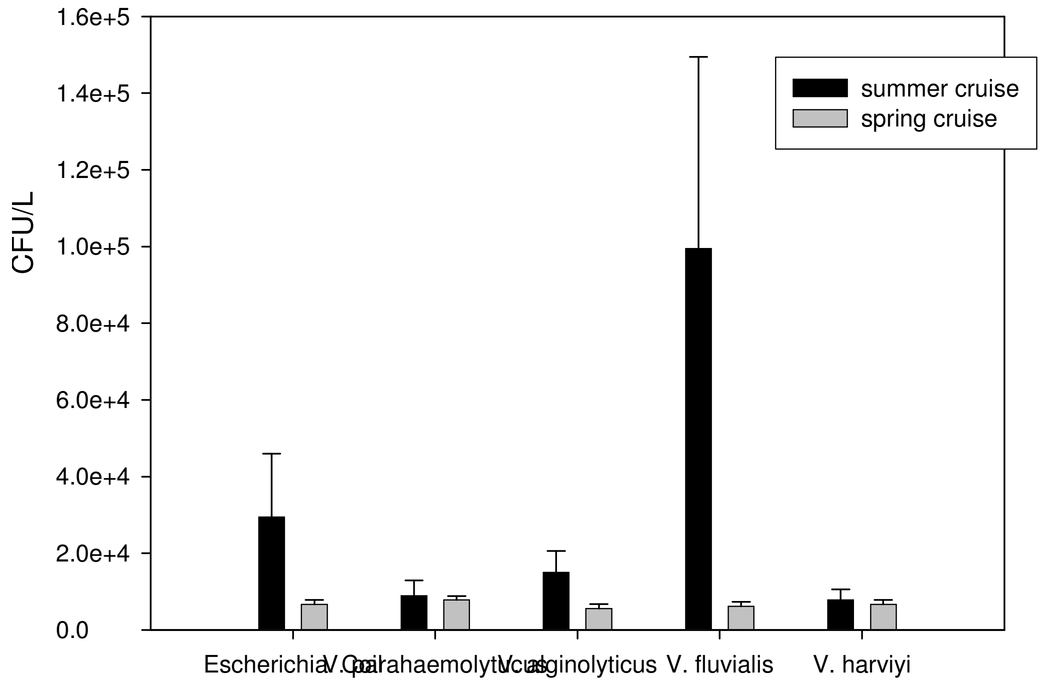

Figure 4. Pathogens in number between the spring and the summer cruise.

On the other hand, the salinity in Boahi Sea ranged from 25.15 to 31.15 % in four seasons and exhibited a trend of increasing from coastal area to central area (Gao et al., 2003). Considering pathogenic *Vibrio*s the favorite moderate salinities, we suppose the salinities a potential important factor to the occurrence and distribution of the pathogens.

In response to decreasing temperature, *Vibrio*s, including *V. cholerae* (Xu et al., 1982), *V. vulnificus* (Nilsson et al., 1991; Brauns et al., 1991; Linder and Oliver, 1989; Whitesides and Oliver, 1997) and *V. parahaemolyticus* (Jiang and Chai, 1996), may enter a viable but non culturable (VBNC) state in estuarine waters which at times reached a temperature of 18℃ (Oliver et al., 1991; Xu et al., 1982; Oliver et al., 1995). However, *Vibrio* spp. could be detected at the Bohai Sea in spring (under 10℃) using protein micro arrays suggesting that *E. coli* and pathogenic *Vibrio*s can inhabit this environment throughout the spring or even the year. Oliver et al. (1995) demonstrated this phenomenon in situ in an estuarine environment by use of the direct enumeration method. However, a small number of pathogenic *Vibrio* pp. has been detected from sea water at temperatures of around 8℃ using a protein micro array technology which possibly could not be detected as CFU. These findings suggest that both organisms can survive in sediment, shellfish and planktonic species in the cold-weather months and later drift from sediment into the water. While in summer the pathogenic *Vibrio*s bloom at high temperature and plentiful nutrition water environment. Future surveys about pathogenic *Vibrio*s would include specific shellfish and planktonic popuatio-l ns. There can also measure the cell viability by CTC or by flow cytometry to know its pathogenicity. These results suggested that a few cells of pathogenic *Vibrio*s in estuarine waters can survive at temperatures below

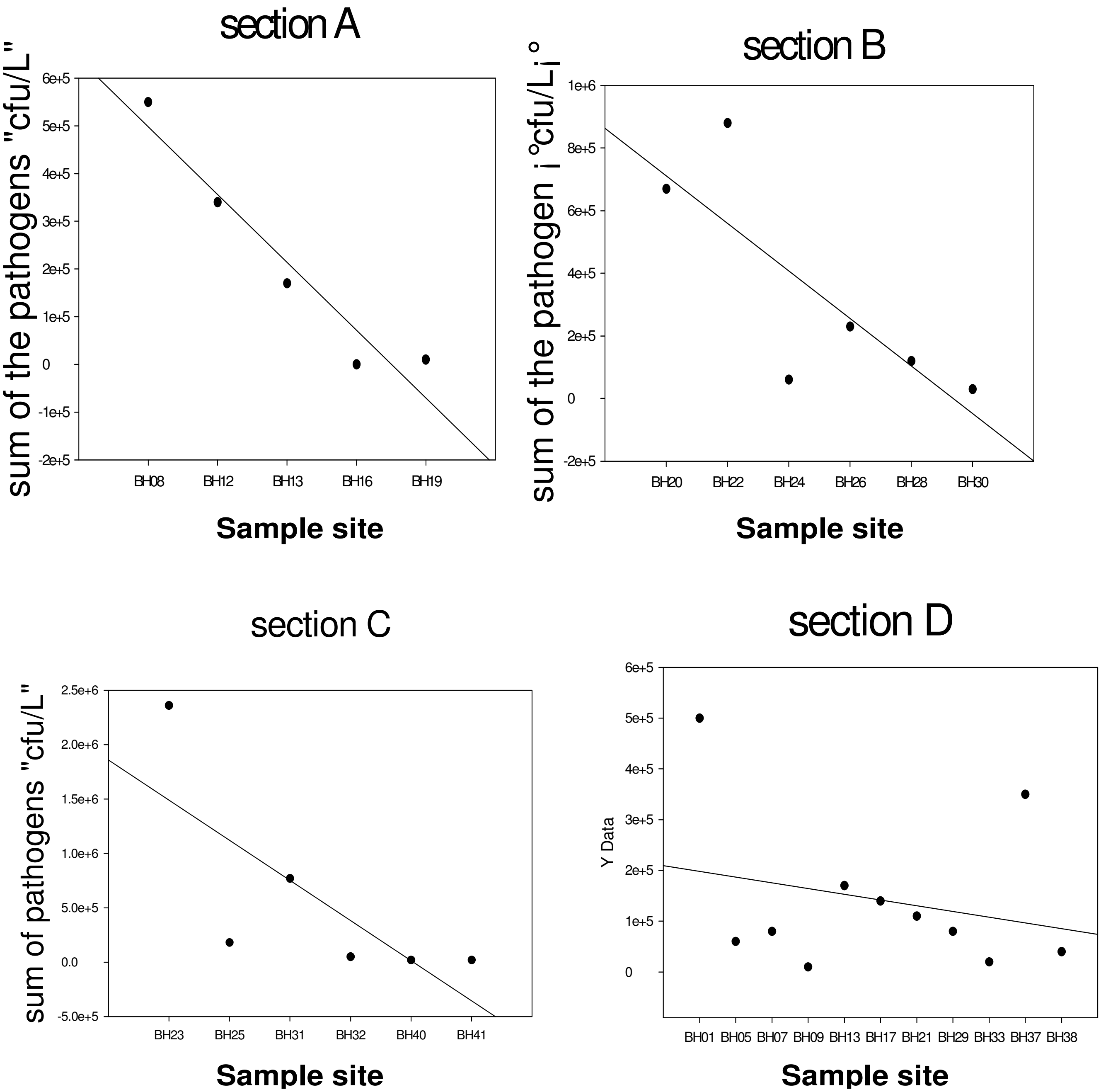

Figure 5. Total pathogens in sample stations of section A, B, C, D.

those previously reported and confirmed that their occurrence in water is strongly correlated with water temperature and salinity, as reported by other researchers (Hoi et al., 1998; Oonaka et al., 2002; O'Neill et al., 1992). However, most of the populations of pathogenic *Vibrio*s died from cold stress and starvation in the cold weather period. This phenomenon was supported by the results that salinity and temperature are interdependent and that lower temperatures may increase the tolerance of *V. vulnificus* to higher salinities (Kaspar, 1993).

Briefly, the present study investigated the distributions of *E. coli, V. parahaemolyticus, V. alginolyticus, V. fluvialis* and *V. harviyi* in Bohai Sea, and this provide the indicator of the sea water quality and referenced background for the local aquicultural industry.

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