CHAPTER 4

RESULTS

4.1 Presumptive *Clostridium perfringens* (Sulphite Reducing Clostridia) Colonies Isolation from TSC and OPSP Selective Media

Presumptive *Clostridium perfringens* (sulphite reducing Clostridia) of various morphology growing on TSC and OPSPS media were generally less than 3mm in diameter (refer Appendix H1 and H2). Yellowish mucotic colonies also frequently grew on the selective media. They were referred as streptococci by the OPSP Supplement Product Info. Compared to OPSP media, TSC media had a lower frequency of harbouring colonies that caused partial or whole plate opaqueness whereby the plate would appear dirty and the features of the black colonies were indistinct (refer Appendix H3). Therefore, colony count and densities calculation of this study were based on reading from TSC media. Morphology of *Clostridium perfringens* (CP) is shown in Appendix H4.

4.2 Pooling Method in Presumptive *Clostridium perfringens* (Sulphite Reducing Clostridia) Isolates Selection

Due to research limitation, pooling method had to be applied whereby only 21 (or less) presumptive CP isolates were selected from four replicates of each water sample to be sub-cultured and subsequently subjected to Nitrate Motility and Lactose Gelatin confirmation tests. It happened that the number of confirmed CP were also mostly low

throughout this study (refer Appendix A7 to A12). The pooling method is therefore considered cost and labour effective.

4.3 Comparison Among Rivers and Study Sites : Mean Sulphite Reducing Clostridia Densities (MBCC), *Clostridium perfringens* Prevalence and Mean *Clostridium perfringens* Densities (MCPC)

MBCC, CP isolation rate (IRt) and MCPC of Sungai Selangor, Sungai Bernam and Tengi Canal are presented in Table 4.1. Arithmetic means of the respective study sites, which are the overall means for the study period were also displayed in Fig. 4.1. Both arithmetic MBCC and MCPC of Site G were about 3 times higher than Site C. This means at downstream level, Sungai Selangor was more polluted with Clostridia (including CP) compared to Sungai Bernam. However, the result was not normalized with catchment area size difference between the two rivers. MCPC fluctuations in Site G were also more drastic, with five non-detection and three high CP densities (>1000 CFU/100ml) in 11 sampling events. MBCC counts in Bernam River were the lowest of the three rivers studied. Tengi Canal showed moderate MBCC but low MCPC. Arithmetic MBCC and MCPC in Sungai Selangor increased towards downstream study site, with density differences of about 6 times higher from Site A to Site F. This is in contrast to the trend of decreasing MBCC and MCPC towards downstream in Sungai Bernam.

Table 4.1 Mean Sulphite Reducing Clostridia Density (MBCC), *Clostridium perfringens* Isolation Rate (IRt) and Mean *Clostridium perfringens* Density (MCPC): Comparison Among Rivers

Sites MBCC [a], CP Isolation Rate (IRt), and MCPC [a], 02 Apr 2007 – 21 Jan 2008

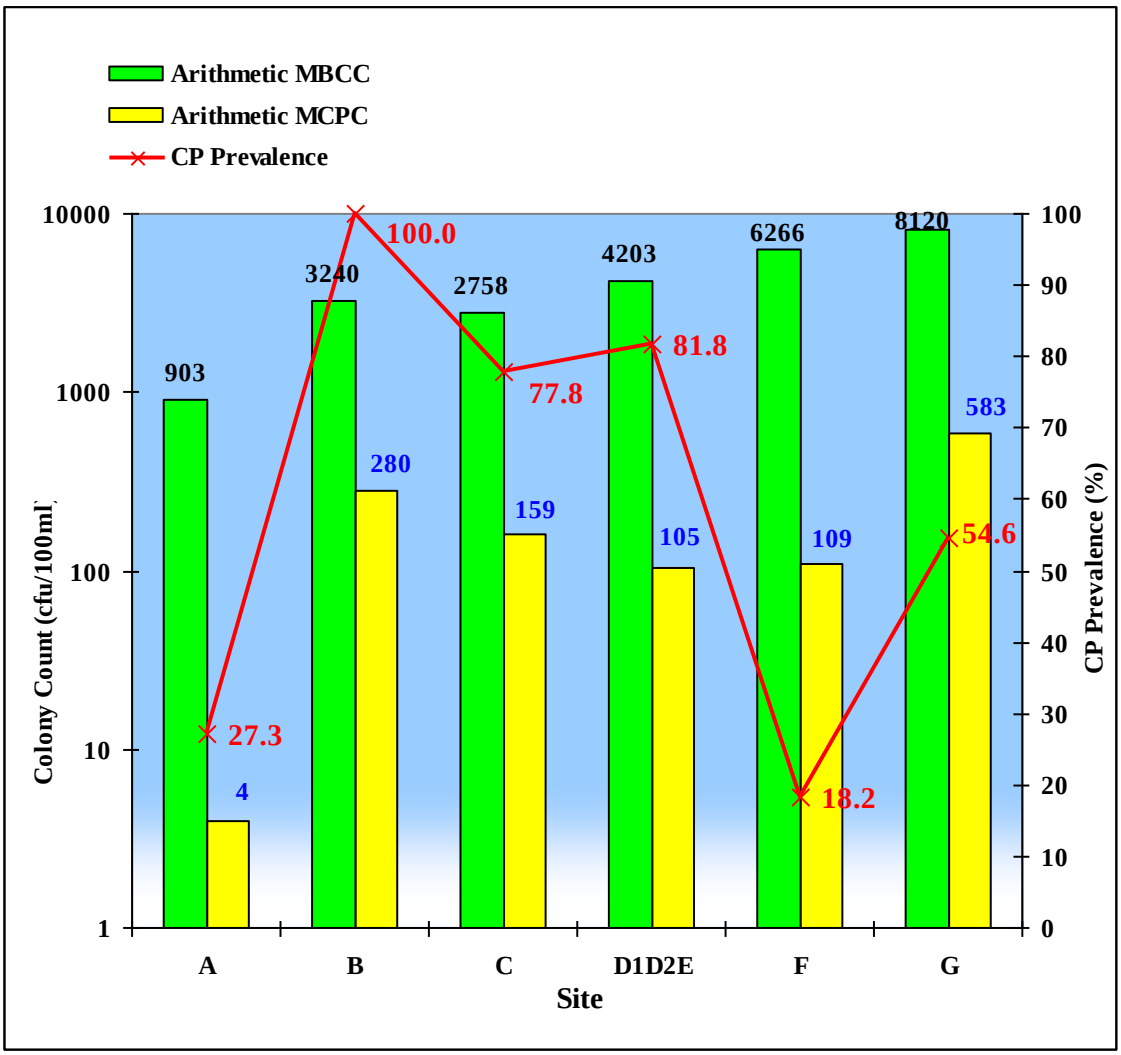
Sungai Selangor	**Site A**			**Site F**			**Site G**		
	MBCC	CP Isolation Rate (IRt)	MCPC	MBCC	CP Isolation Rate (IRt)	MCPC	MBCC	CP Isolation Rate (IRt)	MCPC
02.04.07	- [c]	-	-	3685	-	-	3942	-	-
16.04.07	220	-	-	2475	-	-	-	-	-
07.05.07	-	-	-	-	-	-	-	-	-
21.05.07	2365	0.00	ND[b]	-	-	-	6233	4.44	277
04.06.07	-	-	-	8965	0.00	ND	10780	25.00	2695
15.06.07	183	5.13	9	5775	0.00	ND	2750	0.00	ND
30.07.07	257	0.00	ND	4015	0.00	ND	4565	0.00	ND
17.08.07	183	0.00	ND	4730	0.00	ND	8562	3.51	301
04.09.07	1283	0.00	ND	8250	5.26	434	7150	0.00	ND
01.10.07	202	5.08	10	2365	0.00	ND	-	-	-
17.10.07	348	6.67	23	4895	0.00	ND	6838	1.85	127
27.10.07	862	0.00	ND	7040	0.00	ND	10028	19.23	1928
27.11.07	807	0.00	ND	11330	0.00	ND	9698	0.00	ND
11.12.07	3465	0.00	ND	12540	0.00	ND	17435	6.25	1090
21.01.08	660	0.00	ND	5390	14.29	770	9460	0.00	ND
Arithmetic Mean	**903**		**4**	**6266**		**109**	**8120**		**583**

Sungai Bernam	**Site B**			**Site C**			**Tengi Canal Site D1D2E**		
	Trip MBCC	CP Isolation Rate (IRt)	Trip MCPC	Trip MBCC	CP Isolation Rate (IRt)	Trip MCPC	MBCC	CP Isolation Rate (IRt)	MCPC
02.04.07	-	-	-	-	-	-	-	-	-
16.04.07	917	-	-	3603	-	-	1503	-	-
07.05.07	-	-	-	-	-	-	1412	4.65	66
21.05.07	1503	10.91	164	1513	12.90	195	-	-	-
04.06.07	-	-	-	-	-	-	2695	0.00	ND
15.06.07	770	15.22	117	2347	9.52	223	1027	10.26	105
30.07.07	1980	1.96	39	2182	0.00	ND	3667	3.64	133
17.08.07	1118	1.79	20	1577	3.57	56	2493	5.66	141
04.09.07	10303	3.64	375	4803	0.00	ND	8983	1.69	152
01.10.07	642	35.00	225	1128	20.51	231	-	-	-
17.10.07	2163	19.61	424	-	-	-	3337	4.00	133
27.10.07	6105	12.50	763	3740	11.76	440	3997	2.00	80
27.11.07	3190	2.08	66	4088	3.39	139	6362	3.33	212
11.12.07	7370	4.76	351	-	-	-	8012	1.64	131
21.01.08	2823	18.87	533	2603	5.66	147	6948	0.00	ND
Aritmetic Mean	**3240**		**280**	**2758**		**159**	**4203**		**105**

[a] : Count was in cfu/100ml

[b] : ND : Non-detected

[c] - : No sampling



Sungai Selangor : A = Ampang Pecah F = Kampung Timah G = Rantau Panjang
Sungai Bernam : B = Tanjung Malim C = Jambatan SKC D1D2E = Tengi Canal

Fig. 4.1 Sites Comparison of Arithmetic Mean Sulphite Reducing Clostridia Density, Arithmetic Mean *Clostridium perfringens* Density and *Clostridium perfringens* Prevalence

Site A reported the lowest MBCC and MCPC whereas Site G showed the highest of both parameters among all the sites. These observations agreed with the initial expectations that Site A would demonstrate naturally existing sulphite reducing Clostridia and CP densities in environment with minimum (but not without) human activity; while Site G would illustrate the effect of urbanization on the two parameters.

Site F, despite its nearby feed lot cattle farming activities, reported the lowest CP prevalence, the second lowest arithmetic MCPC, and a pattern of high MCPC upon isolation (refer Fig. 4.2). Interestingly, its arithmetic MBCC was the second highest, thus suggesting that non-CP sulphite reducing Clostridia might be the main microorganism in cattle shedding, rather than CP.

Site B was a good example of focused human settlement. Compared to Site F, Site B reported high MCPC but low MBCC, whereas the CP prevalence was 100% (refer Fig. 4.1). This implied that CP is indeed a good indicator of human shedding. Meanwhile, postulation may be made that sulphite reducing Clostridia densities per capita in cattle might be higher than in human. However, this can only be verified if both the animal and human population densities are known.

During the study period from 02/04/2007 to 21/01/2008, there were seven trips that managed to sample all the study sites. MBCC and MCPC for these seven trips are presented in Fig. 4.2, and the isolation rates (IRt) in Fig. 4.3 and Fig.4.4. An absent bar representing MCPC or isolation rate in a study site meant non-detection of CP. Results showed that CP was actually isolated less frequently in Sungai Selangor compared to Sungai Bernam and Tengi Canal, although Sungai Selangor had higher arithmetic MCPC and MBCC. However, CP upon detection in Sungai Selangor usually reported high value.

Since samplings for Site F and Site G were performed on the same day, it was interesting to note that CP was never detected simultaneously in both the sites (refer Fig. 4.4). These could mean that transport time of CP between Site F and Site G took more than one day; and CP sources detected in Site G were independent from Site F.

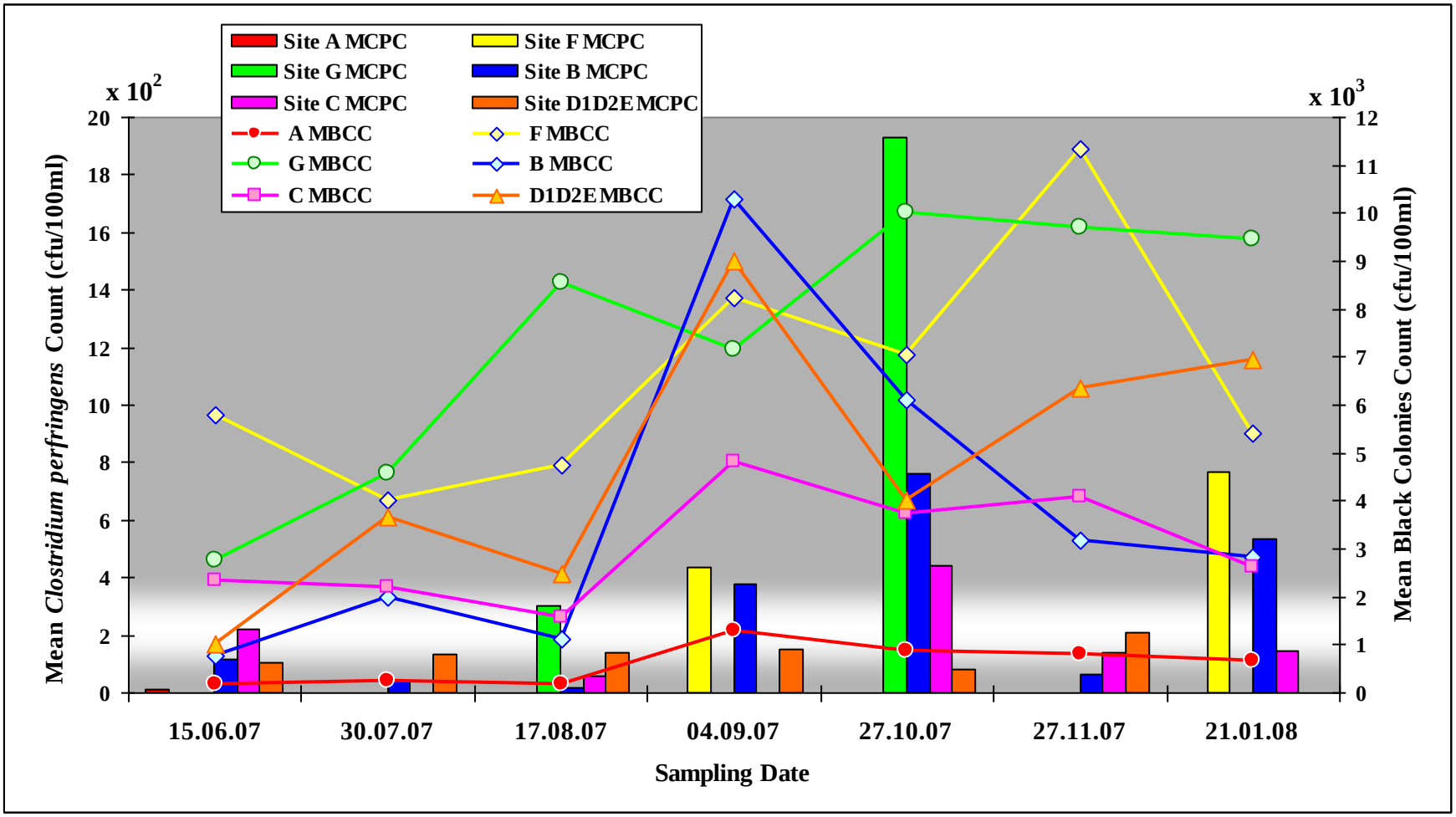


Fig. 4.2 Sites Comparison of Mean *Clostridium perfringens* Densities (MCPC) and Mean Sulphite Reducing Clostridia Densities (MBCC)

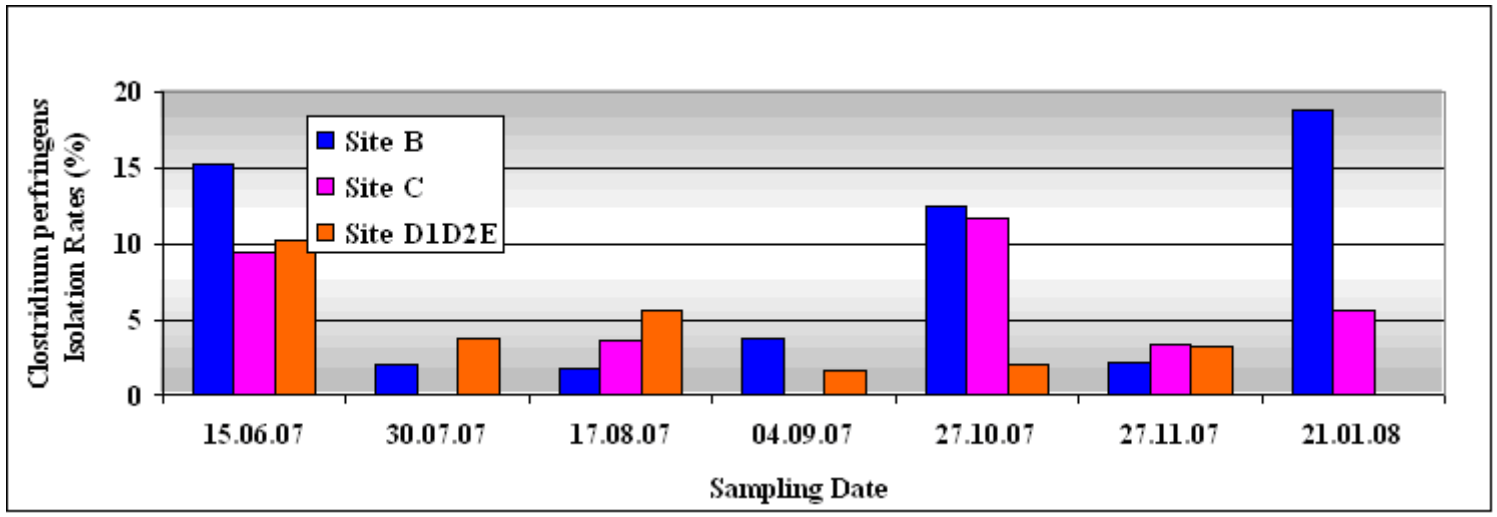


Fig. 4.3 Sungai Bernam and Tengi Canal *Clostridium perfringens* Isolation Rate (IRt)

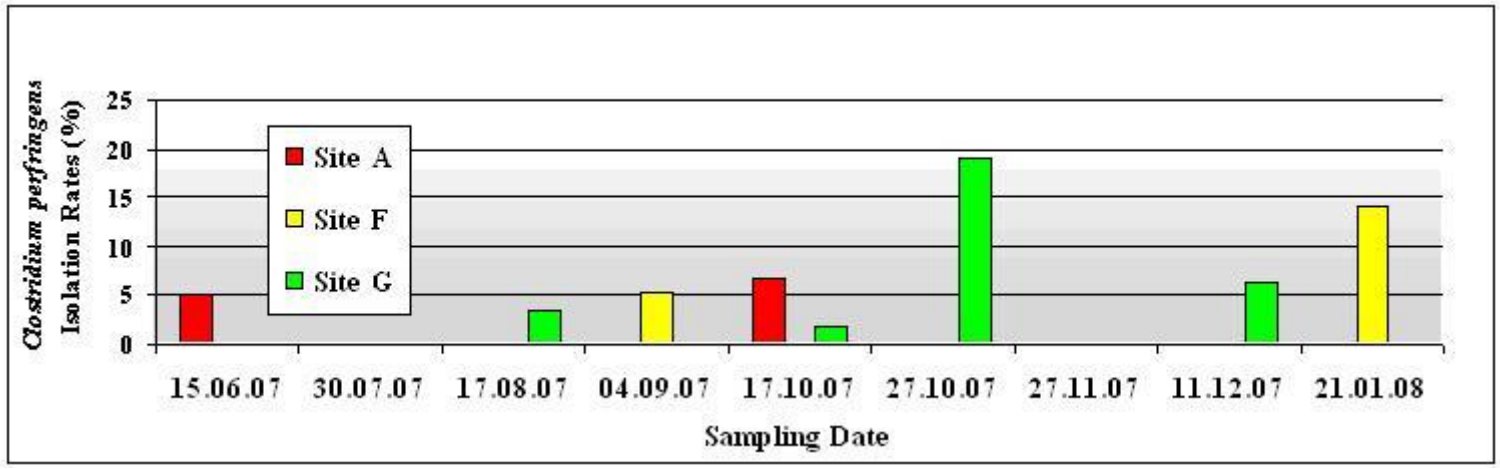


Fig. 4.4 Sungai Selangor *Clostridium perfringens* Isolation Rate (IRt)

4.4 Correlation Between Mean *Clostridium perfringens* Densities, Mean Sulphite Reducing Clostridia Densities, and Mean River Discharge

Mean river discharge (Q) was only normally distributed ($p>0.05$) in Site A and Tengi Canal (refer Appendix B). This seemed reasonable as Site A was situated in water catchment area whereas river discharge of Tengi Canal was regulated through the Ibu Empangan Sungai Bernam water dam. Relationship between mean river discharge and MBBC in Sungai Bernam, Sungai Selangor and Tengi Canal were illustrated in Fig. 4.7 to Fig. 4.12.

In all study sites, sulphite reducing Clostridia colony forming unit (CFU) of water sample replicates cultured on the same type of media did not show statistical difference ($p>0.05$, Table 4.2 and Appendix C). There were also no significant difference between sulphite reducing Clostridia CFU detected on TSC and OPSP media ($p>0.05$) except those from Tengi Canal (Table 4.2 and Appendix C). Meanwhile, the sulphite reducing Clostridia CFU of water sample replicates taken from Tengi Canal, Site F and Site G (Sungai Selangor) were normally distributed ($p>0.05$, Table 4.2 and Appendix C). MBCC of the first, middle and third quarter point across the rivers were not significantly different ($p>0.05$) except in Site B (Table 4.2 and Appendix D). This was due to the sewage outfall at both left and right bank of Site B. Temporal difference of black colonies CFU was significant in all study sites (Table 4.2 and Appendix E).

Table 4.2 Sulphite Reducing Clostridia : Replicates Normality, Statistical Differences and Temporal Difference

	Shapiro- Wilk Normality Test Significance		**Wilcoxon Signed Ranks / Paired T-test** (Asymptotic Significance, 2-tailed)		**Friedman Test** (Monte Carlo Approximation Significance)		**Kruskal-Wallis Test** (Monte Carlo Approximation Significance)	
	Sulphite Reducing Clostridia CFU		Difference Between TSC 1 and TSC 2	Difference Between OPSP 1 and OPSP 2	Difference Among TSC1, TSC2, OPSP1 and OPSP2	MBCC Difference Among First, Mid and Third Quarter Point Sampling	Temporal difference of Sulphite Reducing Clostridia CFU in First, Mid and Third Quarter Point Sampling	
Site A	TSC 1	0.000	0.675	0.265	0.099	0.976	First Quarter	0.012
	TSC 2	0.000					Mid	0.004
	OPSP 1	0.006					Third Quarter	0.041
	OPSP 2	0.000						
Site B	TSC 1	0.011	0.685	0.893	0.258	0.036*	First Quarter	0.000
	TSC 2	0.000					Mid	0.000
	OPSP 1	0.000					Third Quarter	0.000
	OPSP 2	0.000						
Site C	TSC 1	0.019	0.906	0.452	0.064	0.735	First Quarter	0.000
	TSC 2	0.149					Mid	0.000
	OPSP 1	0.034					Third Quarter	0.000
	OPSP 2	0.195						
Site D1D2E	TSC 1	0.227	0.981	0.487	0.046*	1.000	D1	0.000
	TSC 2	0.231					D2	0.000
	OPSP 1	0.996					E	0.000
	OPSP 2	0.064						
Site F	TSC 1	0.271	0.593	0.734	0.587	-	Grab Sampling	0.000
	TSC 2	0.096						
	OPSP 1	0.299						
	OPSP 2	0.245						
Site G	TSC 1	0.222	0.069	0.556	0.395	0.691	First Quarter	0.000
	TSC 2	0.060					Mid	0.000
	OPSP 1	0.015					Third Quarter	0.000
	OPSP 2	0.646						

* Correlation is significant at the 0.05 level (2-tailed)

Table 4.3 Correlation Between Mean *Clostridium perfringens* Densities (MCPC), Mean Sulphite Reducing Clostridia Densities (MBCC) and River Discharge (Q)

Sites	Sampling Point	Correlation Between Sectional MCPC and MBCC (cfu/100ml)	Correlation Between MCPC and MBCC (cfu/100ml)	Correlation Between MCPC and Q	Correlation Between MBCC and Q
Site A	First Quarter	- 0.523	- 0.501	- 0.272	- 0.070
	Mid	- 0.090			
	Third Quarter	- 0.590			
Site B	First Quarter	0.875**	0.509	- 0.255	0.322
	Mid	- 0.041			
	Third Quarter	0.330			
Site C	First Quarter	- 0.184	- 0.276	- 0.335	0.483
	Mid	- 0.342			
	Third Quarter	0.577			
Site D1D2E	D1	0.134	0.269	- 0.251	- 0.329
	D2	0.011			
	E	- 0.012			
Site F	Grab Sampling	0.054	0.054	0.175	**0.681***
Site G	First Quarter	0.621*	**0.620***	- 0.191	0.006
	Mid	0.223			
	Third Quarter	0.408			

* Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)

The correlations between MCPC and Q were weak in all study sites with correlation coefficients in the range of $-0.4 < r < 0.2$ (Table 4.3 and Appendix F). The weak correlations were probably due to the generally low MCPC in all study sites. However, MCPC and Q in Site A yielded a significant cubic equation ($r^2 = 0.751$, $p = 0.016$) while quadratic equation ($r^2 = 0.451$, $p = 0.091$) were found in Site B. Other sites reported equation with $r^2 < 0.3$ (data not

shown). Hence, weak MCPC and Q correlation might not necessarily negate a curve estimation equation that probably has $r^2 > 0.5$. Since this study considered Site A as a pristine area and Site B as a town settlement, it seems that land uses will probably determine whether or not CP and river discharge can be numerically linked. Nevertheless, this postulation only took spatial variations into consideration, and the potential temporal differences were ignored.

Significant MBCC and Q correlations were reported in Site F ($p<0.05$, Table 4.3). The two parameters were significantly linked with sigmoid equation ($r^2 = 0.365$, $p = 0.029$, data not shown). Site B and Site C which had MBCC and Q correlations of $0.3 < r < 0.5$ (Table 4.3) reported cubic equations between MBCC and Q with $r^2 = 0.535$ ($p = 0.091$) and $r^2 = 0.656$ ($p = 0.123$) respectively (data not shown). Hence, MBCC and Q seemed to be linked with meaningful curve estimation equation when there was a positive MBCC and Q correlation. No meaningful equation was found for other sites ($r^2 < 0.2$) when there were weakly negative or no MBCC and Q correlations.

MCPC and MBCC was negatively correlated in Site A ($r = -0.5$, $p>0.05$) whereby both the parameters were the lowest among the study sites (Fig. 4.1). As values of both MCPC and MBCC increased, they became positively correlated, such as shown by Site B ($p>0.05$). The correlation was significant ($p<0.05$) in Site G which had the highest MCPC and MBCC (Table 4.3). Based on the bacterial counts and correlations of Site A and Site G, this study postulate that MBCC and MCPC correlations of $r < -0.5$ is expected typically when MBCC and MCPC is below 1×10^3 cfu/100ml and 10 cfu/100ml respectively; whereas $r > 0.6$ is expected when MBCC and MCPC is more than 8×10^3 cfu/100ml and 500 cfu/100ml (Table 4.1). Relationship between MCPC and MBCC could be affected by land use. Further detail about land use characteristics in the study sites may help elucidate the observations.

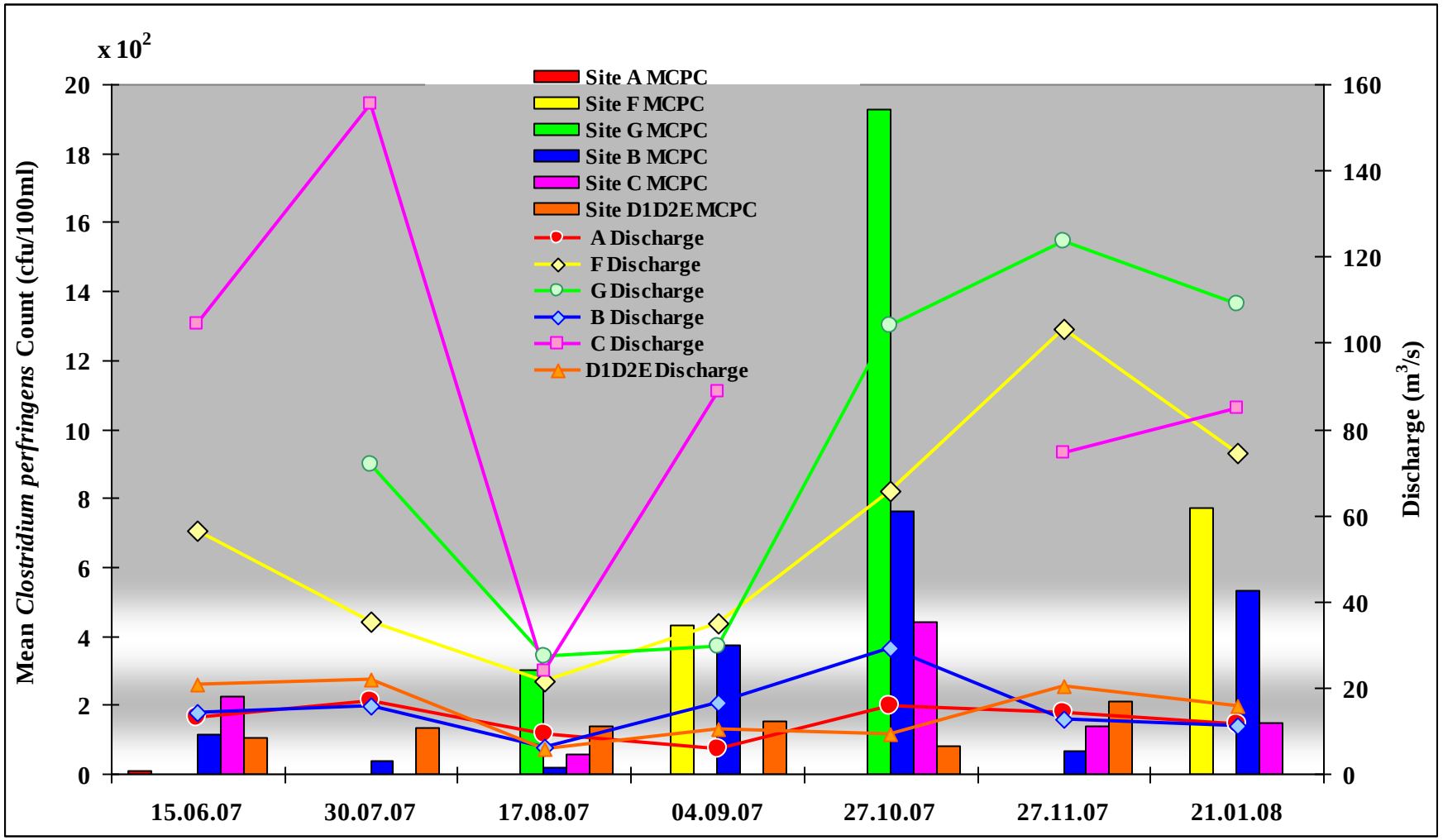


Fig. 4.5 Sites Comparison for *Clostridium perfringens* Densities (MCPC) and Mean River Discharge

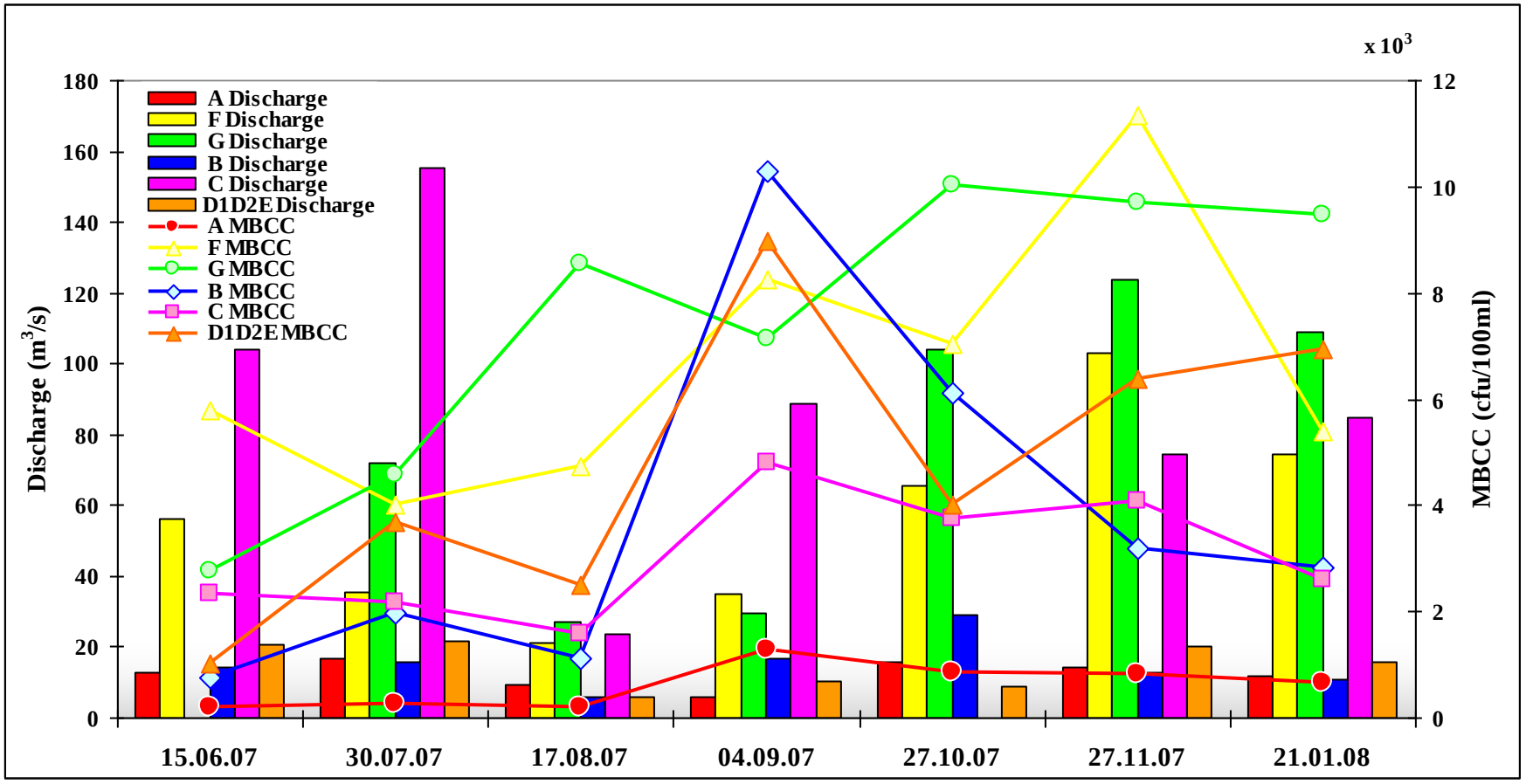


Fig. 4.6 **Sites Comparison for Mean Sulphite Reducing Clostridia Densities (MBCC) and Mean River Discharge**

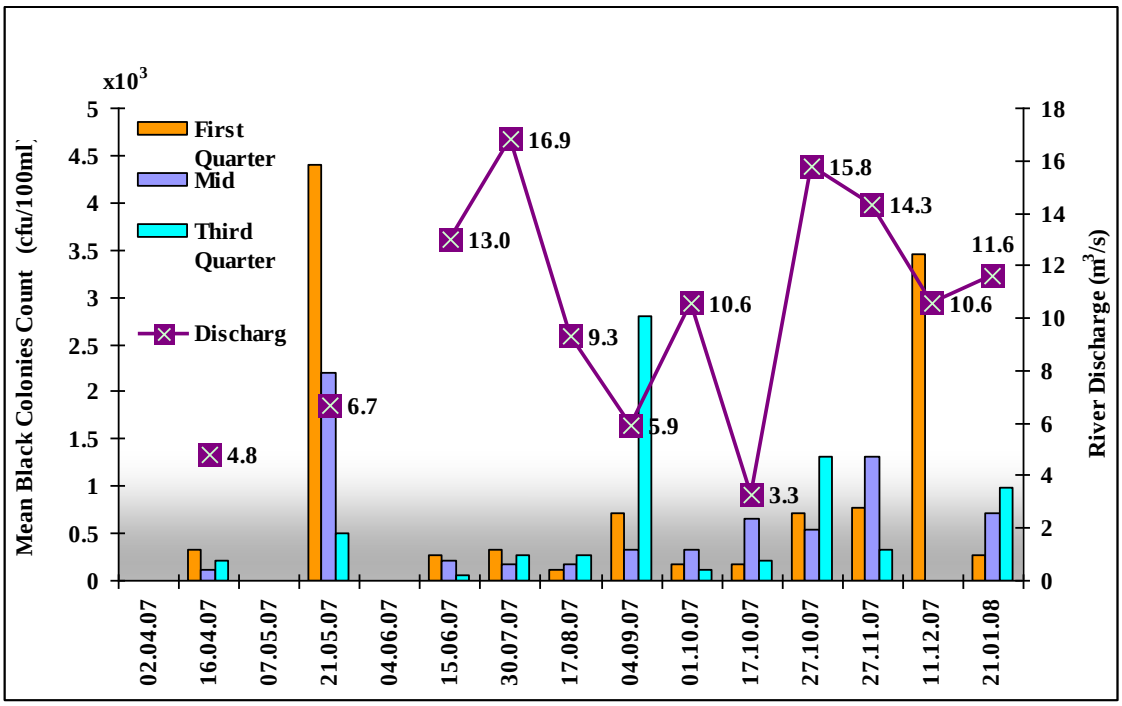


Fig. 4.7 **Site A River Discharge and First, Mid & Third Point Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)**

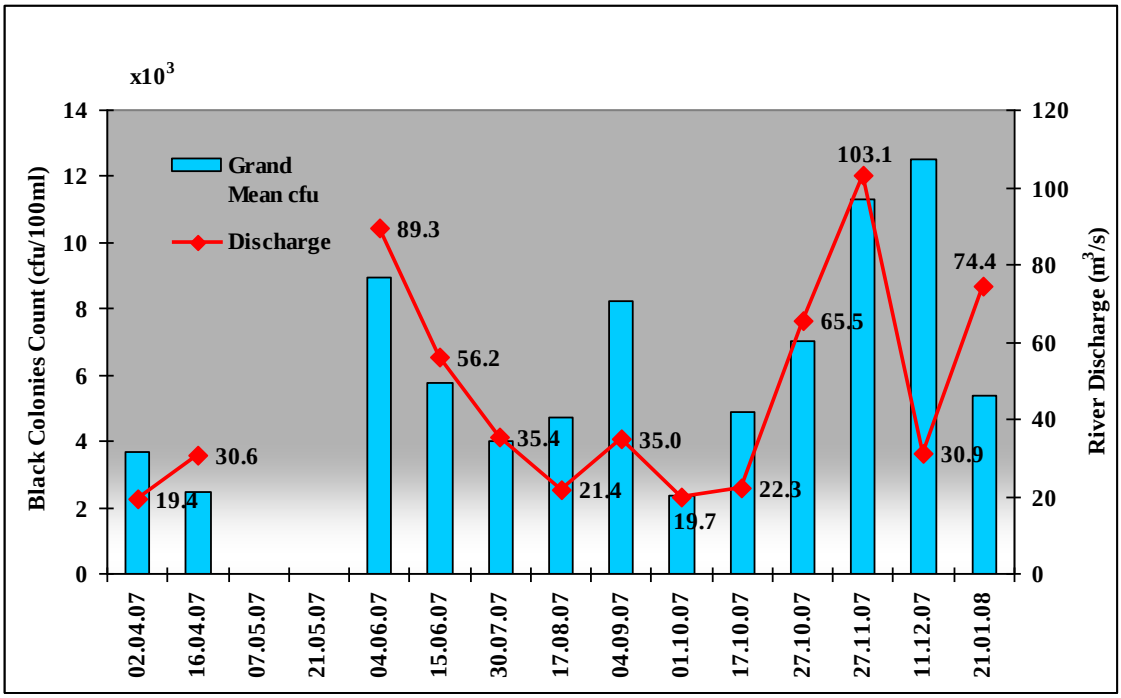


Fig. 4.8 **Site F River Discharge and Grand Mean Sulphite Reducing Clostridia Densities (MBCC)**

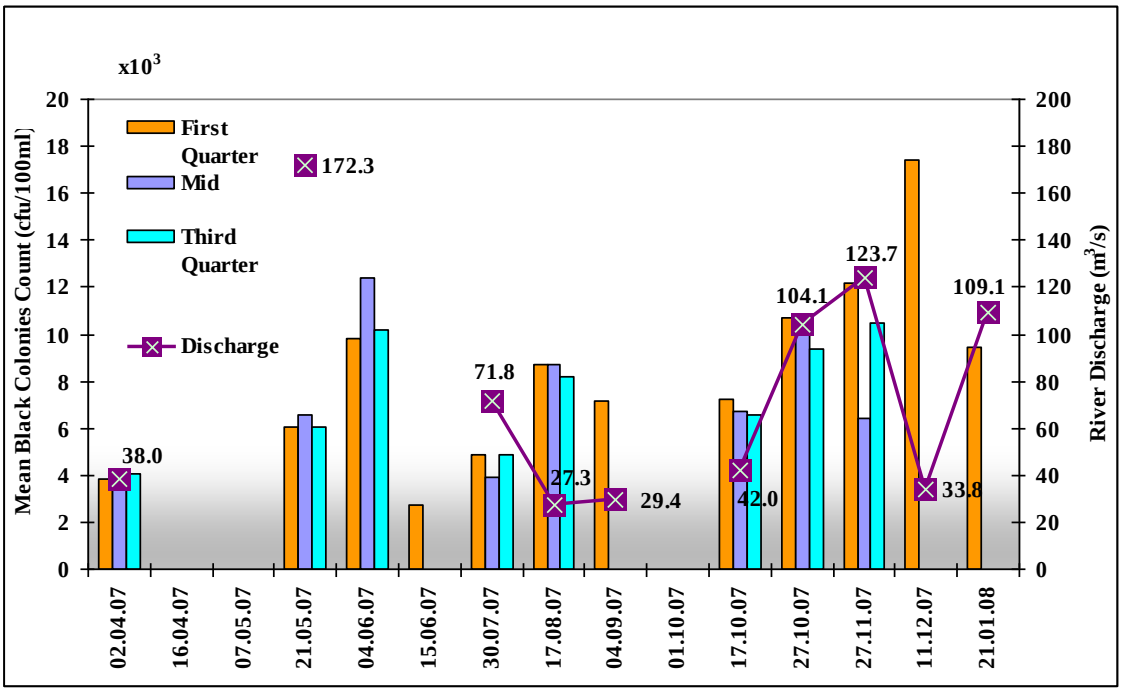


Fig. 4.9 **Site G River Discharge and First, Mid & Third Point Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)**

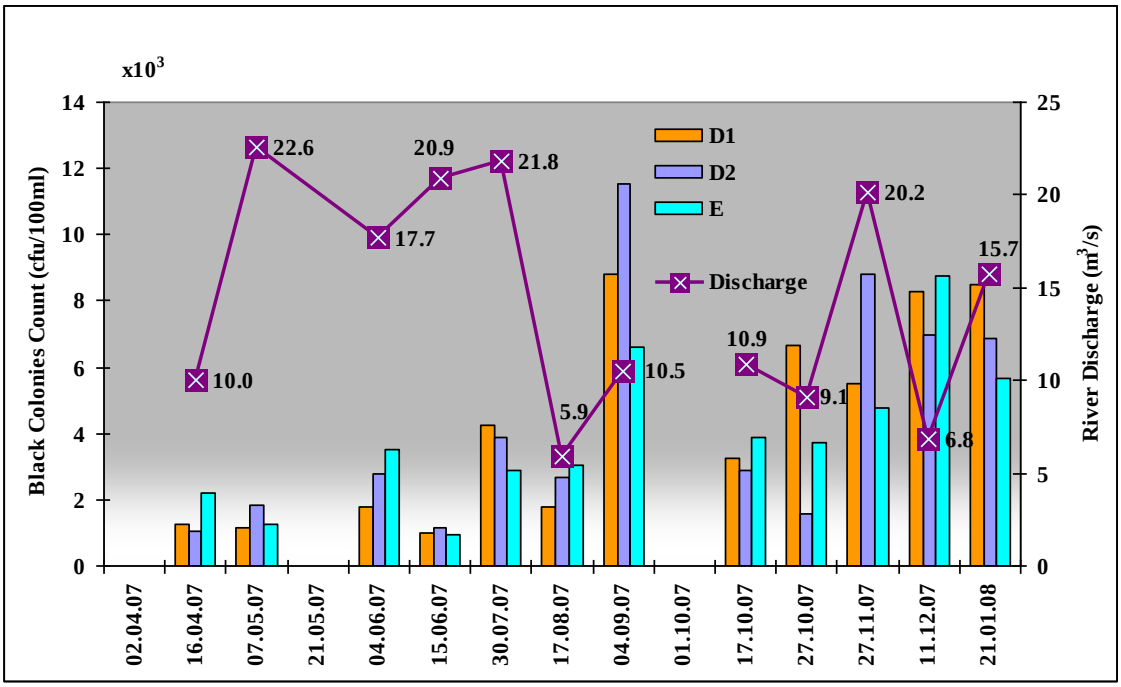


Fig. 4.10 **Site D1D2E River Discharge and Grab Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)**

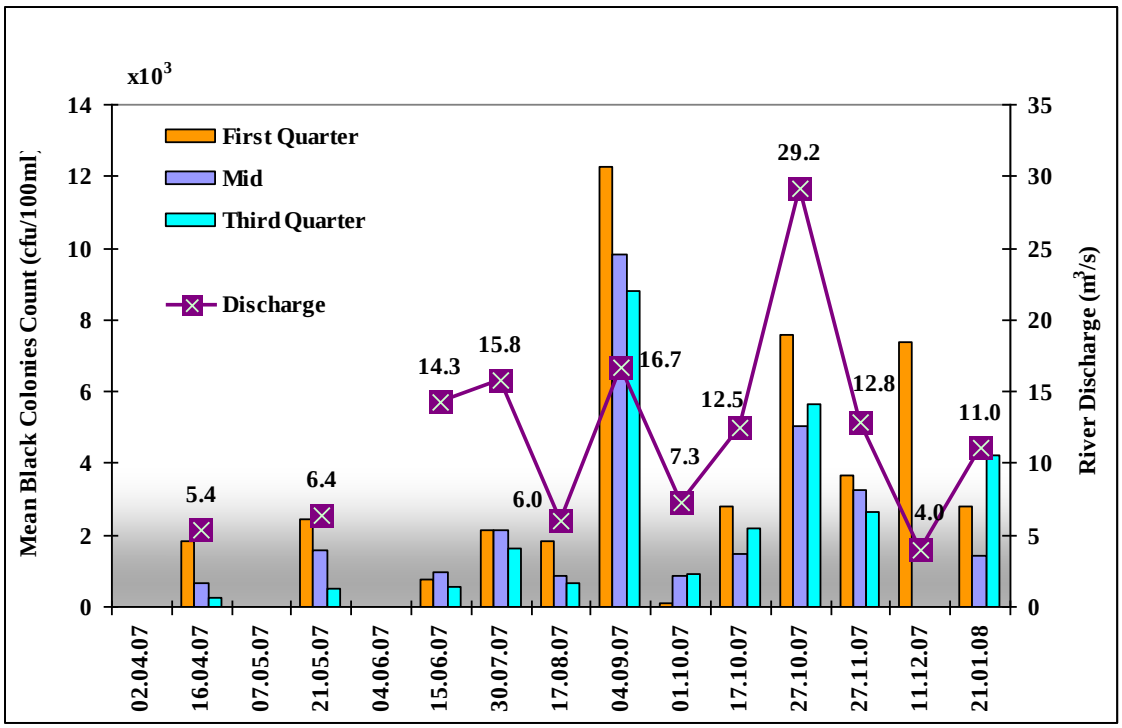


Fig. 4.11 Site B River Discharge and First, Mid & Third Point Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)

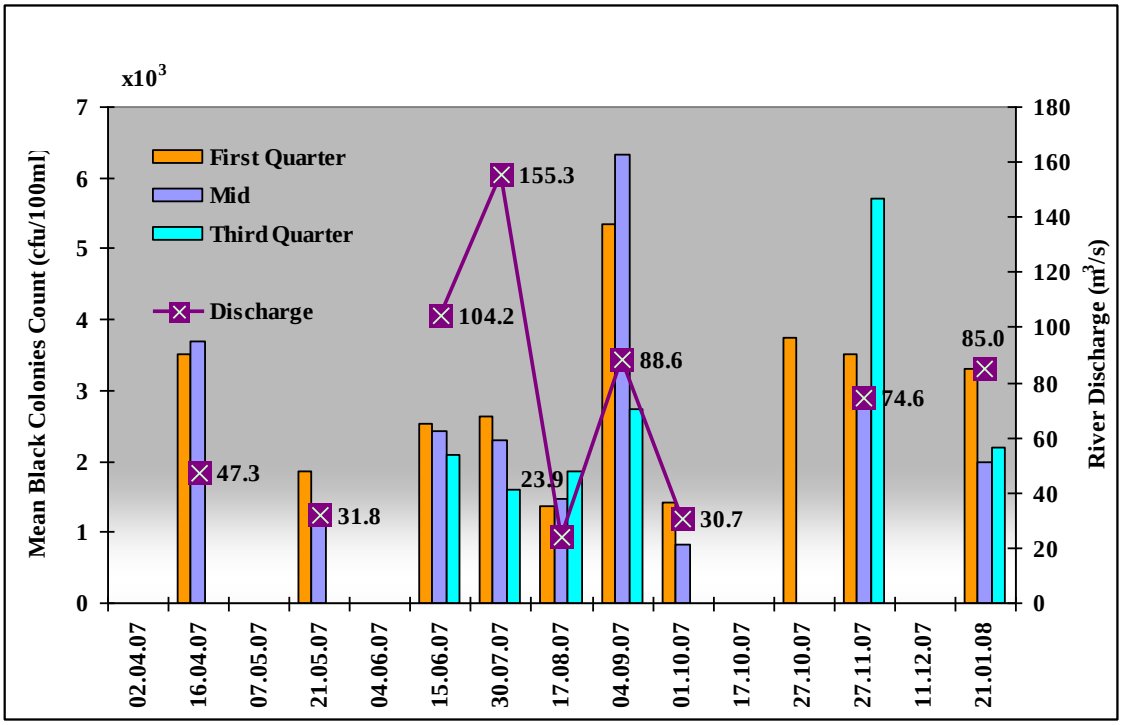


Fig. 4.12 Site C River Discharge and First, Mid & Third Point Sampling Mean Sulphite Reducing Clostridia Densities (MBCC)

Table 4.4 Sulphite Reducing Clostridia Densities Correlations in the First, Mid and Third Quarter Point Across River

Sites	Sampling Point (river section)	Mean Sulphite Reducing Clostridia Densities (cfu/100ml) Correlations Coefficient
Site A	First & Mid Quarter	0.449
	First & Third Quarter	0.583
	Mid & Third Quarter	0.436
Site B	First & Mid Quarter	0.854**
	First & Third Quarter	0.799**
	Mid & Third Quarter	0.743**
Site C	First & Mid Quarter	0.879**
	First & Third Quarter	0.771
	Mid & Third Quarter	0.657
Site D1D2E	D1 & D2	0.781**
	D1 & E	0.907**
	D2 & E	0.818**
Site G	First & Mid Quarter	0.611
	First & Third Quarter	0.976**
	Mid & Third Quarter	0.635

** Correlation is significant at the 0.01 level (2-tailed)

MBCC correlations across the rivers are presented in Table 4.4 (also refer Appendix F). Significant correlations between MBCC in the first, middle and third quarter point were found only in Site B and Site D1D2E ($p<0.01$), contrasting the finding that black colonies CFU were homogenous (no significant differences) across river in all study sites but Site B (Table 4.2). Hence significant MBCC correlations across river may still exist although MBCC densities are significantly different, whereas indifferent counts may not warrant good correlations across the river.

4.5 Correlation of Mean *Clostridium perfringens* Densities (MCPC), Mean Sulphite Reducing Clostridia Densities (MBCC), and Mean River Discharge (Q) Along Sungai Bernam and Sungai Selangor

Correlations of MCPC, MBCC and river discharge along Sungai Bernam and Sungai Selangor are summarized in Table 4.5 (Appendix G1 and G2).

In Sungai Bernam, MCPC was positively correlated ($r = 0.510$, $p>0.05$) between Site B and Site C. The same was also observed for MBCC, and also river discharge. Since MCPC and MBCC in Site C were lower than Site B, this could mean that the dilution of CP and sulphite reducing Clostridia in Sungai Bernam can probably be modeled.

Compared to the positive MCPC correlation in Sungai Bernam, MCPC in the three sampling sites of Sungai Selangor were all negatively correlated. The increasing MCPC towards the downstream of Sungai Selangor (Fig. 4.1) and also the negative correlations of MCPC along the river, collectively suggested that CP in Site G was not brought down from Site F, but was instead contributed by nearby land uses in Site G. Meanwhile MBCC, and also river discharge in Sungai Selangor was significantly correlated. This showed that modeling of MBCC and river discharge in Sungai Selangor is largely feasible, but could be more challenging for MCPC.

Table 4.5 Correlations of Mean River Discharge, Mean Sulphite Reducing Clostridia Densities (MBCC) and Mean *Clostridium perfringens* Densities (MCPC) Along Sungai Bernam and Sungai Selangor

BETWEEN SITES CORRELATION							
Sungai Bernam				**Sungai Selangor**			
Sites	**Mean River Discharge (Q)**	**Mean Sulphite Reducing Clostridia Densities (MBCC)**	**Mean *Clostridium perfringens* Densities (MCPC)**	**Sites**	**Mean River Discharge (Q)**	**Mean Sulphite Reducing Clostridia Densities (MBCC)**	**Mean *Clostridium perfringens* Densities (MCPC)**
Site B and Site C	**0.817***	**0.745***	**0.510**	**Site A and Site F**	**0.655***	**0.743****	**-0.319**
				Site A and Site G	**0.333**	**0.523**	**-0.157**
				Site F and Site G	**0.800****	**0.736****	**-0.461**

** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

4.6 Physico-chemical Parameters of River Water

Water temperature of the study sites during sampling events ranged from 20 to 25 ºC; conductivity spanned between 17 to 60 uS/cm while pH varied between 6.8 and 7.5. These were based on seven set of physico-chemical parameters records which were retrieved from the DID data bank for the sampling period of this study. Relationship between physico-chemical parameters and CP densities were not analyzed because of insufficient data.

4.7 DNA Quantification

Representative DNA was quantified using Invitrogen Low DNA Mass Ladder and Type A *Clostridium perfringens* control strain ATCC 13124. Three microlitre of extracted DNA was loaded and the result is depicted by Fig. 4.13. Comparison between lane 2 and lane 5 shows that each µl of DNA extracted in this study was about 1 ng.

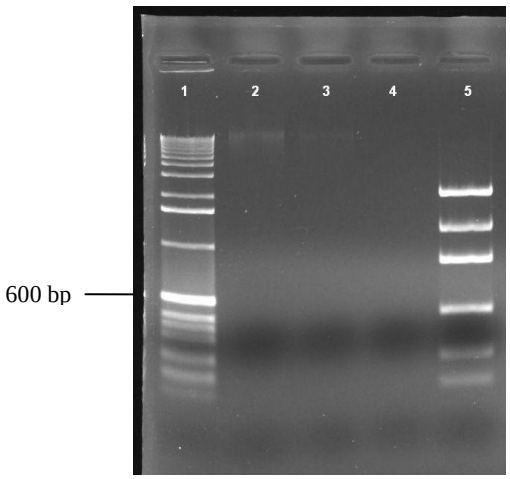


Fig. 4.13 DNA quantification

Lane 1 : 100 bp ladder with the first intense band at 600 bp
Lane 2 : DNA of ATCC 13124 Control Strain
Lane 5 : Low DNA mass ladder of 5, 10, 20, 40, 60 and 100 ng

4.8 Detection of Alpha, Beta, Epsilon, Iota and CPE Toxin Gene in *Clostridium perfringens* by Polymerase Chain Reaction (PCR)

This study confirmed presumptive CP (sulphite reducing Clostridia) as true CP by the presence of alpha toxin gene. A total of 142 CP isolates was detected using Set 1 alpha toxin gene primers. Surprisingly, further monoplex PCR showed that none of the 142 isolates harboured beta, epsilon or iota toxin genes (refer Fig. 4.14). This means that all CP isolates belonged to Type A. Nevertheless, five of them harboured CPE toxin gene. They were isolates number B/19, B/44, B/45, D/21 and J/11. Since agarose gels for toxin gene detections in this study were pre-stained and had small dimension of wells, bands in the gels were observed to be curved.

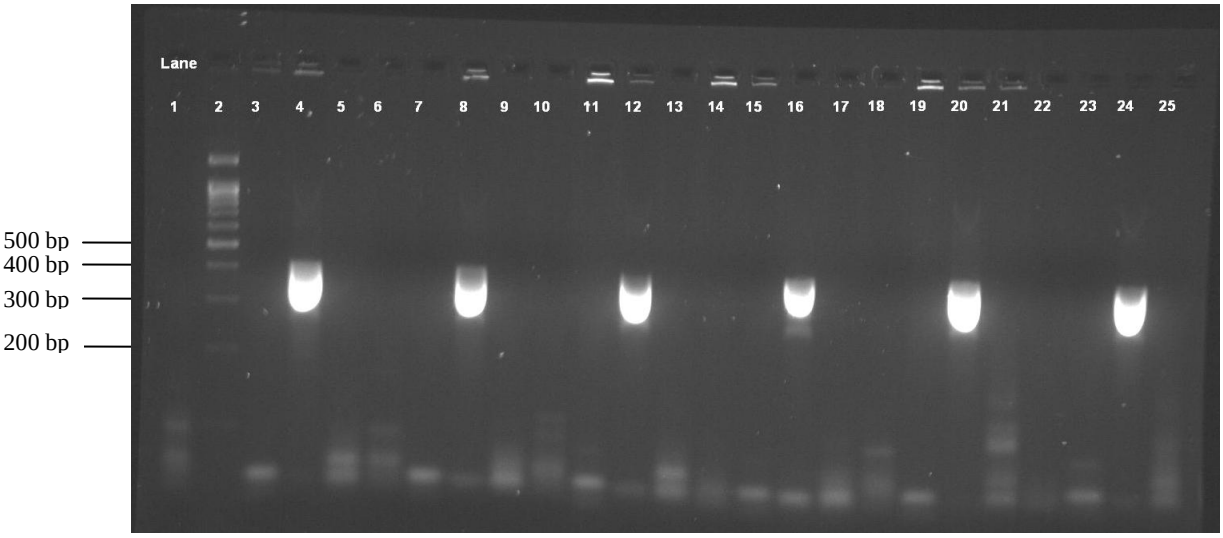


Fig. 4.14 Representative gel of monoplex PCR detection for beta, epsilon, alpha and iota toxin gene using Set 1 Primers

Lane 1, 6, 10, 14, 18, 22 : Non-detection of beta toxin gene
Lane 2 : DNA ladder with the first intense band at 500 bp
Lane 3, 7, 11, 15, 19, 23 : Non-detection of epsilon toxin gene
Lane 4, 8, 12, 16, 20, 24 : Alpha toxin gene positive **(402 bp)**
Lane 5, 9, 13, 17, 21, 25 : Non-detection of iota toxin gene

4.9 Duplex PCR of Alpha and CPE toxin gene Using Set 2 Primers

Duplex PCR for CP isolates harbouring alpha and CPE toxin genes performed with Set 2 Primers produced only two targeted bands, as illustrated in Fig. 4.15.

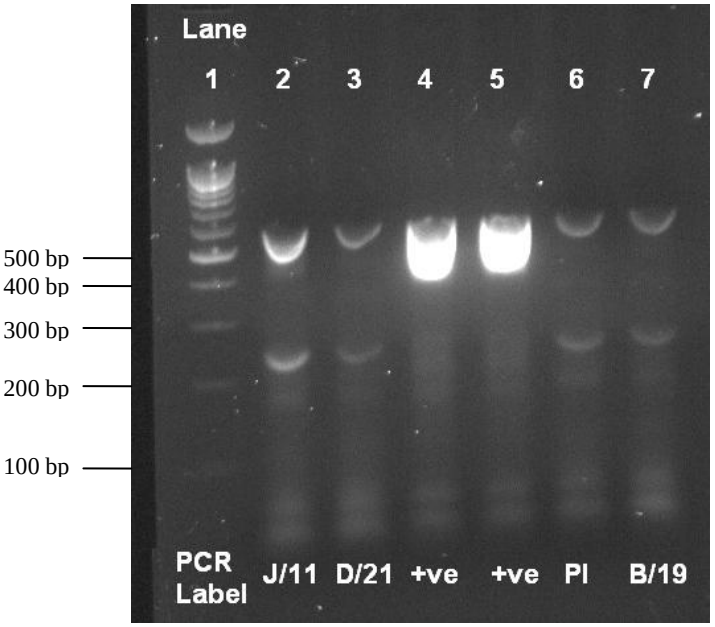


Fig. 4.15 Duplex PCR of alpha and CPE toxin gene

Lane 1 : DNA ladder with the first intense band at 500 bp

Lane 2, 3, 6, 7 : Duplex PCR detection using Set 2 Primers for alpha **(617 bp)** and CPE **(262 bp)** toxin genes

Lane 4 : Duplex PCR of positive control ATCC 13124 with 2 µl DNA template

Lane 5 : Duplex PCR of positive control ATCC 13124 with 1 µl DNA template

4.10 Alpha and CPE Toxin Gene Sequencing Results

Representative sequencing result of amplicons produced with alpha and CPE toxin gene primers (isolate number E/40 and J/11) are presented in Appendix I1 to I5. Appendix I1 and I2 were produced by Set 1 alpha toxin gene primers; Appendix I3 and I4 by Set 2 alpha toxin gene primers; and Appendix I5 by Set 2 CPE toxin gene primers. Blast results against nucleotide sequences in GenBank showed above 97% matching between the PCR amplicon sequences and alpha or CPE toxin gene sequences in *Clostridium perfringens*.