Jurnal Biosains, 18(2), 35–44, 2007

BACTERIOLOGICAL COMPARISON OF COCKLES FROM THREE PRODUCING AREAS IN PENINSULAR MALAYSIA

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Abstrak: Kuala Sg. Jarum Mas, Kuala Sepetang, muara sungai di Perak dan Kuala Juru di Pulau Pinang telah dikenal pasti sebagai kawasan utama penghasilan kerang dibawah program Perseimbangan Dagangan (BOT) bagi kerang-kerangan. Oleh sebab penilaian bakteriologi terhadap kerang dari Kuala Sepetang dan Kuala Sg. Jarum Mas belum pernah dijalankan, kajian ini dimulakan bagi membuat perbandingan antara kualiti bakteriologi dari kawasan ini dengan kerang dari Kuala Juru yang telah banyak dikaji. Kerang telah dipungut pada bulan Februari, April dan Jun 2004 dan diuji bagi hitungan plat standard (SPC), koliform total (TC), koliform najis (FC), *Escherichia coli* (EC) dan kehadiran patogen (*Salmonella* spp., *Vibrio cholerae* dan *Vibrio paraheamolyticus*). Pengambilan sampel dan analisis telah dijalankan menurut kaedah mikrobiologi standard. Keputusan menunjukkan bahawa kerang dari Kuala Juru dan Kuala Sepetang melebihi had keselamatan bagi SPC (5×10^5 CFU/g), FC (< 300 MPN/100 g) dan EC counts (< 230 MPN/100 g) sementara keputusan dari Kuala Sg. Jarum Mas adalah jauh rendah daripada had keselamatan bagi parameter yang sama. Analisis statistik menunjukkan perbezaan yang signifikan ($p < 0.05$) bagi SPC di ketiga-tiga buah kawasan. Sementara itu, perbezaan signifikan diperhatikan pada hitungan TC, FC dan EC antara Kuala Juru dengan Kuala Sepetang dan Kuala Sg. Jarum Mas ($p < 0.05$). *Vibrio parahaemolyticus* didapati hadir dalam sampel dari semua kawasan yang diuji manakala V. *cholerae* hanya terdapat dalam kerang dari Kuala Juru pada bulan Februari. Kehadiran *Salmonella* dalam sampel dari Kuala Juru dan Kuala Sepetang menunjukkan bahawa sample tersebut tidak memenuhi saranan had keselamatan. Keputusan kajian ini menyarankan agar kerang yang dipungut dari Kuala Juru dan Kuala Sepetang melalui dekontaminasi sebelum pemasaran dan penggunaan seterusnya.

Abstract: Kuala Sg. Jarum Mas, Kuala Sepetang, the river estuaries in the state of Perak and Kuala Juru in Penang have been identified as major cockle producing areas under the Balance of Trade (BOT) program for mollusc. Since the bacteriological assessment of cockles from Kuala Sepetang and Kuala Sg. Jarum Mas has not been carried out before, this study was initiated to compare the bacteriological quality of cockles from these areas as compared to the extensively studied area, Kuala Juru. Cockles were collected in February, April and June 2004 and examined for Standard Plate Count (SPC), total coliform (TC), fecal coliform (FC) counts, *Escherichia coli* (EC) counts and presence of pathogens (*Salmonella* spp., *Vibrio cholerae* and *Vibrio parahaemolyticus*). Sample collection and analyses were carried out according to standard microbiological methods. The results indicated that cockles from Kuala Juru and Kuala Sepetang exceeded the safety level for SPC (5×10^5 CFU/g), FC (< 300 MPN/100 g) and EC counts (< 230 MPN/100 g) while, result from Kuala Sg. Jarum Mas falls below the safety level for the same parameter. Statistical analyses showed significant difference for the three areas ($p < 0.05$) for SPC. Meanwhile significant differences were observed in TC, FC and EC counts

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between Kuala Juru with Kuala Sepetang and Kuala Sg. Jarum Mas ($p < 0.05$). *Vibrio parahaemolyticus* was present in samples from all locations examined, whereas *cholerae* was only detected in cockles from Kuala Juru in February. Presence of *Salmonella* in samples from Kuala Juru and Kuala Sepetang showed that it does not comply with the safety recommendations. The results from this study strongly recommend that cockles harvested in Kuala Juru and Kuala Sepetang to undergo decontamination before sale and eventual consumption.

Keywords: *Anadara granosa,* blood cockles, bivalve, total coliform, fecal coliform, *Vibrio paraheamolyticus, E. coli, Salmonellla*

INTRODUCTION

The bivalve *Anadara granosa* (blood cockles), locally known as kerang are commercially cultured in the tidal mudflats along the western coast of Peninsular Malaysia. It is a popular gourmet seafood in Malaysia and its cultivation is a good revenue earner. In 2001, approximately 6,750 hectares of cockle-rearing grounds produced about 70,816 metric tonnes of cockles (Anon 2001). Kuala Sg. Jarum Mas, Kuala Sepetang, the river estuaries in the state of Perak and Kuala Juru in Penang have been identified as major cockle producing areas under the Balance of Trade (BOT) program for mollusc. In addition, Kuala Sg. Jarum Mas, has also been selected as the study site for producing hepatitis A virus-free cockles under the cooperation program with Agri-Food and Veterinary Authority (AVA), Singapore. Since the bacteriological assessment of cockles from Kuala Sepetang and Kuala Sg. Jarum Mas has not been carried out before, this study was initiated to compare the bacteriological quality of cockles from these areas compared to the extensively studied area, Kuala Juru. Shellfish harbor microorganisms in their tissue from surrounding waters during the filter-feeding process and are recognized as the reservoir for various microbial pathogens (Potasman *et al*. 2002). Besides accumulating naturally occurring microorganisms such as *Vibrio vulnificus*, *Vibrio parahaemolyticus* and their pathogenic forms, these molluscan shellfish are also prone to contamination by fecal pathogens, primarily, *Salmonella* spp., *Shigella* spp., *Escherichia coli* and enteric viruses from sewage-polluted waters (Roberts *et al*. 1990). Enumeration of fecal bacteria in shellfish is the common method of assessing the potential health hazards to consumer and effects of sewage disposal near to waters in which shellfish are grown (West 1985). The objective of this study, as part of a larger overall project on improving the safety and quality of whole fresh cockles was to generate information on the incidence of a range of bacterial pathogens on fresh cockle from several major cockles producing area.

MATERIALS AND METHODS

Samples collection of cockles, ($n = 5$) were freshly harvested from Kuala. Sg. Jarum Mas, Kuala Sepetang in Perak and Kuala Juru, Penang. Samples were collected in disposable sterile containers using standard collection procedure,

transported in ice-cooled insulated box, brought to the laboratory immediately and analyzed within 24 hours. Samples were analyzed by standard procedures (APHA 1992). Bacteriological quality parameter determined were Standard Plate Count (SPC), TC, FC and EC counts as well as the presence of *Salmonella* spp., *V. cholerae* and *V. parahaemolyticus.*

SPC

A sample of 25 g blood cockle meat with 250 ml 'of sterilised 0.1% peptone water was put into a sterile blender (Waring USA) and homogenized for 1 minute. From this mixture 2, 3, 4 and 5 dilutions were obtained by mixing with sterilised 0.1% peptone water (serial dilution). A 1 ml aliquot from each diluent was applied to a melted (45^{o}C) sterilised standard plate count agar (PCA, OXOID CM 325) in a universal bottle. After mixing it was poured into the sterile plates. The plates, in triplicate, were incubated at 23^{o}C for 48 hours. The colonies grown on the plates were counted using colony counter and standard plate count values were indicated as colony forming units (CFU) per gram of cockle meat.

TC, FC and EC Count

Enumeration of total coliform, fecal coliforms and *E. coli* in shellfish tissue was carried out by using the 3-tube Most Probable Number (MPN) technique. A total of 25 g of the shellfish tissue and fluid were aseptically transferred to a sterile blender (Waring, USA) and 1:10 dilution was made with a sterile 0.1% peptone water followed by blending for 1 minute. From this mixture 2 and 3 dilutions were obtained. A 1 ml aliquot from each diluent was applied to 9 ml sterile lauryl tryptose broth (LST Broth, OXOID CM451). Presumptive total coliforms were obtained using LST (incubated at 35°C for 24–48 hours) followed by confirmation with brilliant green lactose bile (2%) broth (BGLB Broth, OXOID CM 31). To enumerate fecal coliforms, a loopful from this positive LST cultures were transferred to E.C broth (E.C Broth, OXOID CM 853) incubated at 44.5 ± 2°C for 24–48 hours and examined for gas formation. A gas positive culture were streaked onto Eosin Methylene Blue Agar (EMBA, OXOID CM 69) and was incubated at 35^{o}C for 24 hours. Confirmation of *E. coli* was carried out by IMViC test. Results were evaluated according to the MPN tables. Values were indicated as Most Probable Number per 100 g (MPN/100 g) of cockle meat.

Prevalence of *Salmonella* spp.

Twenty-five grams of shellfish meat was homogenized with 225 ml of Nutrient Broth. The homogenate was incubated at 35 ± 2°C for 24 hours. The incubated homogenate (1 ml) was transferred to a 10 ml selenite broth base (OXOID CM 699) with sodium biselenite (OXOID LP 0121A). The broths were incubated overnight at 35 ± 2°C. A loopful of sample was streaked onto bismuth sulphite agar (BSA, OXOID CM 201) and incubated at 35^{o}C for 48 hours. Brown–grey–black colonies surrounded by a brown–black zone with metallic sheen were regarded as typical *Salmonella* colonies and appropriate confirmatory tests were performed.

Prevalence of *V. cholerae* and *V. parahaemolyticus*

Fifty grams of shellfish meat were homogenized with 450 ml of alkaline peptone water (pH 8.5) (APW, OXOID L37). The homogenate was incubated at 35 ± 2°C for 18 hours. A loopful from APW was then streaked onto Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS, OXOID CM 333) and the plate was incubated at 35^{o}C for 24 hours. Typical *V. cholerae* and *V. parahaemolyticus* colonies were picked from this agar and transferred onto trypticase soya agar plates for further identification and biochemical tests as described in the standard method.

Statistical Analysis

Results (SPC, FC, and EC) were analysed using ANOVA and means were separated by the least significant difference test (SPSS 11.5) at a probability level of 0.05 as the level of significance.

RESULTS AND DISCUSSIONS

SPC

SPC or total plate count or aerobic plate count is commonly used to determine 'total' number of microorganisms in a food product. The U.S. Food and Drug Administration (2001) microbial standard for SPC in freshly processed bivalve meats is < 500,000 CFU/g, with > 1,000,000 CFU/g considered to be substandard quality. Figure 1 shows the result of SPC. In this study, average microbial growth of sample from Kuala Juru and Kuala Sepetang exceeded the recommended level with cockles from Kuala Juru recorded the highest counts ranging from 3.2×10^6–6.5×10^6 CFU/g followed by sample from Kuala Sepetang 5.4×10^6–5.8×10^6 CFU/g. However, the average SPC for Kuala Sg. Jarum Mas was below the recommended level ranging from 3.4×10^4–5.7×10^4 CFU/g. From statistical analyses the mean result showed a significant difference ($p < 0.05$) between the three locations. Means SPC for Kuala Sepetang (5,626,667) is the highest followed by Kuala Juru (4,717,556) and Kuala Sg. Jarum Mas (463,555) the lowest.

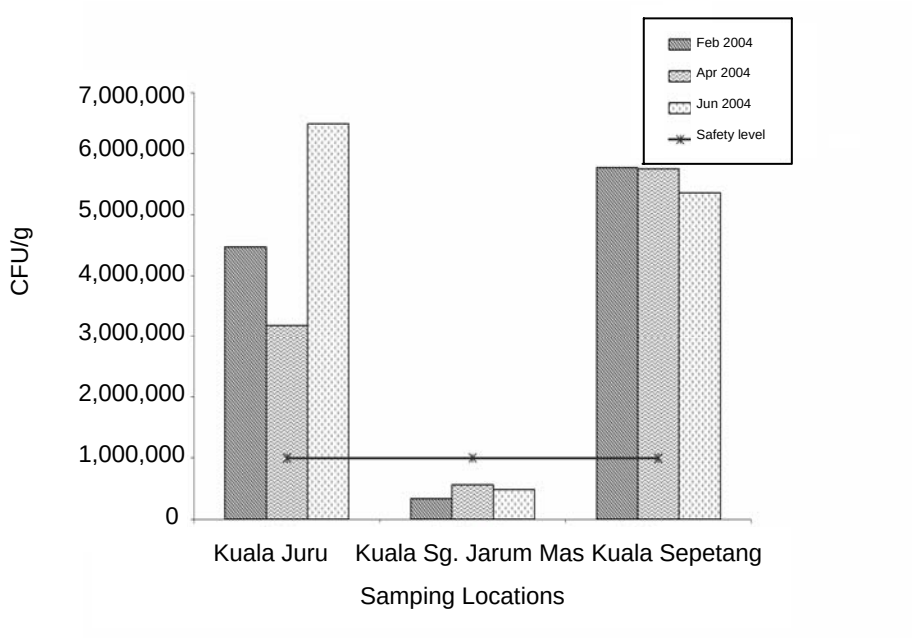

Figure 1: SPC recorded in cockles from the three study sites.

TC Count

The TC group comprised of aerobic and facultative anaerobic, gram negative, non-spore forming rod shaped bacteria that ferment lactose to yield acid and gas within 48 hours at 35^{o}C (Andrews 1992). As the result shown in Figure 2, total coliform count for Kuala Juru was the highest for every sampling month. The value range from 16,320 –28,400 MPN/100 g followed by sample from Kuala Sepetang 900–2,300 MPN/100 g. Meanwhile, cockles from Kuala Sg. Jarum Mas showed the lowest result for every sampling month, ranging from 700–800 MPN/100 g. Result from statistical analyses showed significant differences ($p < 0.05$) between Kuala Juru (22,907), Kuala Sepetang (1,647) and Kuala Sg. Jarum Mas (733). Hence there was no significant difference between Kuala Sepetang and Kuala Sg. Jarum Mas. The significance of the coliform group as an indicator organism has been questioned because some genera within the group are frequently associated with surface runoff rather than fecal pollution (Greenberg & Hunt 1985). Therefore it was not frequently used as a reference for indicator.

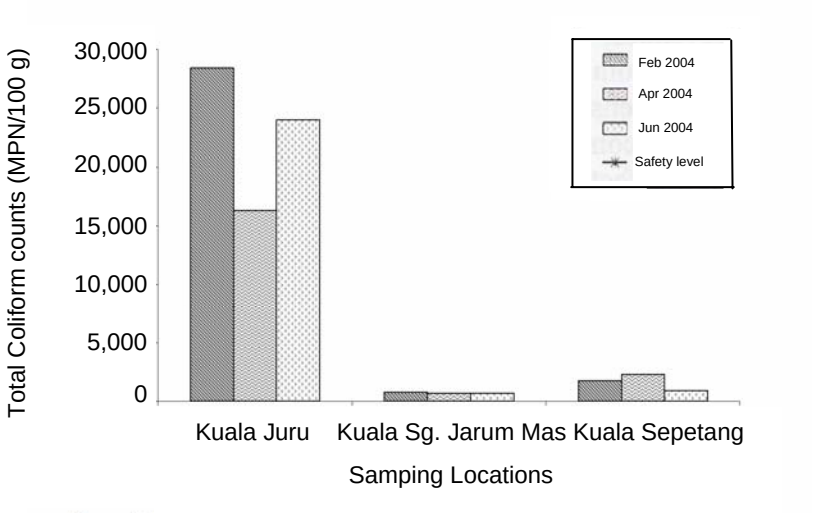

Figure 2: TC count in cockles from the three study sites.

FC Count

FC are enteric pathogens. They are the coliforms which produce acid and gas from lactose in EC medium at a higher temperature of 44.5°C when incubated within 48 hours (Andrews 1992). Average FC counts in cockles for the three months of sampling are shown in Figure 3. All the sampling locations harbored FC counts exceeding the recommended limit of < 300 MPN/100 g. Cockles from Kuala Juru recorded the highest counts of FC ranging from 14,180–21,060 MPN/100 g, followed by Kuala Sepetang ranging from 400–900 MPN/100 g. Although the result from Kuala Sg. Jarum Mas were 400 MPN/100 g for every sampling month, the lowest counts of FC recorded but it is still a little bit above the recommended level. There were significant differences between Kuala Juru (17,787) with Kuala Sepetang (733) and Kuala Sg. Jarum Mas (400). However there was no significant difference between Kuala Sepetang and Kuala Sg. Jarum Mas in term of means value at $p = 0.05$. Urban run-off, effluent from heavily populated rivers, drainage from sewage and livestock farm are the major factors identified in elevating the FC and EC counts (Wan Norhana & Nor Ainy 2004). High FC counts in cockles from Seberang Perai and Penang have been reported earlier (Table 1).

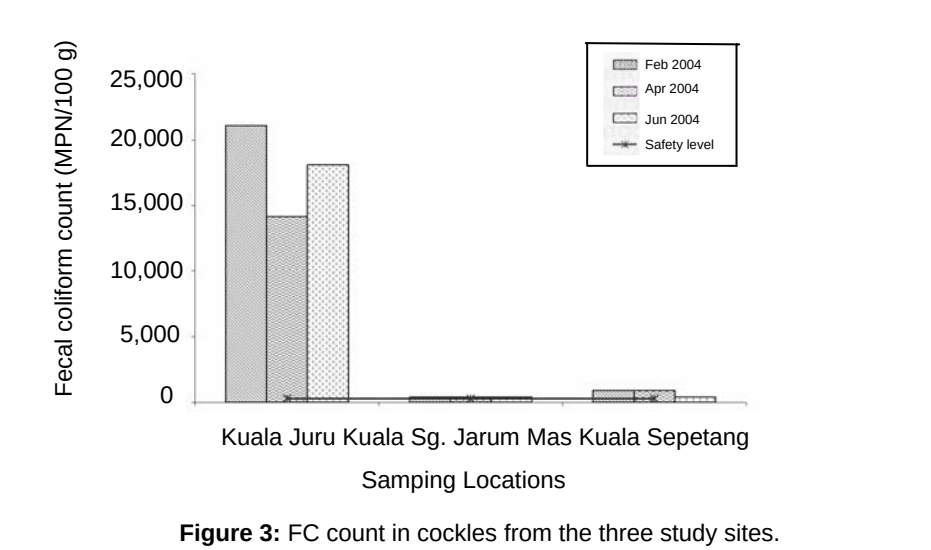

Figure 3: FC count in cockles from the three study sites.

Table1: FC counts in shellfish reported by previous workers.

Bivalves	Location	Geometric Mean Concentration (FC MPN/100 g)	References
A. granosa	Penang	31,800	Ismail, H. I (1991)
	Perak	4,100	
	Selangor	1,700	
A. granosa	Bukit Tambun, Penang	80–200	Wan Norhana (1996)
A. granosa	Bukit Tambun	510	Wang (1996)
	Kuala Juru (seabound)	2,290	
	Kuala Juru (estuary)	3,500	

EC Count

EC is the most widely used indicator of fecal contamination. It may also be the most versatile of human pathogens. Average EC counts in cockles observed for the three months sampling are illustrated in Figure 4. Generally high EC counts in cockles correspond with high counts of FC. Only two of the stations examined recorded EC counts exceeding the recommended limit of < 230 MPN/100 g for every sampling month. The highest EC counts were observed in cockles from Kuala Juru (2,700–3,100 MPN/100 g) followed by Kuala Sepetang (300–340 MPN/100 g). Meanwhile Kuala Sg. Jarum Mas recorded the lowest EC counts with an average of less than 230 MPN/100 g for every sampling month.

The same results were obtained from statistical analyses. There was significant difference between Kuala Juru (2,873), Kuala Sepetang (313) and Kuala Sg. Jarum Mas (230). But no significant difference was observed between Kuala Sepetang and Kuala Sg. Jarum Mas in term of means value at $p = 0.05$.

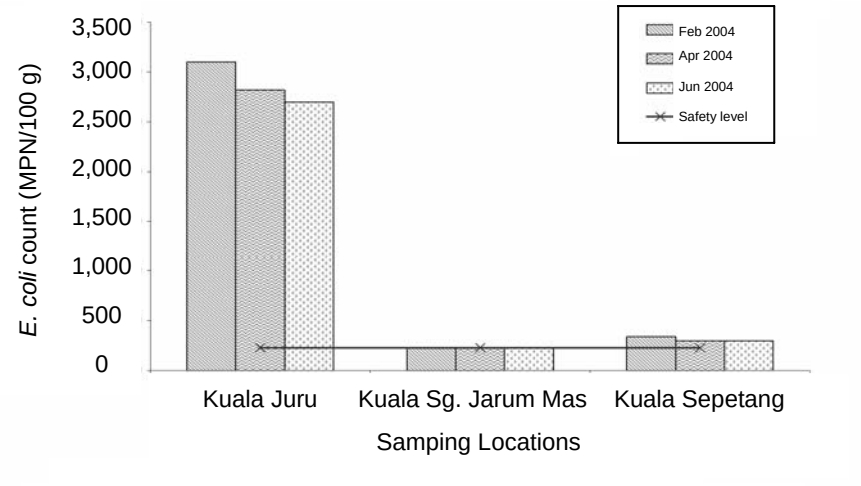

Figure 4: *Escherichia coli* count in cockles from the three study sites.

Vibrio cholerae and *V. parahaemolyticus*

Substantial evidence has been provided showing that *Vibrio* species are natural inhabitants of marine aquatic environments in both temperate and tropical regions, with most human infections acquired by exposure to such environments or to food derived from them. In previous study, observed that *Vibrios* are commonly isolated from cockles compared to other shellfish examined (Wan Norhana & Nor Ainy 2004). Occurrence of *V.* parahaemolyticus in shellfish was more predominant compared to *V. cholerae* (Table 2). *Vibrio parahaemolyticus* was detected in cockle, two times from both locations, Kuala Sepetang and Kuala Juru. However, only one detection was observed in cockle from Kuala Sg. Jarum Mas from the three sampling times. *Vibrio cholerae* was only detected in cockles from Kuala Juru in February.

Table 2: Prevalence of Vibrios in shellfish examined.

	Vibrio parahaemolyticus			*Vibrio cholerae*		
Locations	Feb 2004	Apr 2004	Jun 2004	Feb 2004	Apr 2004	Jun 2004
Kuala Sepetang	d	d	nd	nd	nd	nd
Kuala Sg. Jarum Mas	d	nd	nd	nd	nd	nd
Kuala Juru	d	nd	d	d	nd	nd

d = detected
nd = not detected

Salmonella spp.

Salmonellae are pathogens with an enormous impact on public health as they infect all types of domestic animals used in the human food chain. *Salmonella* infections lead to a variety of diseases known as salmonellosis. The presence of *Salmonella* in cockle from Kuala Juru and Kuala Sepetang showed that it does not comply with the safety recommendations (Table 3). Meanwhile, *Salmonella* spp. was not detected in samples Kuala Sg. Jarum Mas. This result is in accordance

with the European Directives criterion indicating that no *Salmonella* spp. should be found in 25 g of live and cooked crustaceae and molluscan shellfish (Harrigan 1998).

Table 3: Prevalence of Salmonella in shellfish examined.

Locations	*Vibrio parahaemolyticus*		
	Feb 2004	April 2004	June 2004
Kuala Sepetang	nd	d	d
Kuala Sg. Jarum Mas	nd	nd	nd
Kuala Juru	d	nd	d

d = detected
nd = not detected

CONCLUSION

The condition of cockles collected during the study period based on 91/492/EEC. Directives showed that none of the cockles produced in Kuala Juru and Kuala Sepetang in the month of February to June 2004 could be used for direct human consumption. Generally cockles produced from Kuala Sepetang could be collected for human consumption only after a treatment in a purification centre or after relaying. Meanwhile cockles from Kuala Juru could be collected but must be placed in the market only after a longer period of relaying combined with purification. In conclusion, high counts of faecal bacteria in cockles were observed throughout the study period for cockles from Kuala Juru and followed by Kuala Sepetang. Cockles from Kuala Sg. Jarum Mas have the best bacteriological quality in terms of SPC, TC, FC, EC and free from pathogenic bacteria. The primary factor influencing the fecal coliform bacteria in shellfish growing areas seems to be related to development and human activity in the surrounding areas.

ACKNOWLEDGEMENTS

We would like to express sincere gratitude to the Director of Research, Mr. Ismail bin Awang Kechik, and Biotechnology Head of program Officer, Mr. Ismail bin Ishak for their valuable support in conducting this study. Appreciation also goes to all staff of Biotechnology and Fisheries Product Laboratory, Institut Penyelidikan Perikanan, Penang; Larut Matang State Fisheries and Kuala Jarum Mas Lembaga Kemajuan Ikan Malaysia staff for their assistance and cooperation.

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