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Investigation on the differences of accumulating *Escherichia coli* in three types of shellfish species, involving in the environmental factors

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ABSTRACT

This study investigated accumulation of *Escherichia coli* and aerobic colony count in three types of shellfish species. The results indicated that the capability of accumulating *E. coli* and aerobic colony count for *Sinonovacula constricta* was stronger than that for *Meretrix meretrix* and *Tegillarca granosa*, and capability of accumulating *E. coli* for *M. meretrix* was slightly stronger than that for *T. granosa*. However, no significant difference was observed in the capability of accumulating aerobic colony count between *M. meretrix* and *T. granosa*. Moreover, accumulation of *E. coli* in *S. constricta* is affected by contaminated seawater and *E. coli* were accumulated much faster and more in *S. constricta* when the seawater contaminated more serious. Meanwhile, the results suggested that the populations of *E. coli* in *S. constricta* changed in accordance with the weather. This is the first study to investigate the differences of accumulating *E. coli* in three types of shellfish species.

1. Introduction

Shellfish are healthy foodstuffs, not only low in calories, rich in unsaturated fats and proteins, but also delicious and low in price. Therefore, shellfish are widely consumed all over the world. However, shellfish are filter feeders, which are able to accumulate bacteria from contaminated waters and may act as passive carriers of human pathogens (Silva et al., 2003; Pereira et al., 2006; De Donno et al., 2008; Kay et al., 2008; Francesco et al., 2013). The ingestion of shellfish contaminated with pathogens may cause a severe disease (Brasher et al., 1998), and which can even be fatal.

European Union (European Parliament, 2004; Campos et al., 2013) and People's Republic of China Ministry of Agriculture-Fisheries Bureau require monitoring of the shellfish breeding sea field annually, and based on an evaluation of sanitary survey carried out subsequent classification of shellfish harvesting areas. They have stated quantities of *Escherichia coli* monitored in shellfish flesh as primary indicator for classification, which decides the means of post-harvest treatment required before shellfish sales. Classification has played important roles in providing an indication for the overall microbial quality of shellfish growing waters and in reducing the incidence of bacterial illnesses from the consumption of contaminated shellfish.

Up to now, classification had been carried out for more than a decade in China. However, the capability of accumulating microbes in different types of shellfish species and relevance research of their growing waters were not investigated. The objective of this study was to evaluate the differences of accumulating *E. coli* in three types of shellfish species (*Tegillarca granosa*, *Sinonovacula constricta* and *Meretrix meretrix*), and explore the environmental factors associated with accumulating *E. coli* in *S. constricta*. Furthermore, an open *S. constricta* bed in southern China had been chosen to investigate the influence of persistent rainfall on accumulating *E. coli*.

2. Materials and methods

2.1. Bacteria strain and media

The strain of *Escherichia coli* ATCC 8739 used in this study was obtained from China Center of Industrial Culture Collection. Lauryl Sulfate Tryptose Broth (LST, pH 6.8) contained (g/L) tryptone, 20.0; NaCl, 5.0; lactose, 5.0; KH_2PO_4, 2.75; K_2HPO_4, 2.75; sodium dodecyl sulfate, 0.1. *E. coli* Broth (EC, pH 6.9) contained (g/L) tryptone, 20.0; No.3 bile salt, 1.5; lactose, 5.0; KH_2PO_4, 1.5; K_2HPO_4, 4.0; NaCl, 5.0. Eosin-Methylene Blue Agar (EMB, pH 7.1) contained (g/L) peptone, 10.0; lactose, 10.0; K_2HPO_4, 2.0; agar, 15.0; eosin γ, 0.4; methylene blue, 0.065. Nutrient Agar (NA, pH 7.3) contained (g/L) beef extract, 3.0; peptone, 5.0; agar, 15.0. Plate Count Agar (PCA, pH 7.0) contained (g/L) tryptone, 5.0; yeast extract, 2.5; glucose, 1.0; agar, 15.0.

LST, EC, EMB and NA were used for *E. coli* culture, PCA used for aerobic colony count. All media were sterilized at 121 °C for 20 min. The biochemical characteristics of *E. coli* were identified by using HBIG13.

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All media and HBIG13 were purchased from Qingdao Hope Bio-Technology Co., Ltd. All other chemicals used in this study were routine reagents.

2.2. Shellfish sample processing

Shellfish were washed with clean tap water, and then surfaces were scrubbed by 75% sterilized cotton ball. Subsequently, shells were opened with a sterile forceps. 25 g of shellfish flesh was collected and placed separately in sterile lab blender bags containing 225 mL of phosphate buffered saline (PBS), which was subsequently homogenized at 10 Hz, 2 min with germfree homogenizer (JN-400i, Ningbo Jiangnan Instrument Factory). Such samples were collected for bacterial analysis.

2.3. Analytical methods

E. coli were analyzed using the 3-tube most probable number method (MPN) in accordance with National Standards of P. R. C. (GB 4789.38-2012) and the FDA's Bacteriological Analytic Manual (FDA, 1998) with a slight modification (Kumar et al., 2005). Three suitable gradients of dilution homogenate were selected and separately inoculated into three tubes of LST at 37 °C for 24–48 h. Durham tubes showing a sign of turbidity and gas were transferred into 45 °C EC broth medium, and then cultured in a water bath at 44.5 °C for 24–48 h. The EC broth tubes with an indication of turbidity and gas were considered be positive. Subsequently, positive tubes were streaked onto EMB agar plates at 37 °C for 18–24 h. A minimum of five typical colonies were selected and purified on NA plates. The purified strains were subjected to standard biochemical tests using HBIG13 for the identification of *E. coli*. Finally, the MPN values were determined by checking the MPN table.

For aerobic colony count analysis, 1 mL volumes of dilution homogenate (three continuous gradients) were separately spread in two sterile petri dishes, then covered with 15–20 mL of PCA maintained at 46 °C. The colonies were counted after 72 h incubation at 30 °C. An ideal count ranged from 30 to 300 colonies was selected and the results were calculated according to the equation:

$$N = \frac{\sum C}{(n_1 + 0.1n_2)d}$$

in which N is aerobic colony count. C is total colony count of all plates. The n_1 and n_2 are the numbers of flat plate corresponding with first and second dilution gradient, respectively. The d is dilution factor of first dilution gradient.

2.4. Assays for accumulating microbes in three types of shellfish species

2.4.1. Experiments for polyculture ponds of shellfish

The study was performed in Yueqing Bay, located in south-central coast of Zhejiang Province, China. Three polyculture ponds of shellfish containing *T. granosa*, *S. constricta* and *M. meretrix* were selected as the sampling sites (Fig. 1). Shellfish were collected monthly from each site from June to August 2015, and placed separately in sterile ziplock bags. Subsequently, all samples were maintained in an icebox at 4 °C and instantly transported to the laboratory. Within 24 h, samples were analyzed to detect *E. coli* and aerobic colony count. All experiments were repeated in triplicate.

2.4.2. Laboratory simulation test

A shellfish culture system simulating ecological pond (Fig. 2) was used in the experiment. The system was mainly composed of a terrarium A (65 × 40 × 35 cm), the pump B, and an automatic seawater supply equipment C. *T. granosa*, *S. constricta* and *M. meretrix* were cultured in the terrarium A and the artificial seawater (49 faecal coliform organisms/100 mL) harvested from shellfish-growing areas, was used for this study. Equipment C slowly and constantly provided fresh artificial seawater for A, which could maintain dynamic balance of microorganism in the system. Air was pumped into the terrarium A to keep the shellfish active by pump B.

Before the experiments, shellfish were relayed for 2 days in purified running seawater in a commercial shellfish farm. After incubation, under the seawater temperature of 22 °C, shellfish were collected from terrarium A at 24 h intervals, and analyzed to detect *E. coli* and aerobic colony count. All samples were monitored for 6 days. Three replicates were used for each sample.

2.5. Uptake of E. coli in S. constricta from different contaminated seawater

The *S. constricta* were transferred to the shellfish culture system (Fig. 2) containing artificial seawater contaminated with 5, 23, 49, and 240 faecal coliform organisms/100 mL, respectively. All artificial seawater was maintained at a same temperature (22 °C). Accumulation of *E. coli* in *S. constricta* was determined at 24 h intervals, and continuously for 6 days. Three replicates were used for each sample.

2.6. The survey of persistent rainfall on accumulation of E. coli in S. constricta

This was a survey conducted “in field” and so necessary to choose a station with a known bacterial contamination. The study was performed

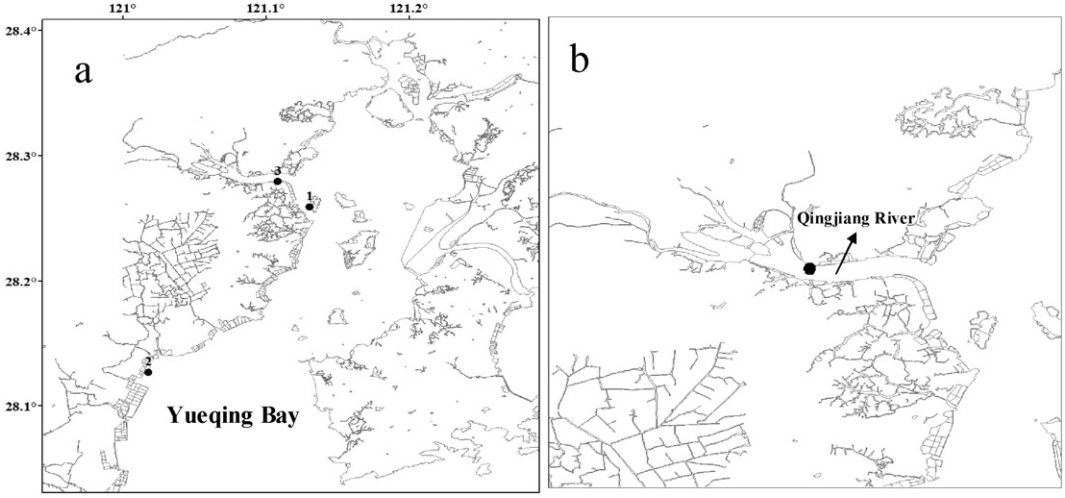

Fig. 1. Sites for shellfish samples collecting. a: three stations of polyculture ponds; b: the station for experiment of persistent rainfall.