CHAPTER 3

MATERIALS & METHODS

This chapter consists of the following main parts, which are (i) study sites, (ii) water sampling, (iii) water sample analysis and culture work, (iv) mean sulphite reducing clostridia (MBCC) and mean *Clostridium perfringens* (MCPC) densities calculation, (v) data analysis, and (vi) PCR detection of toxin genes in *Clostridium perfringens*.

3.1 Study Sites

Sungai Bernam was chosen in this study to represent a cleaner river. Compared to Sungai Bernam, Sungai Selangor has a larger catchment area upstream, but a denser population downstream that would probably accumulate more pollutants. Tengi Canal, a man-made counterpart was also examined. Sungai Selangor was studied at Ampang Pecah, Kampung Timah and Rantau Panjang; Sungai Bernam at Tanjung Malim and Jambatan SKC; and Tengi Canal at Ibu Bekalan Sungai Bernam and Jambatan Mergastua. These sites are actually also the gauging stations of the Department of Irrigation & Drainage Malaysia (DID) in the respective rivers, at the following locations (Fig. 3.1) :

1) Sungai Selangor at Ampang Pecah (DID Malaysia station no. 3516424)
   (upstream, 03 32 25, 101 39 52) – Site A
2) Sungai Selangor at Kampung Timah (DID Malaysia station no. 3415433)
   (midstream, 03 29 08, 101 32 21) – Site F

3) Sungai Selangor at Rantau Panjang (DID Malaysia station no. 3414421)
(downstream, 03 24 10, 101 26 35) – Site G

4) Sungai Bernam at Tanjung Malim (DID Malaysia station no. 3615412)
(upstream, 03 40 45, 101 31 20) – Site B

5) Sungai Bernam at Jambatan SKC (DID Malaysia station no. 3813411)
(downstream, 03 48 15, 101 21 50) – Site C

6) Tengi Canal at Ibu Bekalan Sungai Bernam and Jambatan Mergastua
(03 41 33, 101 20 32; 03 40 03, 101 20 49) – Site D1D2E

Site A, is located in Ampang Pecah, Kuala Kubu Bharu near a school and hostel. The river water was usually clear, although there was a period when the water turned slightly yellowish but still clear. There were quarry and logging activities within 10 km upstream from Site A. Kuala Kubu Bharu is the heart of Sungai Selangor Water Supply Scheme Phase 3, whereby an area of 197 km^2 upstream from Sungai Selangor Dam has been gazetted as water catchment reserve (Syarikat Pengeluar Air Sungai Selangor Sdn Bhd, Protection of Raw Water Source).

Site F, the mid-stream sampling site of Sungai Selangor, is located in Kampung Timah. The immediate 5 km vicinity of Site F is a feed lot farming area. Hence, high level of faecal pollution was expected. Besides cattle farming, aquaculture and patches of swamp area are also observed. Soil in the vicinity is mostly exposed, with some abandoned housing development.

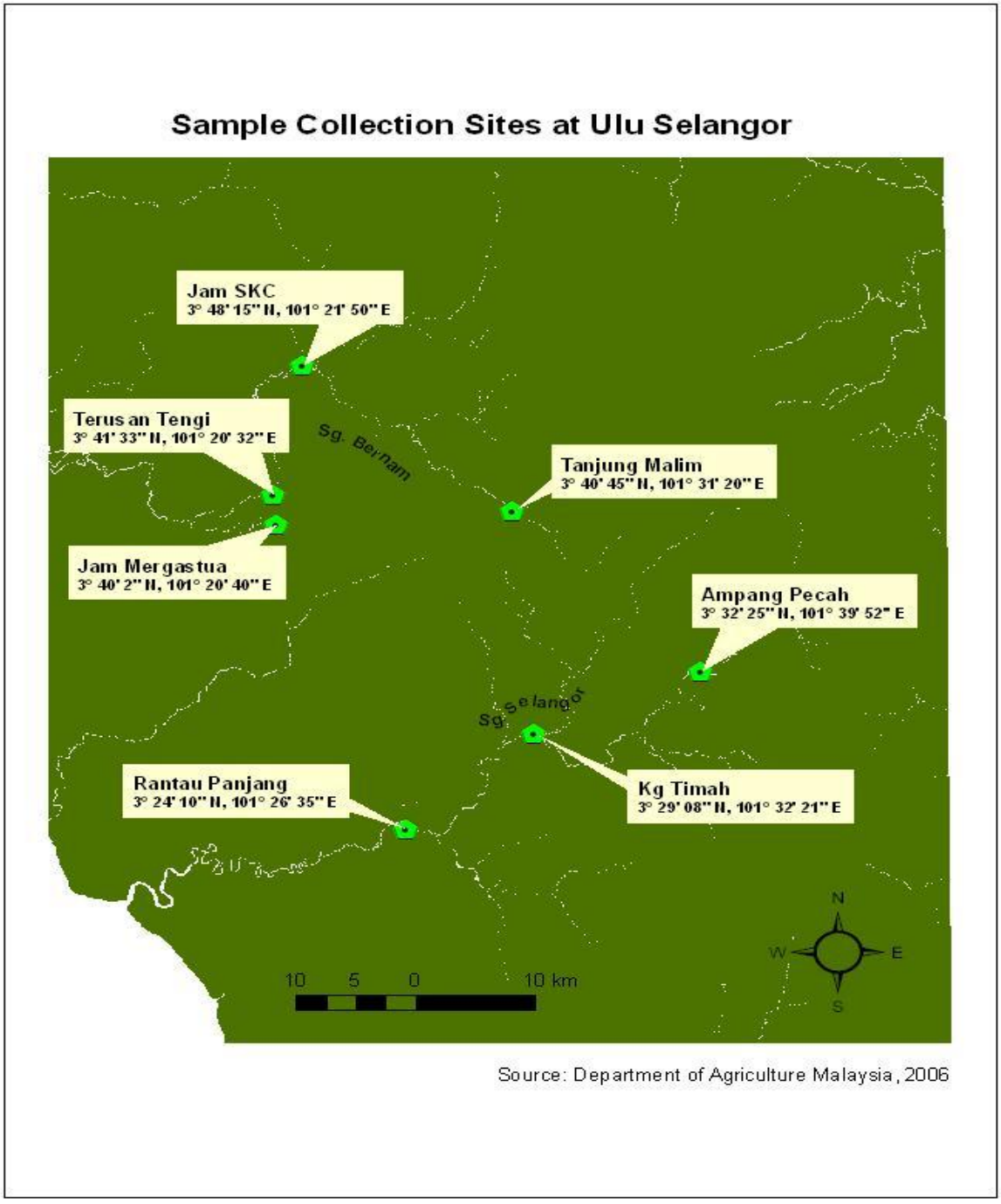

Fig. 3.1 Study Site Locations

Site G is situated in Rantau Panjang, in a detached village housing settlement. River diameter differences of about ten meters were noticed during the sampling period. The village next to Site G occasionally gets flooded. There is a siren set up by the DID Malaysia to warn nearby villagers about possible flooding.

Site B in Tanjung Malim is the upstream sampling site of Sungai Bernam. This site marks the border between the Selangor and Perak state, and is situated in the Tanjung Malim town. Hence, appreciable level of faecal pollution was expected in Site B although it is an upstream site. There are two sewage discharge outlets just a few meters below Site B, near the Tanjung Malim bridge (Appendix H5).

Site C is located at Jambatan SKC, the downstream of Sungai Bernam. It is also a village housing settlement like Site G. There are palm oil plantations in the vicinity. Fishing activities are also observed. River diameter in Site C did not vary that much as compared to Site G, and less domestic wastes were seen.

Sampling site at Ibu Bekalan Sungai Bernam and Jambatan Mergastua (Appendix H6) of Tengi Canal are collectively referred as Site D1D2E, since the two sites are only a few minute's car drive apart. Ibu Bekalan Sungai Bernam (D1D2) is a water dam whereas Jambatan Mergastua (E) is an artificial river drainage created for downstream paddy field irrigation. Fresh water fisheries product is abundant in Tengi Canal.

3.2 *Clostridium perfringens* and Sulphite Reducing Clostridia Detection

CP belongs to the family of sulphite reducing Clostridia that utilizes sulphite and produces sulphide. Two selective media were used to detect CP in this study, the Tryptose Sulphite Cycloserine (TSC) and Oleandomycin Phosphate Sodium Sulphadiazine (OPSP) media. These media contain ferric ammonium ions that react with sulphide so that true CP colonies appear black or grey. However, some sulphite reducing but non-CP Clostridia are able to grow despite the use of selective antibiotics in the media. Therefore, biochemical tests need to be carried out to confirm the presumptive CP as true CP. The most important tests for CP identification are the nitrate, motility, lactose and gelatin tests. CP is non-motile, reduces nitrate to nitrite, consumes lactose and gelatin (Health Canada, 2001). Alternatively, CP identification may also be confirmed by Polymerase Chain Reaction (PCR) using specific primers to detect alpha toxin gene of CP (Yoo *et al*., 1997; Augustynowicz *et al*., 2002; al-Khaldi *et al*., 2004a). This is based on the understanding that most, if not all CP strains possess the alpha toxin gene (Gurjar *et al*., 2008)

3.3 Water Sampling

Fortnightly water samplings were carried out at the study sites with assistance from the Selangor DID personnel. This sampling frequency was in accordance to the proposed fortnightly water quality sampling for bathing water in the European countries (Figueras *et al*., 1997). In Ampang Pecah (Site A), Rantau Panjang (Site G), Tanjung Malim (Site B) and Jambatan SKC (Site C), depth-integration method water sampling was performed at the first, middle and third quarter points across the river (Moody and Troutman, 1992). This yielded a total of three replicates per site that provided a better picture of microbial

variations in the river. However, the simple surface grab sampling was also applied in the remaining sites due to limited facilities. Water samples were kept in 1242 ml Nasco Whirl Pack at room temperature. Physico-chemical parameters of the river water were measured by DID Selangor personnel.

3.4 Water Sample Analysis and Culture Work

3.4.1 Water sample analysis. Water samples were analyzed within three days of acquisition. Each water sample was subjected to 1/10 three-step serial dilution followed by membrane filtration using Sartorious 0.45 μm cellulose nitrate filter membranes. Initial examination showed that presumptive CP (sulphite reducing Clostridia, SRC) counts of below 80 colony forming unit (CFU) were achievable within three dilution steps. For each water sample, membrane filtration was performed four times to produce four replicates. The replicates were plated on 2 TSC media (without egg yolk) and 2 OPSP media (Oxoid) plates, which were then incubated in anaerobic jars (Oxoid Anaerogen sachets and anaerobic indicator) at 37 ºC for two days.

3.4.2 Presumptive CP enumeration, subcultures and biochemical confirmation tests. Colony forming unit of presumptive CP (sulphite reducing Clostridia, SRC) on all filtered membranes were enumerated. For each water sample, twenty one (or less) well developed black or grey colonies were pooled from the four filter membranes of second-step dilutions and subcultured on freshly prepared TSC agar (Merck). Enumeration and subcultures were completed within three hours of removal from anaerobic jar. The subcultured plates were then incubated anaerobically for two days. After two days, well developed and detached presumptive CP colonies of at least 2 mm were picked for lactose, gelatin, nitrate and

motility tests (Amyl Media). Nitrate Motility test was performed with stab wire whereas 1 µl disposable loops were used for Lactose gelatin tests inoculation. Inoculated tubes were incubated overnight at 37 ºC. Readings were taken the following day. The expected CP characteristics are non-motile growth along stab line, reducing nitrate to nitrite (Microgen Nitrate A and B), and consuming both lactose and gelatin (Health Protection Agency, 2005).

3.5 Mean Sulphite Reducing Clostridia (MBCC) and Mean *Clostridium perfringens* (MCPC) Densities Calculation

3.5.1 Mean sulphite reducing Clostridia densities (MBCC) in cfu/100ml. Sulphite reducing Clostridia (SRC) density in the unit of cfu/100ml was calculated for each water sample replicate (Health Canada, 2008). SRC density of two replicates cultured on TSC media were then averaged to produce "TSC Sulphite Reducing Clostridia Density" (TSC MBCC) per water sample (refer Appendix A1 to A6). Finally, TSC MBCC of three water samples in the same study site were then averaged to produce the "Mean Sulphite Reducing Clostridia Density" (or simply MBCC) of that study site.

3.5.2 *Clostridium perfringens* isolation rate (IRt), mean *Clostiridium perfringens* density (MCPC) in cfu/100ml and *Clostridium perfringens* prevalence. The pooling method used in selecting presumptive CP for biochemical confirmation tests (refer section 3.3.2) had produced CP isolation rate (IRt), as shown in the first equation below. Mean *Clostridium perfringens* density (MCPC) in cfu/100ml of individual study site was subsequently calculated from the mean sulphite reducing Clostridia density (MBCC) and

IRt using the second equation (Health Canada, 2001). CP prevalence of a study site was produced with the third formula. These data could be read from Table 4.1 and Appendix A7 to A12.

$$\text{CP Isolation Rate (IRt)} = \frac{\text{Total number of confirmed CP isolates per site}}{\text{Total number of selected sulphite reducing Clostridia isolates per site (Nt)}} \times 100\ \% \quad \text{.....(1)}$$

$$\text{Mean } \textit{Clostridium perfringens} \text{ Density (MCPC)} = \frac{\text{IRt}}{100} \times \text{MBCC} \quad \text{.....(2)}$$

$$\text{CP Prevalence} = \frac{\text{Number of CP Detection}}{\text{Number of Sampling Trips}} \times 100\ \% \quad \text{.....(3)}$$

Total number of selected sulphite reducing Clostridia isolates per site (Nt) was the sum of all SRC selected for confirmation tests from all three water samples of a study site. Similarly, "total number of confirmed CP isolates per site" was the sum of all confirmed CP from the three water samples. "CP detection" meant at least one CP isolate was detected among water sample(s) of the sampling site.

3.6 Data Analysis

Mean river discharge (Q) data were acquired from the Malaysian Drainage and Irrigation Department. Data analysis was conducted using SPSS 11.5 software. Normality of densities was checked with the Shapiro-Wilk test. Differential tests on densities among water sample

replicates and also site replicates involved the Friedman test. Temporal differences of the densities were also examined with the Kruskal-Wallis Test. The Spearman Rho's test was used to evaluate correlation between river discharge, MCPC and MBCC within the same study site, and also river discharge and densities across and along the rivers. Relationship between river discharge, MBCC and MCPC were assessed with Curve Estimation.

3.7 *Clostridium perfringens* Toxin Gene Detection

3.7.1 DNA Extractions. DNA of biochemically confirmed CP isolates were extracted using the Bio-Basic BS423 EZ-10 Spin Column Genomic DNA Kit for Bacterial Samples (Bio-Basic Inc.) according to manufacturer's instructions.

3.7.2 Toxin Gene Primers. Two sets of multiplex toxin genes primers (Bio-Basic Inc.) were used for this study. Primers Set 1 was adopted from Yoo *et al.* (1997) that detects four toxin genes which were the alpha, beta, epsilon and iota (refer Table 3.1). The enterotoxin (CPE) gene was detected with forward primer 5'-AGGAGATGGTTGGATATTAGG-3' and reverse primer 5'-CCATCACCTAAGGACTGTTC-3' as described by Meer and Songer (1997) but with slight differences (GenBank reference no.X81849). Meanwhile, a new set of multiplex primers, the Primers Set 2, were designed using the Invitrogen Designed D-Lux^{TM} Primers primer design software to detect six toxin genes including CPB2 and CPE (refer Table 3.2). PCR amplicons of these six toxin genes have size differences of about 100 basepairs that would produce easily distinguishable bands on agarose gels during electrophoresis. The toxin gene fragments used for designing Primer Set 2 were based on Gene Bank reference numbers as reported by Meer and Songer (1997).

3.7.3 Polymerase Chain Reaction (PCR) and Gel Electrophoresis. PCR master mixes were prepared from Bioron products. This included DFS-Taq DNA Polymerase 5000 units/ml enzyme, 4 dNTPs Mix (10mM of each), 100 bp Blue Extended DNA Ladder, and Loading Buffer DNA IV (10x). The DFS-Taq DNA Polymerase enzyme were supplied with 1 ml of Reaction Buffer (x10) Complete that contained 160 mM $(NH_4)SO_4$, 670mM TrisHCl pH 8.8, 0.1% Tween-20 and 25mM $MgCl_2$. Lyophilized primers were re-hydrated into 100 µM stock solution as prescribed, and then diluted into 10 ρmol/µl working concentration.

PCR in this study were performed using Eppendorf MasterCycler® Gradient, Applied Biosystems 2720 Thermal Cycler or Perkin Elmer GeneAmp PCR System 2400, whichever was available during the experiments. MJ-105 Mini Horizontal Gel Electrophoresis System and MP250N Power Supply from Major Science were used in gel electrophoresis. PCR products in agarose gels were captured with AlphaImager HP (AlphaInnotech) system.

Table 3.1 Primers Set 1 (adopted from Yoo *et al*., 1997)

Primer Name	Toxin Gene	Product Size (bp)	Location in Gene	Primer Sequence	Primer bp	Tm Calculation (°C) = 2(A+T) + 4(C+G)
CPB-F3	Beta	236	589-613	5'- ACTATACAGACAGATCATTCAACC - 3'	24	2(10+5) + 4(7+2) = 66
CPB-R3			824-801	5'- TTAGGAGCAGTTAGAACTACAGAC - 3'	24	2(9+5) + 4(4+6) = 70
EtxD-F3	Epsilon	541	436-459	5' - ACTGCAACTACTACTCATACTGTG – 3'	24	2(7+7) + 4(7+3) = 68
EtxD-R3			976-953	5' - CTGGTGCCTTAATAGAAAGACTCC – 3'	24	2(7+6) + 4(6+5) = 70
CPA-F3	Alpha	402	511-535	5' - GTTGATAGCGCAGGACATGTTAAG – 3'	24	2(7+6) + 4(3+8) = 70
CPA-R3			913-889	5' - CATGTAGTCATCTGTTCCAGCATC – 3'	24	2(5+8) + 4(7+4) = 70
iA-F3	Iota	317	563-586	5' - GCGATGAAAAGCCTACACCACTAC – 3'	24	2(9+3) + 4(8+4) = 72
iA-R3			879-856	5' - GGTATATCCTCCACGCATATAGTC – 3'	24	2(6+ 7) + 4(7+4) = 70

Table 3.2 Primers Set 2

Primer Name	Gene Bank Number	Product Size (bp)	Location in Gene	Primer Sequence	Primer Bp	Tm Calculation (°C) = 2(A+T) + 4(C+G)
CPB1-F2	L13198: Beta Toxin Gene 1	356	1147-1502 = 356 bp	c t g c t c c t a a t g g t a c t g a a g	21	2(5+6) + 4(5+5) = 62
CPB1-R2				t g a g g c t t c t t a g g t t g a c a g	21	2(4+7) + 4(3+7) = 62
EtxD-F2	M95206: Epsilon Toxin	442	252-693 = 442 bp	g c a t c a g c g g t g a t a t c c a t C	21	2(5+5)+ 4(6+5) = 64
EtxD-R2				c t t g t a t c g a a g t t c c c a c a g	21	2(5+6) + 4(6+4) = 62
CPE-F2	X81849: Enterotoxin Gene	262	410-671 = 262 bp	c t a c a a c t g c t g g t c c a a a t g	21	2(6+5) + 4(6+4) = 62
CPE-R2				g t a g c a g c a g c t a a a t c a a g g	21	2(8+3) + 44+6) = 62
CPA-F2	X13608: Alpha Toxin Gene	617	1137-1753 = 617 bp	t a c c t g a c a c a g g g g a a t c a C	21	2(7+3) + 4(6+5) = 64
CPA-R2				T g g g t t g t c c a t t t c c c a t t c	21	2(2+9) + 4(6+4) = 62
iA-F2	X73562: Iota Toxin Gene	707	2012-2718 = 707 bp	A t g a a c c a g g t a a t g g c g a T G	21	2(7+4) + 4(3+7) = 62
iA-R2				t c c t g c a t a a c c t g g a a t g g C	21	2(5+5) + 4(6+5) = 64
CPB2-F2	AJ537530: Beta Toxin Gene 2	202	531-732 = 202 bp	C A c a a g c a a t t g g g g g a g t t t a	22	2(7+5) + 4(3+7) = 64
CPB2-R2				g c a g a a t c a g g a t t t t g a c c A T	22	2(7+6) + 4(4+5) = 62

3.7.4 Alpha Toxin Gene Detection and Optimization. Alpha toxin gene detections in this study were performed using alpha toxin gene primers of Primers Set 1 (Yoo *et al*., 1997). The following optimized PCR conditions had produced specific band. PCR ingredients in 25 µl assay are also shown.

Optimized PCR conditions, at 25 cycles:

1) Initial Denaturing : 5 min, 94 °C
2) Denaturing : 20 s, 94 °C
3) Annealing (Tm) : 20 s, 60 °C
4) Extension : 20 s, 72 °C
5) Final Extension : 5 min, 72 °C
6) Holding : 15 °C

PCR ingredients and concentrations in 25 µl assay:

1) DNA : 1.0 µl (about 1 ng)
2) Forward and Reverse primers : 0.4 ul each (4 ρmol in 25 µl reaction)
3) dNTP mix : 0.4 µl (160 µM in 25 µl reaction)
4) Reaction Buffer (x10) : 2.0 µl (2 mM of $MgCl_2$ in 25 µl reaction)
5) Taq DNA Polymerase : 0.2 µl (1 Unit in 25 µl reaction)
6) Distilled Water : 21.0 µl

To perform gel electrophoresis, 2% agarose gels of 25-well (2.8 mm x 1.5 mm) or 17-well (4.0mm x 1.0 mm) were prepared from Pronadisa Low-EEO Agarose and Bio Basic TBE 10x powder diluted into 1x concentration. The MJ-C01 combs of Mini Horizontal Gel Electrophoresis System were used for gel casting. Gels were pre-stained with Cambrex GelStar Nucleic Acid Gel Stain with the ratio of 1 µl stock stain to 10 ml of agarose. 9 µl of each PCR products were loaded. Electrophoresis of PCR products were performed at 120 V with 1x TBE.

3.7.5 Beta, Epsilon, Iota and CPE Toxin Gene Detection. Beta, epsilon, and iota toxin genes were detected using Set 1 Primers (Yoo *et al*., 1997) whereas CPE toxin gene was detected with the adapted primers from Meer & Songer (1997). Monoplex PCR was performed for 45 cycles with the following conditions :

1) Initial Denaturing : 5 min, 94 °C
2) Denaturing : 30 s, 94 °C
3) Annealing (Tm) : 30 s, 60 °C
4) Extension : 30 s, 72 °C
5) Final Extension : 7 min, 72 °C
6) Holding : 15 °C

3.7.6 Duplex PCR of Alpha and CPE Toxin Gene Detection. CP isolates harbouring alpha and CPE toxin gene from monoplex PCR were subjected to alpha and CPE toxin gene duplex PCR using Set 2 Primers. From the duplex PCR master mix, each isolates received 2.5 µl of complete reaction buffer while DNA template was increased to 2 µl

(equivalent to 2 ng of DNA). Concentration of other ingredients remained the same (refer section 3.6.4). PCR was performed for 35 cycles, with conditions as stated in section 3.6.5.

3.7.7 PCR Product Sequencing. Representative toxin gene PCR bands were excised, purified with commercial kit (Bio-Basic) and submitted for commercial sequencing. Sequencing results were read with Chromas software programme and blasted against nucleotide sequence in GenBank.