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Establishment of a shellfish model farm: a case study in Sg. Jarum Mas, Perak, Malaysia

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Abstract: This study evaluates the sanitary and physico-chemical quality of Sg. Jarum Mas shellfish waters in order to establish its suitability as a model farm. Seawater and shellfish from nine stations (4 shellfish harvesting waters, 4 surrounding waters and 1 control site) were collected and analyzed monthly from September 2004 - September 2005. The results show that shellfish harvesting waters in Sg. Jarum Mas can be classified as 'approved' and 'conditionally approved'. Hepatitis A virus was not detected in any of the shellfish examined. *Dinophysis caudata* and *Pseuodonitzshia* spp. were the most common harmful alga species observed. Harmful species that are known to produce toxins and cause shellfish poisoning such as *Alexandrium* spp., *Gymnodinum* spp., *Pyrodinium* sp. and *Prorocentrum* spp. were not detected. The physico-chemical characteristics of shellfish waters in Sg. Jarum Mas imply that they are suitable for aquaculture activity of moderately tolerant species such as shellfish.

Keywords: shellfish, model-farm, Sg. Jarum Mas and sanitation

Introduction

The shellfish industry offers great potential to the country in terms of providing food for the people, increasing the income of small-scale fishermen faced with dwindling catches, providing livelihood for people in coastal areas as well as exchange earnings from export of shellfish. Shellfish such as cockles, mussels and oysters are good sources of relatively cheap and high quality protein. Their shells have long been used to produce handicrafts, ornaments, medicinal and cosmetic products. Recent findings have indicated the possibility of using the shells to produce higher value products such as anti-cancer peptide (Leng et al., 2005), bone spare-parts, cements for bone structures and surgical glue (from the byssal thread). Shellfish are also ideal species to culture because of their low position in the food chain. More importantly shellfish culture is practically a "green" and sustainable industry. This is due to the act of shellfish feeding (biofiltering) which improves water quality by removing particulates (organic matter, nutrients, silts, bacteria, virus etc.) in the water column thus making it an effective way to counter eutrophication (Shunway et al., 2003).

However due to their filter feeding nature, shellfish tend to concentrate viruses and bacteria present in water. Thus, harvesting shellfish from areas exposed to fecal pollution may pose a health hazard. Food-borne diseases have been well associated with the consumption of raw or undercooked shellfish (Ripabelli et al., 1999; Less, 2000). The enumeration of fecal bacteria group or species such as total coliforms, fecal coliforms and/ or *Escherichia coli* in shellfish is the internationally accepted method of assessing the potential health hazards to consumers (West et al., 1985). Meanwhile, the resistance patterns to certain antibiotics in fecal bacteria may give some indication of the source of fecal contamination (Parveen et al., 1997; Harwood et al., 2000).

Traditionally the shellfish industry in Malaysia has been carried out on a small-scale basis and exists as a free enterprise. In order to realize the potential of this industry, it has to be transformed to a more systematic, well-managed, competitive industry. With this intention, a program has been planned and developed by the Department of Fisheries (DOF), Malaysia with both macro and micro action plans designed specifically to boost the industry to be more competitive in order to achieve a positive balance of trade to Malaysia. Four strategies have been identified which focus on the improvement and enhancement

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of infrastructure and logistic, quality and safety of products, adoption of new technology and better support services.

Sg. Jarum Mas, Larut-Matang, Perak is one of the locations chosen as potential shellfish culture area under this program. Sg. Jarum Mas was selected because of its suitable location which is protected from prolonged flooding, strong winds and waves, with a high natural productivity of the water and moderate tidal current flow for the transport of phytoplankton and oxygen and elimination of wastes as well as being far from industrial and densely populated areas. Besides that, sufficient logistic and good collaboration that exist among the culturists and fisheries officials have made it to be selected as a location for developing a model farm under the program. Although Sg. Jarum Mas is geographically suitable, there are other factors that need to be examined to ascertain its suitability for shellfish growth, survival and safe production. To our knowledge, there are no data or reports on the sanitary status of shellfish or shellfish water from this area. Hence a one-year study (September 2004-September 2005) was carried out to evaluate the suitability of this location as model shellfish farm. The specific objectives of the study were to evaluate the fecal contaminations in shellfish tissue and shellfish waters of Sg. Jarum Mas, to screen the presence of harmful algae/phytoplankton in the waters and to correlate the physico-chemical quality of shellfish waters and the numbers of fecal bacteria in them. The results obtained were used to classify the Sg. Jarum Mas shellfish waters according to the United States National Shellfish Sanitation Program Programme Model Ordinance (US NSSP), (1999) and determine the bacteriological quality of shellfish produced, based on European Committee regulation (Directives 91/492/EC).

Materials and Methods

Fecal bacteria counts

Seawater and shellfish sample

Seawater samples from 9 stations located around Sg. Jarum Mas (Figure 1) were collected monthly from September 2004 to September 2005. During the same time, shellfish samples (blood cockles (*Anadara granosa*); oysters (*Crassostrea* spp.); green mussels (*Perna viridis*)) were also collected from stations raft, 2, 5 and 6. Seawater was collected using 500 ml sterile bottle and shellfish in a sterile bag. Each shellfish sample consisted of 30-40 cockles, 10-12 oysters and 12-15 mussels. Samples were transported in ice-cooled insulated box and brought to the laboratory and analyzed within 24 h. Shellfish which gaped open or dead were discarded. Samples were washed in tap water and shucked using a sterilized knife. 25 g of the shellfish meat and inter-vulvular fluid were aseptically transferred to a sterile blender (Waring, USA) and 1:10 dilution was made with a sterile phosphate buffer solution, followed by blending for 1 min.

Enumeration of Fecal Coliform (FC) and Escherichia coli *(EC) in seawater and shellfish*

The analysis of FC in water was carried out based on the Most Probable Number (MPN) method

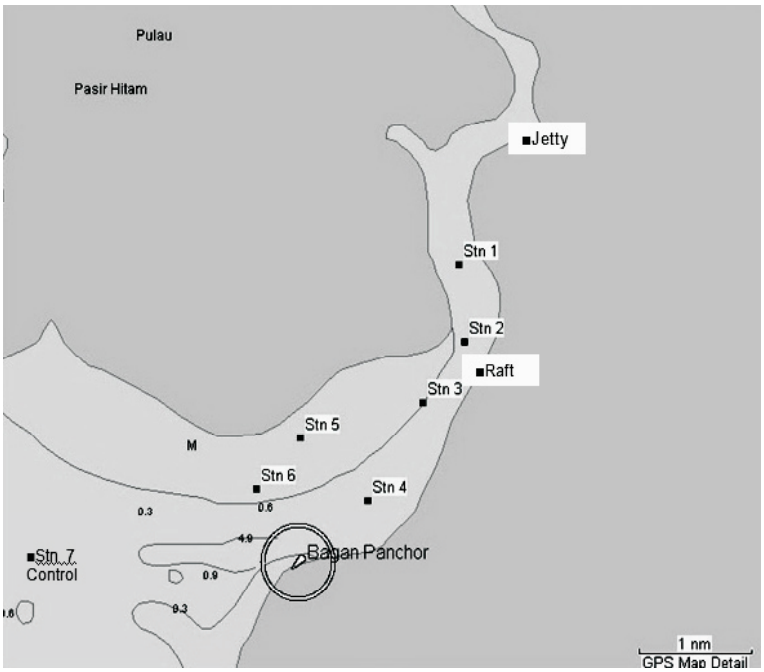

Figure 1. Sampling stations at Sg. Jarum Mas

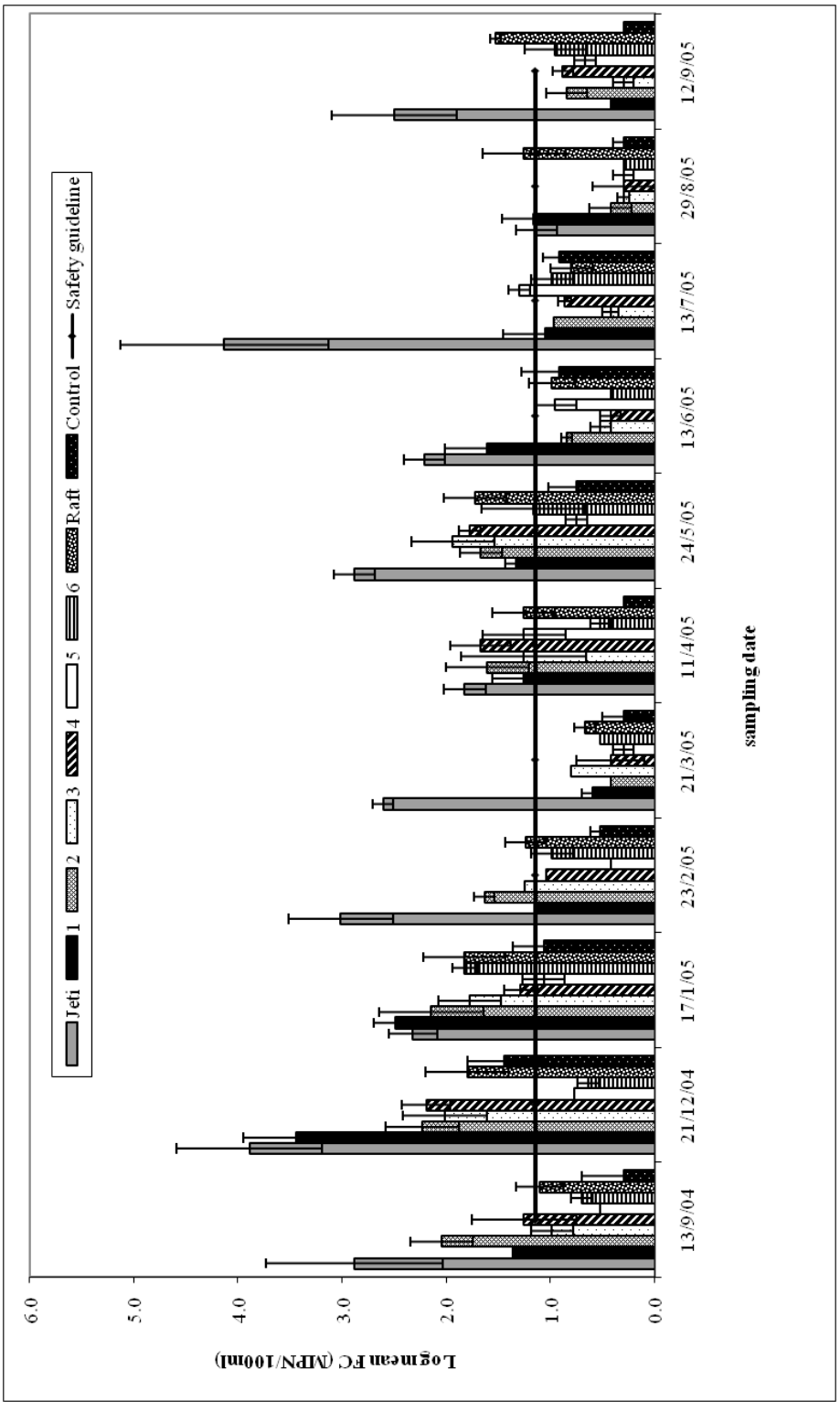

Figure 2. Mean FC counts in seawater samples from nine stations in Sg. Jarum Mas

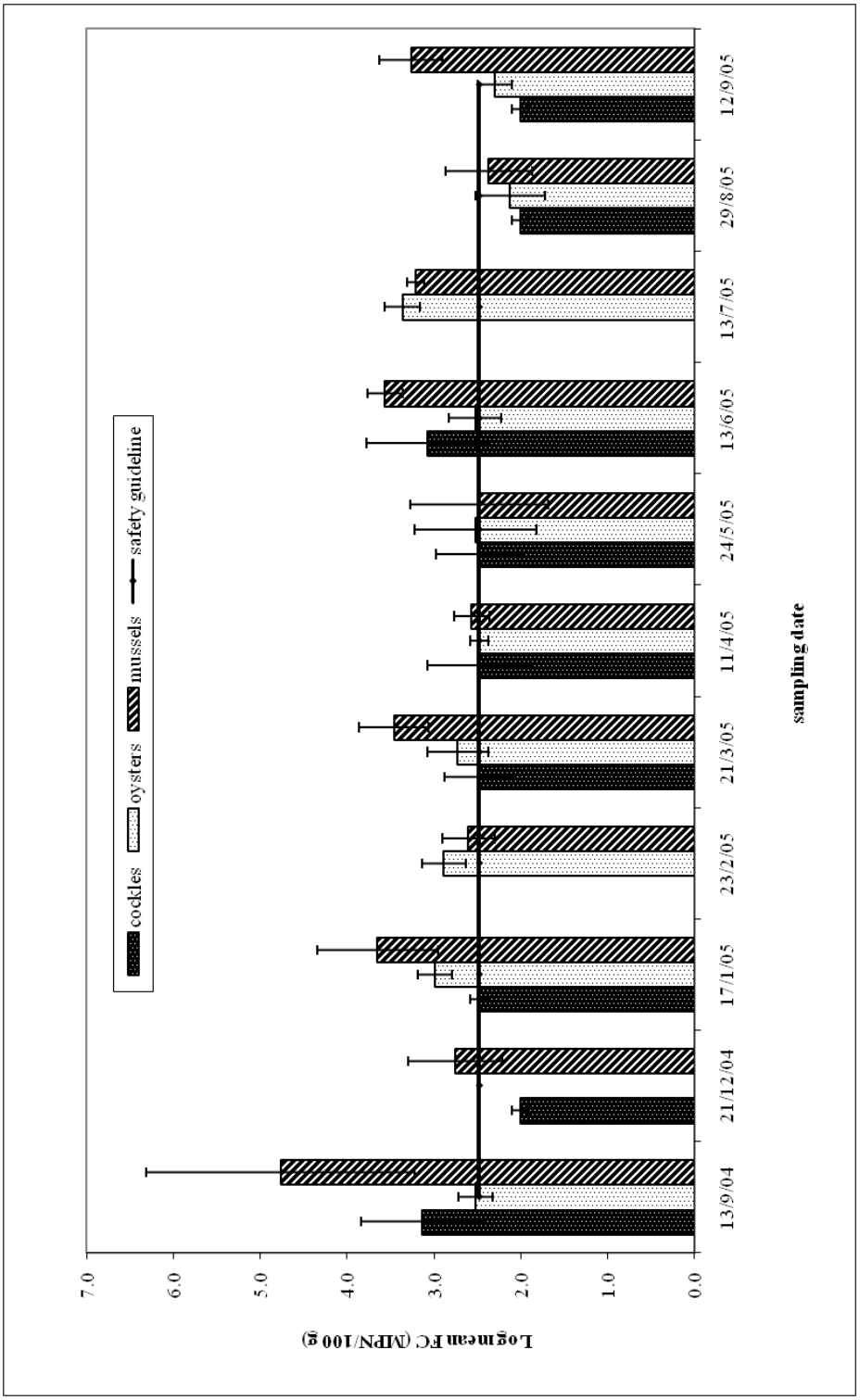

Figure 3. Mean FC counts in shellfish from Sg. Jarum Mas

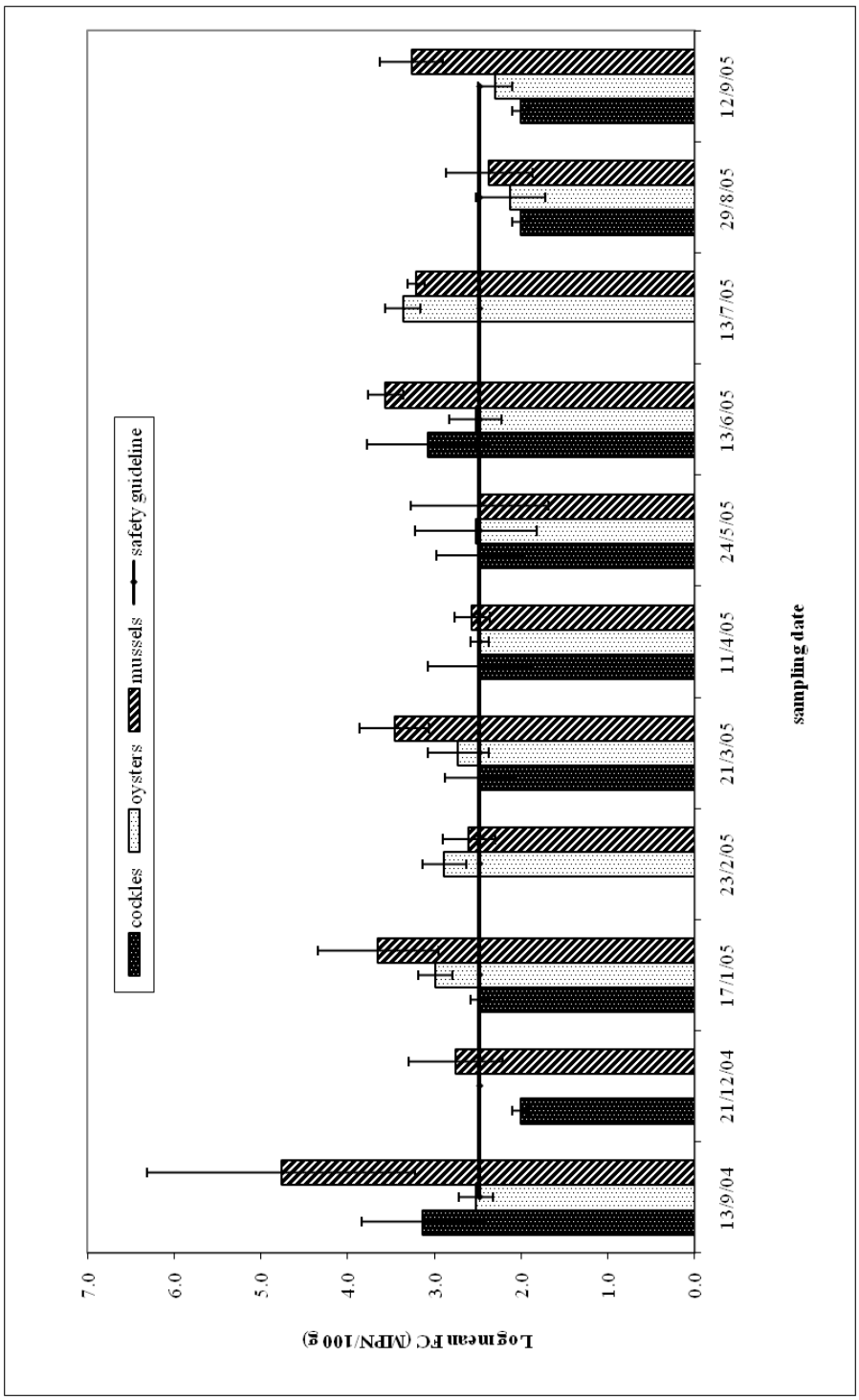

Figure 4. Mean EC counts in shellfish from Sg. Jarum Mas

as described in the Standard Methods for the Examination of Water and Wastewater, American Public Health Association (APHA), (1998). FC and EC in shellfish were also determined according to MPN methods as detailed in the Compendium of Method for Microbiological Examination of Foods, (2001).

Classification of shellfish waters and determination of shellfish safety

The FC counts in seawater obtained were compared to the US NSSP of the Food and Drug Administration (Table 1) to classify the shellfish waters in Sg. Jarum Mas. Meanwhile, FC and EC counts in shellfish obtained in this survey were compared to the recommended levels set by the European Committee (Directives 91/492/EEC of 15 July 1991- Laying down the health conditions for the production and the placing on the market of live bivalve shellfish) (Table 2). The determination of the safety condition of shellfish harvested was carried out as outlined by the same document.

Determination of antibiotic resistant profile

A total of 69 *E. coli* isolates from shellfish and shellfish waters from 9 sampling stations in Sg. Jarum Mas were used in this study. The isolates were first identified by selective plating on eosin-methylene blue agar (OXOID, Basingtoke, UK). Typical *E. coli* colonies (green and metallic sheen or dark/purple centered colonies) were verified by using the IMViC series of test (Indole, Methyl-Red, Voges-Proskauer, and Citrate). Isolates exhibiting ++-- IMVC profiles were confirmed as *E. coli*. Control *E. coli* strains were obtained from the Institute for Medical Research, Kuala Lumpur and the Public Health Laboratory, Ipoh.

Antibiotics chosen in this study were from those commonly used in clinical applications and/ or added to animal feed. *E. coli* isolates were tested for their susceptibility to fourteen antibiotics using the disc-diffusion method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (1999). E. coli were grown in a shaking water bath at 37°C until a 0.5 McFarland turbidity standard was attained. A volume of 0.1 ml of the culture containing approx. 10^5 colony forming unit/ml (CFU/ml) was spread over Brain Heart Infusion (OXOID) agar plates. Ampicillin (A) (2 µg), chloramphenicol (C) (10 µg), doxycyclinehydrochloride (DO) (30 µg), erythromycin (30 µg), furazolidone (FR) (15 µg), kanamycin (K) (5 µg), nalidixic acid (NA) (30 µg), neomycin (N) (10 µg), oxolinic acid (OA) (2 µg), oxytetracycline (0T) (30 µg), penicillin G (1 i.u.), streptomycin (S) (10 µg), sulphonamide (Su) (300 µg) and tetracycline (TE) (5 mg) antibiotic disc (OXOID) were placed on the surface of the inoculated agar. The agar was incubated at 35°C for 24 hr and zones of inhibition around each disc were measured. Using NCCLS measurement guidelines each E. coli was classified resistant, intermediate or susceptible to specific antibiotics. All experiments were carried out in triplicates to ensure reproducibility of the data.

Detection of Hepatitis A virus (HAV)

Hepatitis A Virus (Enterovirus 72), cytopathic HM 175 (Clone 2) in infected cell lysates obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were used as positive controls. Viral RNA extraction was carried out using the RNeasy® kit (Qiagen, Hilden, Germany) while the Qiagen® One-Step RT-PCR kit (Qiagen, Hilden, Germany) was used for the detection of HAV in shellfish tissue. A total of 30 samples of shellfish collected from stations 2, 5, 6 and raft were used in this study. The samples were transported in ice-cooled insulated box to the laboratory and processed immediately.

At the laboratory, the shellfish were washed and scrubbed thoroughly in running water and opened aseptically. Shellfish flesh was shucked and the stomach and digestive diverticula were dissected. Viral RNA extraction was carried out according to the protocol supplied with the kit. Briefly, the shellfish digestive tissue was homogenized with a homogenizer (IKA Ultra Turrax T25, Staufen, Germany) for 1 min in lysis buffer supplemented with 1% B-mercaptoethanol (Sigma, St. Luis, MO, USA). After a brief incubation at 4°C, the mixture was centrifuged at 12, 000 rpm for 4 min at 4°C. RNA was extracted from the supernatant by a spin column method and finally eluted in 50 µl of RNAse-free water. The total RNA yields (µg/ml) and purity ($A_{260}/_{A280}$) were determined spectrophotometrically (Biophotometer, Eppendorf, Germany).

The sequence of the RT-PCR primers used in this study was based on the sequence of wild type HAV (strain HM-175). This primer amplified 489 bp regions (nucleotides 6256 to 6744) (Goswami et al., 1993). The sequence of the forward primer was 5'-ATGCTATCAACATGGATTCATCTCCTGG-3'. The sequence of reverse primer was 5'-CACTCATGATTCTACCTGCTTCTCTAATC-3'). The primers were synthesized by the Research Biolabs Sdn. Bhd., Ayer Rajah Crescent, Singapore and stored at -20°C. RT-PCR was carried out in a volume of 50.0 µl reaction mixtures containing 10.0 µl 5X Qiagen One-Step RT-PCR buffer (12.5 mM $MgCl_2$),

Table 1. Shellfish water classification criteria according to the US NSSP (cited from Jackson and Ogburn (1999)).

Classification	Criteria
Approved areas	When under the most unfavourable meteorological, hydrographic, seasonal or point-source conditions, the FC counts do not exceed 14/100 ml and ≤ 10% of the samples exceed a FC MPN of 43/100 ml, for a five-tube decimal dilution test. At least 15 samples must be analysed.
Conditionally approved areas	When there are specific, predictable events (such as rainfall) that can cause an area to exceed the water quality standards. The area is approved for shellfish harvest unless such an event occurs, at which time it is closed for harvest for a period of time pre-determined by the State. Areas with conditionally approved status must meet the standards set forth by the NSSP (see under approved status) outside of the specific, predictable events. Shellfish harvest is allowable in these areas when a closure is not in place.
Restricted areas	When the waters are subjected to limited amounts of pollution such that shellfish must be depurated or relayed prior to sale. Under the most unfavourable meteorological, hydrographic, seasonal or point-source conditions, water samples should not have total coliforms levels in excess of 700 per 100 ml with less than 10% of samples exceeding 2,300/100 ml for a 5 tube MPN. In addition, FCs must not exceed 88/100 ml with ≤ 10% of samples exceeding 260 per 100 ml for a 5 tube MPN.
Conditionally restricted areas	When the waters are subject to intermittent pollution which make them temporarily unsuitable as a source of shellfish for depuration or relaying. The waters are closed for harvesting until they can meet the sanitary criteria for restricted areas.
Prohibited areas	When the level of pollution is such that shellfish are likely to be unfit for human consumption even after depuration or relaying. The harvesting of shellfish is banned from such waters.
Unclassified areas	When no sanitary survey has been conducted. Harvesting of shellfish from such areas is banned.

Table 2. EC shellfish directives 91/492/EEC

Classification		Permitted levels	Outcome
A	<230	Less than 230 EC/100g flesh or Less than 300 FC/100g flesh	May go direct for human consumption
B	<4,600	Less than 4,600 EC/100g flesh (in 90% of the samples) or Less than 6,000 FC/100g flesh (in 90% of the samples)	Must be depurated, heat treated or relayed to meet category A requirement
C	<46,000	Less than 60,000 FC/100g flesh (in 90% of the samples) Less than 46,000 EC/100g flesh	Must be relayed for a period of at least 2 months, followed where necessary by treatment in a purification centre to meet category A requirements
Above 60,000 FC			Unsuitable for production

Table 3. Antibiotic resistance (AR) profiles for individual *E. coli* isolates

AR Profiles (No of antibiotics)	Stations	No. of isolates
A-C-DO-E-K-NA-N-OA-OT-P-S-Su-TE (13)	Raft	3
A-C-DO-E-K-NA-OA-OT-P-S-Su-TE (12)	Jetty	1
A-C-DO-E-K-N-OA-OT-P-Su-TE (11)	Raft	1
A-C-E-K-NA-N-OA-OT-P-Su-TE (11)	Raft	1
A-DO-E-K-N-OT-P-S-Su-TE (10)	Raft	1
E-K-NA-N-OA-OT-P-S-Su-TE (10)	Raft	1
A-C-E-OT-P-S-Su-TE(8)	Raft	1
E-K-NA-OA-OT-P-Su-TE (8)	Raft	1
A-E-NA-OA-P-S-Su (7)	Raft	1
A-E-OT-P-S-Su-TE (7)	Raft	1
A-C-E-P-Su-TE (6)	Raft	1
E-NA-OA-OT-P-TE (6)	Raft	1
E-OT-P-S-Su-TE (6)	3, raft	3
A-E-OT-P-TE (5)	2	1
E-OT-P-S-TE (5)	Jetty, 1, raft	5
E-OT-P-Su-TE (5)	Raft	2
C-DO-E-K-P (5)	3	1
E-P-Su-TE (4)	4	1
E-N-P (3)	4	1
E-P-S (3)	Jetty, 1, 2, 3, raft control	15
E-P (2)	Jetty, 1, 2, 3, 4, raft, control	26

Note: Ampicillin (A) (2 µg), chloramphenicol (C) (10 µg), doxycyclinehydrochloride (DO) (30 µg),erythromycin (E) 30 (µg) kanamycin (K), (5 µg), nalidixic acid (NA) (30 µg), neomycin (N) (10 µg), oxolinic acid (OA) (2 µg), oxytetracycline (OT) (30 µg), penicillin G (P) (1 i.u.), streptomycin (S) (10 µg), sulphonamide (Su) (300 µg) and tetracycline (TE) (5 mg)

Table 4. Presence of harmful algae/phytoplankton in Sg. Jarum Mas, Perak

Sampling date	Toxic algae of potential
September 2004	*Pseudonitzschia* sp.
December 2004	*D. caudata, Pseudonitzschia* sp.
January 2005	*D. caudata*
February2005	*D. caudata*
March 2005	*Dinophysis* sp., *Pseudonitzschia* sp
April 2005	*Dinophysis* sp., *Pseudonitzschia* sp
May 2005	*D. caudata* (at 2 sites)
June 2005	*D. caudata* (at 2 sites -1 site observed both during high and low tides)
July 2005	*D. caudata* (2 sites during high tides), *Noctiluca* sp
August 2005	Not detected
September 2005	Not detected

Table 5. Ranges of physico-chemical parameters of nine stations in Sg. Jarum Mas from September 2004-September 2005

Physico-chemical parameters	Stations								
	Jetty	1	2	3	4	5	6	Raft	Control (7)
Temp. (°C)	27.66-32.95a[A]	28.26-33.02a	27.88-32.27a	28.12-32.29a	29.29-32.71a	29.6-30.94a	29.25-30.82a	28.51-32.76a	28.66-31.66a
Salinity (ppt)	9.98-18.8a	22.77-31.68b	23.02-32.81b	23.41-32.66b	24.89-31.66b	27.16-31.30b	27.9-31.84b	23.7-31.78b	25.73-32.23b
DO (mg/l)	1.87-6.93a	2.03-5.89a	2.14-7.26a	2.65-7.59a	2.87-6.48a	3.4-6.9a	5.02-6.32a	2.25-6.13a	3.61-8.86b
pH	6.17-7.89a	6.98-8.13a	7.16-8.12a	7.13-8.16a	7.51-8.55a	7.78-8.33a	7.9-8.45a	7.18-8.19a	7.44-8.45b
NH_3-N (mg/l)	0.0-0.84a	0.0-0.6a	0.0-0.85a	0.0-0.48a	0.0-0.53a	0-0.62a	0.0-0.68a	0.01-0.56a	0.0-0.85a
NO_2-N (mg/l)	0.01-0.05a	0.01-0.30a	0.0-0.5a	0.0-0.8a	0.0-0.08a	0-0.007a	0.0-0.02a	0.00-0.04a	0.0-0.08a
T.D.S (mg/l)	29.1-31.07a	23.55-31.77a	23.81-32.69a	24.17-32.58a	25.54-31.66a	27.63-31.42a	28.27-31.82a	24.44-31.78a	10.62-12.18b

[A] values in the same row with the same lowercase letter are not significantly different ($p>0.05$)

2.0 µl dNTP Mix (10 mM of each dNTP), 10.0 µl Q solution, 0.3 µl of each forward and reverse primer (0.6 µM), 2.0 µl Qiagen One-Step RT-PCR Enzyme Mix, RNA template (1 pg-2 µg) and RNAse free water to make up to 50.0 µl. RT-PCR was performed with a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: reverse transcription at 50°C/30 min; initial PCR activation at 95°C/15 min; 3-step cycling-denaturation at 94°C/40 s, annealing at 49°C/40 s and extension at 72°C/60 s for 25-40 cycles and left at 4°C until the next step.

To evaluate the effect of potential RT-PCR inhibitors, thirty (30) mg of shellfish tissue was also spiked with 10 ul of the HAV stock (positive control; 10-1 dilution) and subjected to the same extraction procedure.

From each RT-PCR product, 10 µl was electrophoresed on 1% agarose gel stained with ethidium bromide and amplicons visualized with gel photo-documentation. Included in each run were a negative control (containing no nucleic acid) and a positive control (RNA from viral stocks). The sample was considered as positive or HAV was considered presence when 489 bp amplicons were detected by gel electrophoresis.

Detection of harmful algae/phytoplankton

Plankton samples were towed by vertical hauling with 20 µm pore size plankton net during low and high tides. Concentrated samples were dispensed into 2 labeled bottles where into one bottle a few drops of Lugol's solution were added while the other remained fresh for immediate examination. A drop of the sample was placed onto a glass slide followed by an examination under the light microscope. Toxic planktons were identified based on several taxonomic manuals (Taylor 1976; Hasle and Syverstsen, 199; Steidenger and Tangent, 1996).

Physico-chemical analysis of seawater

Basic physico-chemical parameters of seawater samples from 9 stations (Fig. 1) were analyzed *in situ* for temperature, salinity, pH and dissolved oxygen (D.O.) and total suspended solids (TSS) using multi parameter water quality probe (YS1 6800, USA). Representative water samples were also brought back to the lab and analyzed immediately for ammoniacal nitrogen (NH_3-N) and nitrite (NO_2-N) spectrophotometrically (HACH DREL 2010, USA). Water quality readings were compared to the proposed Interim National Water Quality Standard for Malaysia (INWQS) (Department of Environment, 2008).

Statistical analysis

All microbial data (FC and EC counts) were transformed to logarithms before analysis. One-way analysis of variance (ANOVA) of means, comparison of means (Tukey's method) and correlation analysis were performed on all data sets using Microsoft Office Excel 2007 at 95% confidence level.

Results

Fecal bacteria counts

Geometric mean of FC counts in seawater samples from nine stations in Sg. Jarum Mas is shown in Figure 2. There is no significant difference ($p>0.05$) between FC counts and sampling dates. However, there is a significant difference ($p<0.05$) in FC counts between the stations examined. FC counts in station jetty were significantly ($p<0.05$) higher than other stations. This station recorded a vast range of FC counts ranging from 7-≥16,000 MPN/100 ml and median of 350 MPN/100 ml. The FC counts in this station were consistently high throughout the study period with more than 90% of samples recorded >100 MPN/100 ml. There were no significant differences ($p>0.05$) in FC counts between stations 1, 2, 3, 4, 5, 6 and raft. Stations 1, 2 and raft recorded median of 23, 22 and 17 respectively, which is slightly higher than the recommended criteria of 14 MPN/100 ml. In addition, more than 10% of the seawater samples from each of these stations exceeded 43 MPN/100 ml. Stations 3, 4, 5 and 6 recorded median FC counts of <14 MPN/100 ml which is in accordance with approved areas criteria under the NSSP. Furthermore, station 7 or control station recorded significantly lowest ($p<0.05$) FC counts throughout the study with median FC counts of 2 MPN/100 ml.

Figures 3 and 4 showed FC and EC counts in cockles, oysters and mussels collected from Sg. Jarum Mas. Cockles were collected from stations 2, 5 and 6 while mussels and oysters were collected from station raft. Statistically there is no significant differences ($p>0.05$) between FC counts and sampling date or between FC counts and type of shellfish. However consistently higher FC counts were observed in mussels compared to oysters or cockles. FC counts in mussel's samples ranged from 300-160,000 MPN/100g with almost 90% of them recorded FC counts of <6,000 MPN/100 g. FC counts in oysters and cockles ranged from <300 - 2,300 MPN/100 g and <300-2,400 MPN/100 g respectively and 100% of the oysters and cockles samples recorded FC counts <6,000 MPN/100 g tissue.

EC counts were below the detection limits in 82%, 70% and 30% of cockles, oysters and mussels samples respectively. Similarly, EC counts were highest in mussels ranging from <300-4,300 MPN/100

g followed by oysters (<300 - 2,300 MPN/100 g) and cockles (<300-900 MPN/100 g respectively). There was a significant difference ($p<0.05$) of EC counts between cockles and mussels as well as oysters and mussels.

Classification of shellfish waters and determination of shellfish safety

Based on the NSSP classification, cockle culture areas at stations 5 and 6 in Sg. Jarum Mas could be categorized as "approved areas" based on their median FC counts which did not exceed MPN of 14/100 ml and not more than 10% of the samples exceeded MPN of 43/100 ml. However the cockle culture area in station 2 could not be categorized as such, as the median FC exceeded 14/100 ml and more than 10% (36%) of the samples exceeded 43/100 ml. Similarly, station raft which is oysters and mussels culture area is categorized as conditionally approved since the median FC is 17/100 ml and more than 10% (24%) of the samples examined exceeded 43/100 ml.

With regard to shellfish safety, according to the Directives 91/492/EC of the European Union, shellfish harvested from Sg. Jarum Mas are classified as Class B where 90% of the samples examined harboured FC and EC counts less than 6,000/100 g and 4,600/100 g respectively. Based on this document, shellfish harvested from Sg. Jarum Mas should not be directly consumed or consumed raw, but must be depurated, relayed or heat treated before being put in the market.

Determination of E. coli antibiotic resistant profile

A total of 69 *E. coli* strains, isolated from seawater and shellfish tissue from various sampling stations were tested for their resistant towards a number of antibiotics. The results demonstrate that all *E. coli* isolates from Sg. Jarum Mas were resistant to erythromycin (100%) and penicillin (100%) while less than quarter of the isolates were resistant to tetracycline (23.2%), oxytetracycline (20.3%) and sulphonamides (20.3%). Lower percentages of resistance were observed for ampicillin (14.5%), streptomycin (14.5%), kanamycin (11.6%), oxalinic acid (11.6%), nalidixic acid (10.1%), chloramphenicol (8.7%), neomycin (8.7%) and doxycyclinehydrochloride (7.2%). All isolates were sensitive to furazolidone.

On the other hand, twenty-six isolates (37.6%) were resistant to 2 antibiotics, sixteen isolates (23.1%) to 3 antibiotics, 1 isolate (1.4%) to 4 antibiotics while another twenty-six isolates (37.7%) were resistant to at least 5 antibiotics. The isolates from station raft exhibited the most antibiotic resistant characteristics with most of the isolates, resistant to more than 5 antibiotics. An isolate with the highest number of resistance (up to thirteen antibiotics) was also found here. Stations jetty, 1 and 3 were another three stations with high number of resistances (≥5) in single isolate.

Detection of HAV

After amplification, HAV was not detected in all shellfish samples examined (Figure 5). The results

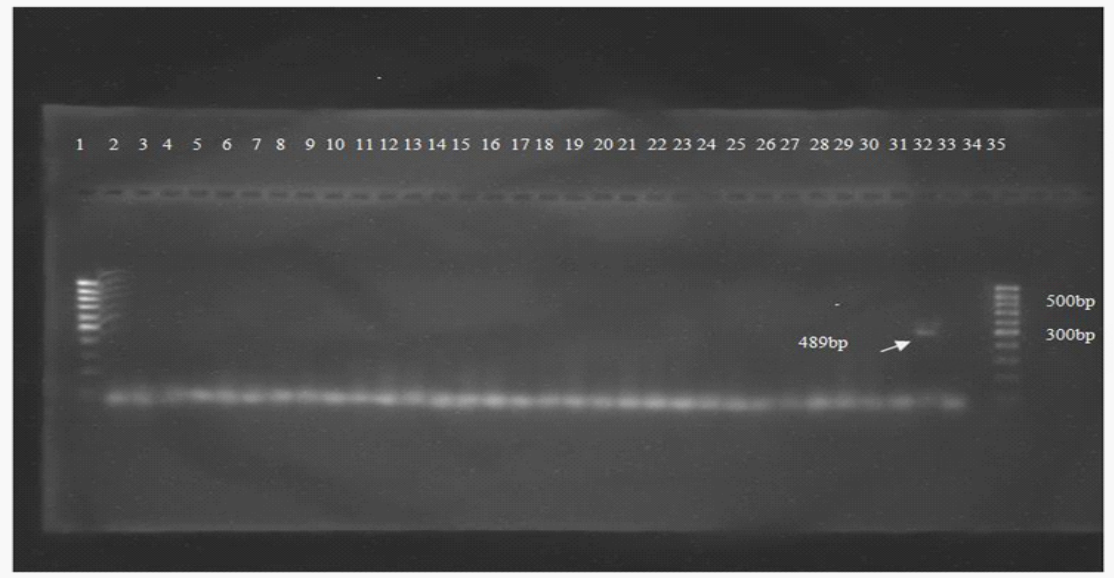

Figure 5. HAV analysis of shellfish from Sg. Jarum Mas by RT-PCR

Lanes 1 and 35: molecular size marker (PCR marker 100 bp); Lanes 2-31 shellfish samples from Sg. Jarum Mas; Lane 32: positive control (30 mg of shellfish tissue seeded with 10^{-1} dilution of HAV stock); Lane 33: negative control; Lane 34: negative control (distilled H_20). Arrows denote 489 bp HAV amplicon

suggest the absence of HAV or very low amount of HAV viral particles in most of the shellfish examined. The subsequent detection of HAV in spiked shellfish samples ensured that the negative results were not due to methodological limitations.

Detection of harmful algae/phytoplankton

Table 4 shows the presence of harmful algae/ phytoplankton in Sg. Jarum Mas during the period of study. The dominant phytoplankton groups found in the area were the diatoms (data not shown). *Pseudonitzschia* spp., one of the harmful diatom genera responsible for the Amnesic Shellfish Poisoning (ASP), was commonly observed in Sg. Jarum Mas shellfish waters particularly during September and December, 2004 and March and April, 2005. Another harmful species regularly detected was *Dinophysis caudata* in December 2004 and January, February, May, June, July 2005. A few other *Dinophysis* spp. was also noted in March and April 2005. The presence of *Noctiluca* sp., another harmful dinoflagellate was also noted in July 2005.

Physico-chemical analysis of seawater

Table 5 list ranges of physico-chemical parameters of seawater samples collected from 9 stations in Sg. Jarum Mas from September 2004-September 2005. In general, all water parameters are within the acceptable ranges for aquaculture activity under the Interim National Water Quality Standard for Malaysia (INWQS). There is no significant difference ($p>0.05$) in the seawater temperature between all stations. On the other hand, there is a significant difference ($p<0.05$) in the salinity, D.O., pH, NH_3-N, NO_2-N and TSS of seawater samples between stations. The D.O. and pH readings were lowest while the TSS was highest at station jetty. On the other hand, the D.O. and pH readings were highest while the TSS was lowest at station 7 (control). There is no particular trend or pattern in NH_3-N and NO_2-N readings among the stations. In addition, there is no negative or positive correlations between the temperature (R^2= 0.54), salinity (R^2= 0.67), D.O. (R^2= 0.42), pH (R^2= 0.44), NH_3-N, (R^2= 0.49), NO_2-N (R^2= 0.32) or TSS (R^2= 0.65) of seawater samples and the FC or EC counts in them. There is also no significant difference ($p>0.05$) between all the physico-chemical parameters examined and sampling dates

Discussions

Although the monitoring of shellfish waters has been carried out regularly in Peninsular Malaysia, it is usually focused on the enumeration of fecal bacteria in shellfish tissue or harvesting waters only. This is the first comprehensive evaluation of shellfish waters which comprises of the determination of fecal bacteria counts (FC and EC) in shellfish waters (harvesting and surrounding areas) and tissue, detection of HAV in shellfish tissue, monitoring of harmful algae species and determination of physico-chemical quality of shellfish waters.

Fecal bacteria contamination is very important in shellfish production where increase in fecal bacteria counts in shellfish waters will lead to closures of shellfish habitat and this could affect the industry. Fecal pollution from non-human (pets, livestock, or wildlife) and human sources is one of the major factors that contribute to the degradation of water quality. From the results, station jetty showed the highest FC counts followed by station 1, 2 and raft. This is not surprising since stations jetty, 1 and 2 are situated in the narrowest part of Sg. Jarum Mas with heavy population on both sides of the riverbanks. This might be the source of constant influx of sewage (mostly from human, livestock and pets) into the waters. Although station raft is not highly populated as stations jetty and 1, the direct untreated fecal material discharged by the farm workers and animals living on the raft could explain the high FC counts recorded at this station. Accordingly, stations 3, 4, 5 and 6 recorded low counts of FC since they are situated away from human settlements. Their location which is close to the river mouth could also bring about better dispersant of fecal bacteria due to the strong mixing of water and current compared to the more "static" state of seawater at stations jetty, 1 and 2.

There are no significant differences between the FC counts and different sampling period or dates although a slightly higher FC counts were noted in December, 2004 compared to other sampling months. Based on the NSSP shellfish water classification criteria, stations 5 and 6 at Sg. Jarum Mas, which are cockles culture areas can be classified as 'approved areas" while stations 2 (cockle culture area) and raft (mussels and oysters culture areas) as "conditionally approved'.

Although FC counts are not very high in the seawater samples, but their counts in shellfish tissue are many times higher. This is probably due to accumulation by the shellfish. In general, FC counts were consistently found to be higher in mussels compared to oysters and cockles. The high counts of fecal bacteria in mussels and oysters observed might be due to greater accumulation factor possess by them as compared to cockles. This observation agrees with Wang (1996) which reported high fecal

coliforms accumulation factor for *P. viridis* (7.5-366.6) and *Crassostrea* spp. (4.8-69.5) as compared to cockles (0.4-1.75). The same observation has also been noted in samples from Melaka and Johor Bharu (Wan Norhana and Nor Ainy, 2004). Based on the EC directives 91/492/EEC, shellfish from Sg Jarum Mas could be classified as Class B which means that shellfish harvested from here should not be consumed raw and must be depurated, relayed or heat-treated before being put in the market.

As mentioned in the introduction, resistance patterns to antibiotics commonly used in particular practices may give some indications of the source of fecal contamination. The differentiation of animal or human sources of fecal contamination will assist water resource managers in developing strategies to protect shellfish harvesting areas and thus reducing the public health risk from these waters. Harwood et al. (2000) reported that fecal bacteria resistant to ampicillin were most often associated with humans, whereas resistance to tetracycline and streptomycin was most commonly associated with animals. In addition, Kelsey et al. (2003) indicated that penicillin resistance was likely to be indicative of human or animal sources. Results of antimicrobial susceptibility test carried out showed that most of the isolates were resistant to antibiotics used in animals and human such as erythromycin, penicillin, streptomycin, tetracycline, oxytetracycline and to a lesser extent ampicillin, thus confirming the earlier assumption that sources of fecal bacteria in Sg. Jarum Mas sampling stations are from human and livestock drainage from the surrounding areas. Results also indicated that complex profiles of antibiotic resistance in *E. coli* are more prominent in stations with higher population density as mentioned above. The results suggest that E. coli resistance pattern is site or station dependant. However there is no clear difference in the antimicrobial resistance pattern between *E. coli* isolated from shellfish or seawater. On the other hand, simpler resistant profiles of *E. coli* were observed in stations 1, 2 and 7 (control). E. coli isolated from station 1 and 2 might originate from wildlife from nearby mangrove which provides habitat appealing to the animals. The dense mangrove acts as a buffer and filters out bacteria run-off entering the waters and thus explaining the more antibiotic-sensitive *E. coli*. As for station 7, lower resistance might be due to its location being far from human or animal sewage influx. High percentages of resistance to antibiotics in *E. coli* isolated from seafood and aquatic environment have been observed and reported elsewhere. Adesiyun (1993) reported resistance as high as 76.7% in *E. coli* isolated from Trinidad seafood. Kumar et al., (2005) has also described *E. coli* isolates from seafood that are resistant to antibiotics in India. Meanwhile Harakeh et al. (2005) also reported high rates of antimicrobial resistance in *E. coli* isolated from Lebanese aquatic environment.

Several studies have demonstrated the successful application of PCR in the detection of HAV in laboratory. However, few studies have been reported on the application of molecular methods for HAV in naturally polluted shellfish (Chironna et al., 2002). This is the first few attempts to analyze HAV in wild and cultured shellfish of Peninsular Malaysia by RT-PCR technique. The results suggest the absence of HAV or very low amount of HAV viral particles in most of the shellfish examined and not due to methodological limitations since the efficiency of the RT-PCR was controlled by running the seeded HAV or positive control. The very low detection of HAV in naturally contaminated shellfish observed in this study is in accordance with other reports. HAV detection using various extraction methods and RT-nested PCR and real time RT-PCR in 30 retail cockles' samples from Serdang, Malaysia also failed to detect HAV (Tek, 2009). Vilariño et al. (2009) was also not successful in detecting HAV in wild and cultured shellfish in France. Others, however, have reported higher HAV prevalence such as 26% in mussels and clams from Tunisia (Elamri et al., 2006), 20-23% in mussels from Italy, (Chironna et al., 2002) and 27.4% in shellfish from Spain (Romalde et al., 2002). The comparison of percentage of HAV detected in all the studies is however, difficult as different conditions (sampling site, extraction method, detection method, sample size etc) employed in each of them tend to yield varying results.

The events of phytoplankton blooms in coastal waters seem to be increasing and receiving growing attention in newspapers, electronic media and scientific literature. As a result, more researchers are now surveying their local waters for the harmful species (Hallegraeff, 2006). For shellfish, harmful effects of these species may include oxygen depletion in the water as a result of phytoplankton respiration at night or decomposition of cells and production of toxins (Martin, 2004). In this study, only a few harmful phytoplankton species have been observed in the shellfish waters of Sg. Jarum Mas. The most common harmful species observed was *Dinophysis caudata*, which caused Diarrhetic Shellfish Poisoning (DSP) and a few other *Dinophysis* spp.. This species is detected in most of the sampling months. Other harmful species detected are the diatoms that are responsible for the Amnesic Shellfish Poisoning (ASP) cases i.e *Pseudonitzschia* spp.. Their presence

is less frequent and only detected in September and December 2004 as well as March and April in 2005. However the identification of the toxic species of *Pseudonitzschia* such as *P. multiseries*, *P. australis* and *P. pungens* were not able to be carried out due to its submicroscopical structures which can only be shown by electron microscopy and not light microscopy as used in our laboratory. *Noctiluca* spp. was also observed occasionally. This species is however more associated with fish kill event. Harmful species that produce toxin and cause shellfish poisoning such as *Alexandrium* spp., *Gymnodinum* spp. and *Pyrodinium* spp. have not been observed in the seawater samples from Sg. Jarum Mas. The finding supports the suitability of Sg. Jarum Mas as model shellfish farm.

In general, the physico-chemical characteristics of 9 selected stations in Sg. Jarum Mas adhered to the INWQS criteria for Class 111 (Fishery 111-Common of economic value and moderately tolerant aquatic species) waters. Water temperature affects a multitude of important processes in aquaculture. The deviations of seawater temperature readings were ± 1.2, which adheres to the recommended level that allows up to ±2ºC. The average pH of seawater samples were 7.73 ± 0.4 which are also within the recommended range 5.0-9.0. Meanwhile the NH_3-N content of seawater samples ranges from 0-0.85 mg/l just below the recommended levels of 0.9 mg/l. NO_2-N and TSS readings were way below the standards (0.4 mg/l and 150 mg/l respectively). The results imply that the physico-chemical characteristics of seawaters in Sg. Jarum Mas especially shellfish waters (stations 5, 6, raft) are suitable for aquaculture activity of moderately tolerant species such as shellfish.

Conclusion

In conclusion, results from this study indicate that the physico-chemical parameters of shellfish waters in Sg. Jarum Mas are well below the standards. HAV is not detected in shellfish samples and only few harmful algae/phytoplankton are observed in the shellfish waters. Although 2 of the shellfish areas examined are classified as "conditionally approved", the FC counts recorded are not even 2 folds higher than the guidelines. Furthermore, this could be overcome by moving the culture area towards the river mouth or away from the highly populated area. Together with its good location, infrastructure and networking support from the Department of Fisheries, Sg. Jarum Mas has a great potential to be groomed as a model shellfish farm.

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