Faculty of Resource Science and Technology

ESCHERICHIA COLI DISTRIBUTION AND PERSISTENCE IN WATER AND SEDIMENT AT SANTUBONG RIVER

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DECLARATION

I hereby declare that no portion of this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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List of Abbreviations

pH	A measurement of the acidity or alkalinity of solution [p stands for "potenz" (this means the potential to be) and H stands for Hydrogen]
^{o} C	Degree Celsius
mL^{-1}	per millilitre
g^{-1}	per gram
d^{-1}	per day
r^{2}	coefficient of determination
CFU	colony-forming unit
DO	Dissolved oxygen
NTU	Nephelometric Turbidity Units
%	percentage
mm	millimetre
cm	centimetre
mg	miligram
EMB	Eosin Methylene Blue
PSA	Particle Size Analysis
LOI	Loss-On-Ignition
HMP	hexamethaphosphate
H_2O_2	hydrogen peroxide
NaCl	Sodium Chloride
$CaCl_2$	Calcium Chloride

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Escherichia coli Distribution and Persistence in Water and Sediment at Santubong River

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ABSTRACT

Various land use and human activities at Santubong River such as residential, fish cage culture, shrimp farming and wood processing factory may contribute to contamination of fecal bacteria. *Escherichia coli (E. coli*) is a common fecal indicator used to monitor fecal pollution of water and sediment. Temperature and characteristic of the sediment are two factors that could affect the persistence of *E. coli* in sediment. In this study, the impact of land use on *E. coli* concentration was investigated and the survival study of *E. coli* in sediment from different human activities was conducted at different temperatures. The water and sediment samples was collected monthly for three month during low tide at five stations where four stations reflected land use activities and one station with no visible of human activity as a control. *In-situ* parameters were recorded during sample collection. Pure *E. coli* ATCC 25922 was grown and inoculated in the sediment for survival study. The highest mean concentration observed in water was 19.77 CFU/mL and the lowest was Station 4(6.67 CFU/mL). In sediment the highest concentration was 41.50 CFU/g wet also from Station 5 and the lowest was Station 1(23.93 CFU/g wet). DO value of Station 5 was the lowest (6.89 mg/L) and the highest was Station 4 (10.22 mg/L). Turbidity of Station 5 was the highest among the stations. The survival study shows that temperature significantly affected the die-off rates. At 25°C, the die-off range from 0.38 to 0.64 d^{-1} and 0.36 to 0.89 d^{-1} at 30°C. As the temperature increases, the die-off rates increased. Station 4 with lower organic matter content showed higher die-off rate compared to Station 3 and 5.

Key words: *E. coli*, concentration, die-off, temperature

ABSTRAK

Pelbagai penggunaan tanah dan aktiviti manusia di Sungai Santubong seperti kawasan perumahan, kultur sangkar ikan, penternakan udang dan kawasan kilang pemprossesan kayu menyumbang kepada pencemaran oleh bakteria najis. Escherichia coli (E. coli) adalah bakteria penanda yang biasanya digunakan untuk mengawal pencemaran air dan mendapan. Suhu dan ciri-ciri mendapan adalah dua faktor yang mempengaruhi keterusanan E. coli di dalam mendapan. Dalam kajian ini, implikasi penggunaan tanah terhadap kepekatan E. coli telah dikaji dan kajian kelangsungan hidup E. coli didalam mendapan yang berlainan telah dijalankan pada suhu yang berlainan. Sampel air dan mendapan di kumpul pada setiap bulan untuk tiga bulan semasa air surut di lima stesen; empat daripadanya menunjukkan pelbagai penggunaan tanah dan satu daripadanya ialah stesen yang tiada kegiatan manusia sebagai kawalan. Parameter in-situ juga direkodkan semasa pengambilan sampel. E. coli asli berstrain ATCC 25922 ditumbuhkan dan diinokulat kedalam sampel mendapan untuk kajian kelangsungan hidup E. coli. Berdasarkan pemerhatian, purata kepekatan yang tertinggi didalam air ialah Stesen 5(19.77 CFU/mL) dan paling rendah ialah Stesen 4(6.67 CFU/mL). Didalam mendapan, kepekatan tertinggi ialah Stesen 5(41.50 CFU/g wet)dan terendah ialah Stesen 1(29.93CFU/g wet). Stesen 5 mencatatkan nilai DO yang paling rendah manakala Stesen 4, nilai DO adalah yang tertinggi. Kajian kelangsungan hidup E. coli menunjukkan signifikasi suhu mempengaruhi kadar kematian. Pada suhu 25°C, kadar kematian dalam lingkungan 0.38 hingga 0.64 d^{-1} dan 0.36 hingga 0.89 d^{-1} pada suhu 30°C. Apabila suhu meningkat, kadar kematian juga meningkat.

Kata kunci: E. coli, kepekatan, kadar kematian, suhu

CHAPTER ONE

INTRODUCTION

The origin of fecal bacteria contamination in water and sediment could be from natural wildlife, agricultural or storm runoff as well as human development and activities (Shah *et al*., 2007; Haller *et al*., 2009). *Escherichia coli* (*E. coli*) is a common indicator of fecal contamination of water and soil (Ling *et al*., 2005). Various land use such as residential area and aquaculture activities become source of fecal bacteria contamination due to sewage discharged (Mudge and Duce, 2005; Kullman *et al*., 2006). Aquaculture activities such as fish cage culture and shrimp farming give impact on water and sediment quality. Santubong River, located at Kuching accommodates shrimp farm and cage culture activities as they provide essential protein for growing population (Ling *et al*., 2010). Wastewater discharge from these activities contributes to high concentration of nutrients loading into the river.

The waste from fish and shrimp feces not only results in chemical but also biological changes in the water and sediment and support greater bacterial growth (Kullman *et al*., 2006). Studies have shown that sediment can act as important reservoir for fecal bacteria in freshwater environments (Anderson *et al*., 2005; Haller *et al*., 2009). Fecal bacteria can survive longer in marine sediment than water column to availability of nutrient and organic carbon, oxygen level, reduced sunlight inactivation and protection from predation (Haller *et al*., 2009; Lee *et al*., 2006). The survival of *E. coli* in soil also influences by several stresses including variation in soil textures, moisture and organic matter content (Ling *et al*., 2003; Ishii *et al*., 2005).

Environmental conditions including variation of temperature and pH of water have important effect on the survival and growth of bacteria. Temperatures below the optimum

typically have a more significant effect on growth rate than temperature above the optimum. The optimum pH for bacterial growth lies between 6.5 and 7.5 (Tchobanoglous *et al*., 2003). Higher water column turbidity has been related to higher counts of faecal bacteria (Ortwine, 2007). Changes in tidal condition have been reported gave effects to the bacteria concentration (Bonilla *et al*., 2007; Mallin *et al*., 2006).

Different land use activities may affect the fecal bacteria concentration. However, there were no recent studies on impact of these activities on *E. coli* concentration at the Santubong River. Therefore, in this study, the impact of different land use activities on *E. coli* concentration was investigated. The significance of this study was to provide information that could contribute to developing an efficient waste management either from agriculture or human waste to reduce fecal contamination of water. The objectives of this study were to determine the impact of different land use on *E. coli* concentration in water and sediment, to compare the *E. coli* concentration among the stations and to study the die-off of *E. coli* in sediment from different human activities at different temperatures.

CHAPTER TWO

LITERATURE REVIEW

2.1 Fecal bacteria

2.11 *Escherichia coli* (*E. coli)*

Fecal bacteria are indicator bacteria which live in gastrointestinal tracts of human and animals (Anderson *et al*., 2005). *E. coli* is Gram-negative facultative anaerobic bacterium correlated with the gastrointestinal tract. Its species name, *coli*, is derived from its usual presence in the large intestine or colon (Batzing, 2002). Generally, more than one million (10^6) *E. coli* cells are present in 1 g of colon material and released to environmental usually through fecal deposition (Ishii and Sadowsky, 2008).

E. coli lives in the intestine of warm blooded animals, form a large percentage of fecal coliform bacteria (Davis *et al*., 2006) and researcher have shown that *E. coli* can grow in water or subtropical water (Anderson *et al*., 2005). Indicator bacteria that adapted living in gastrointestinal tract in which pass to a different habitat such as wastewater collection system may be leaving primary habitat and entering secondary habitat (Gordon *et al*., 2002). The versatility in energy acquisition of *E. coli* gives it ability to survive and grow in the environment (Ishii and Sadowsky, 2008). Fecal bacteria are not all pathogen to humans, it only provides an indication of the amount of fecal bacterial load present.

2.12 Bacterial pollution sources

Several studies reported that human activities and sewage effluents can be sources of fecal bacteria in water and sediment (Mallin *et. al*, 2006; Mudge and Duge, 2005). The origin of fecal indicator bacteria has always presumed to be anthropogenic such as sewage, agricultural and urban runoff (Evanson and Ambrose, 2005). Aquaculture activities such as shrimp farming and fish cage culture can contribute to bacterial pollution (Ling *et. al*, 2010). Kullman et al. (2007) reported that aquaculture waste caused not only chemical changes but also biological changes and support better growth of bacteria and algal bloom.

Besides of anthropogenic sources, the wildlife and water also contributed to fecal counts (Ksoll *et. al,* 2007). Choi et al. (2003) reported that sewage, birds, marsh, sediment and barn runoff as predominant sources of enterococci in seawater at Huntington Beach, CA. According to Whitman and Nevers (2003), *E. coli* counts were correlated with the number of birds (gulls) in the morning and afternoon in the water at Lake Michigan beach. Ksoll et al. (2007) reported the presence and sources of fecal coliform bacteria in epilithic periphyton communities of Lake Superior.

2.2 Factors of bacterial pollution

2.21 Environment factors

Environmental factors including waste matter, temperature variations, pH, oxygen levels, ultraviolet radiation and predators such as protozoan and virus may contribute to bacterial growth (Haller *et al.*, 2009 and Mallin *et al.*, 2006). Many studies have reported that decreases in pH result in increases in die-off rates (Ling *et al.*, 2005). The optimum pH for bacterial growth lies between 6.5 and 7.5 (Tchobanoglous *et al.*, 2003). Most microorganisms including pathogen microbial grow between 25°C and 40°C (Batzing, 2002). Changes in air temperature, cloudiness or wastes discharged into water from households may become factors that affect water temperature (Tchobanoglous *et al.*, 2003).

Higher water column turbidity has been related to higher counts of faecal bacteria (Ortwine, 2007). Tidal conditions have been reported gave effects to the bacteria concentration (Bonilla *et al.*, 2007; Mallin *et al.*, 2006). Anne et al. (2006) reported that higher abundances of fecal coliform were recorded at low tide due to urban drainage and agricultural run-off influence. Low concentration of fecal bacteria during high tide might be because of rapid decrease of nutrient caused by tidal flushing (Mallin *et al.*, 2006).

2.22 Nutrients

The key nutrients of bacterial growth are nitrogen and phosphorus (Mallin *et al.,* 2006). Large quantities of nutrients released into the river through the sewage wastewater may result in nutrient enrichment stimulating eutrophication, caused dissolved oxygen depletion due to break down of organic matter in the water by microorganisms. Previous study reported that high concentration of nitrogen-nitrate ranged from 0.6 mg/L to 3.5 mg/L and phosphorus ranged from 0.02 mg/L to 0.38 mg/L at Santubong River effluent from shrimp farming and cage culture area (Ling *et al.,* 2010). The wastewater discharged from these activities increased the nutrients loading and promote the growth of bacteria.

2.3 Fecal bacteria concentration

2.31 In the water column

A study done by Mallin et al. (2006) on water and sediment quality which sewage effluent at urbanized estuary reported that the highest fecal bacteria counts in water column was 2.7×10^5 CFU 100 mL^{-1} meanwhile the fecal bacteria counts ranged between 1.5×10^4 to 2.1×10^4 CFU 100 mL^{-1} and 2.0×10^3 to 3.2×10^3 CFU 100 mL^{-1} in water column of the creek and decreased after a few days. Another study on distribution of indicator bacteria in Canyon Lake, Davis et al. (2005) reported that the mean concentration of fecal bacteria was 1047 CFU 100 mL^{-1} of *E. coli* was recorded. Based on Fernandez et al. (2007) study, *E. coli* density ranging from 2.55 MPN/100 ml to 6250 MPN/100 ml in recreational water of the Sauce Grande lagoon, Argentina.

Nevers et al. (2007) reported that *E. coli* concentration at Kintzele Ditch which drains a watershed that includes natural, residential and urban areas of Michigan City was log mean, 2.91 ± 0.37 whereby at Trail Creek which is a larger watershed that drains urban Michigan City and upstream residential and agricultural areas. According to Edge and Hill (2007) in study to identify the sources of fecal pollution at a freshwater beach in Hamilton Harbour, Lake Ontario reported that *E. coli* concentration in ankle-depth water was 177,000 CFU/100 mL which the sources were predominantly from bird droppings rather than municipal wastewater.

2.32 In the sediment

The characteristics of sediment such as particle grain size, nutrient and organic matter content give impact on fecal bacterial survival (Haller *et al.,* 2009). Lee et al. (2006) who studied the persistence of fecal bacteria in Santa Monica Bay beach sediment proposed that sediment organic content is an important determinant of fecal indicator bacteria survival in overlaying water and reported that the concentration of *E. coli* is 1.5×10^5 MPN $100g^{-1}$ in wet sediment.

Numerous studies reported that the survival of *E. coli* was influenced by sediment characteristics, which may reflect the intrinsic differences between the sediment types (Ling *et al*., 2003; 2008; Craig *et* al., 2001; 2004; Ishii *et al*., 2005; 2009; Anderson *et al*., 2005; Jamieson *et al.,* 2005; Lee *et* al., 2006; Muirhead *et* al., 2006; Haller *et* al., 2009; Garzio-Hadzick *et al*., 2010).

Lower decay rate of *E. coli* was reported by Craig et al. (2001) in coastal sediment compared to overlaying water due to the characteristic of the sediment influenced the decay rates with high organic carbon content and small particle size which this condition found to be more

conducive to *E. coli* persistence. Ling et al. (2003) reported that the particle size of the soil was more important than the organic matter content in the determination of the survival of *E. coli* in soil. There is evidence that fecal bacteria can survive longer in sediment than water column since sediment provide favourable nutrient condition, protection from sunlight inactivation and predation by protozoan (Haller *et al.*, 2009).

Haller et al. (2009) reported that *E. coli* were still present at concentration between 10^2 and 10^3 CFU 100 g^{-1} after 50 days. According to Ishii et al. (2005) study, the greatest cell densities of *E. coli* is up to 3 x 10^3 CFU g^{-1} and the lowest is $\leq$ 1 CFU g^{-1} soil in soil of temperate environment in three Lake Superior watershed. Whitman et al. (2006) reported that *E. coli* densities in stream sediment of Dune Creeks ranged from 10^2 to 10^3 CFU 100 mL^{-1}.

2.4 Sediment analysis

2.41 Particle-size analysis (PSA) and organic matter content

Particle size analysis (PSA) is defined as a measurement of the size distribution of individual particles in a soil sample (Gee and Bauder, 1986). The particle size, using the USDA classification scheme is divided into three major size classifications: sand (2.0-0.05 mm), silt (0.05-0.002 mm), and clay (<0.002 mm) (Gee and Bauder, 1986). PSA is accomplished by dispersion of soil aggregates into discrete units, followed by fractionation and quantification of each particle-size interval by sieving and sedimentation. Pipette method is considered as standard method of PSA for sedimentation procedures (Gee and Bauder, 1986). Chemical dispersion in pipette procedures uses hydrogen peroxide (H_2O_2) and sodium hexamethaphosphate (HMP).

Hydrogen peroxide oxidizes organic matter, lead to binding of soil particles into aggregates while the function of HMP is to complex any Ca^{2+} in solution and to replace Ca^{2+} with Na^{+} on the ion-exchange complex of soil particles which result in dispersion of individual soil particles and causing breakdown of soil aggregates (Kettler et al., 2001). This method is based on Stokes' Law which relates the time of settling to the size of particles remaining in suspended solution (Gee and Bauder, 1986).

Organic matter content may be indirect or directly estimated (Nelson and Sommers, 1996). Direct estimation of organic content can be accomplished by destruction of organic matter, after which the loss in weight of the soil is taken as a measure of the organic content. Loss-On-Ignition (LOI) method is a standard measurement of organic matter content in soil (Nelson and Sommers, 1996) and LOI is widely use to determine organic matter in the marine sediment and freshwater ecosystem (Gretel et al., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study site is located along Sg. Santubong where there are residential area (village/urban) along the river and logging area. There are also aquaculture activities such as shrimp farming and fish cage culture.

Table 1: Location of the stations

Station	Location
1	Santubong Village (Pa Lawai River), with human population about 400 people.
2	Shrimp farm (Sulai River)
3	Cage culture (Buah River)
4	Clean area (Buntal River) located in the middle between aquaculture activities wood processing factory
5	Wood processing factory (Serai River)

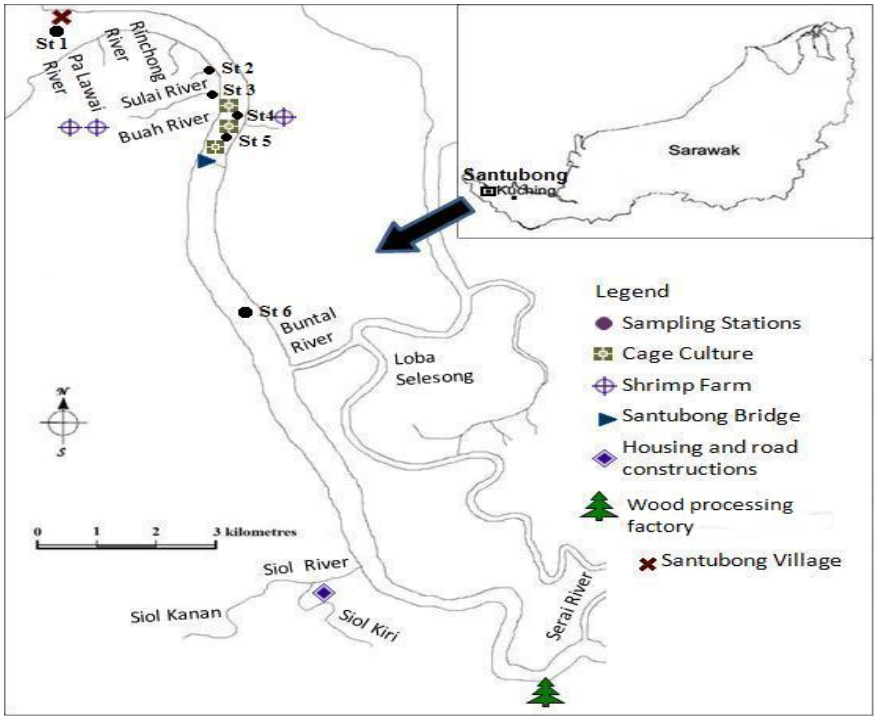

Figure 1: Santubong River and sampling stations (courtesy of Michelle Christine Miod)

3.2 Sample collection

The sample was collected on 3rd February 2010, 1st March 2010 and 10th April 2010 during low tide. The sample of water and sediment from the Santubong River were collected in triplicate at five stations which reflected land use activities including residential area, factory, shrimp farm and fish cage culture and one station with no visible human activities as a comparison. The water and sediment samples were collected at three different sites for each station and mixed to form composite to reduce variation of bacterial concentration. Then, the each composite was divided into three replicates.

3.21 Water sample collection

Water sample was collected from 30 cm below surface water using glass bottle and the sample was placed on ice and analyzed within six hour of collection (Mallin *et al.*, 2006). The parameter of water such as temperature, pH, turbidity and dissolved oxygen were measured *in situ* using YSI Multiparameter Water Quality Probe (Corbin and Gaylard, 2005).

3.22 Sediment sample collection

The sediment sample was collected on the surface of the sediment using spade and placed into sterile plastic bag. The sample was placed on ice and further analysis was carried out in the laboratory (Kullman *et al*., 2006).

3.3 Fecal bacteria concentration

3.31 Water sample

For microbiological analysis, *E. coli* counts were performed with spread plate method (Setty and Sreekrishna, 2007). Serial dilution of 1:10 was prepared. The serial dilution was performed until 10^{-2} to avoid the water sample from too much dilution. Then, 9 ml of sterile saline (0.8% NaCl) was aseptically pipette into each tube. One ml of water sample was transferred to tube first tube labeled 10^{-1} and mixed. One ml from first tube was taken using using sterile tip and transferred to second tube (dilution 10^{-2}) and mixed. The same steps were repeated for each replicate of each station. After that, 0.1 ml from each dilution was placed in separate Eosine Methylene Blue (EMB) agar. The bent glass rod was sterilized with ethanol, flamed and leaved for few minutes to cool it down. The rod was placed gently on the surface of EMB agar and the sample was spread over the agar medium by rotating the petri plate. The plates were incubated in inverted position at 37º C for 24 hours.

3.32 Sediment sample

Serial dilution of 1:10 was performed. One gram of sediment sample was placed on first tube containing 9 ml of sterile saline and mixed by vortexing. After vortexing, the suspension was allowed to settle for few minute. Then, 1 ml of the supernatant was taken and transferred to second tube (dilution 10^{-1}) and mixed by vortexing. One ml from second tube was pipette using new sterile tip and placed into third tube (dilution 10^{-2}) and vortexed to mix. After that, 0.1 ml from each dilution was placed in separate Eosine Methylene Blue (EMB) agar. The bent glass rod was sterilized with ethanol, flamed and leaved for few minutes to cool it down. The rod was

placed gently on the surface of EMB agar and the sample was spread over the agar medium by rotating the petri plate. The plates were incubated in inverted position at 37º C for 48 hours. Due to none of *E. coli* colony was detected from dilution 10^{-1} and 10^{-2} from the first sampling, this method proceeded without serial dilution for water sample and only dilution 10^{-1} was performed for sediment sample for the next sampling.

3.4 Die-off of *E. coli*

3.41 Inoculum preparation

Pure culture of *E. coli* strain ATCC 25922 was used for the study. The *E. coli* suspension for the inoculation was prepared after two-stage culture process. Single colony of pure *E. coli* culture was introduced in 20 ml nutrient broth and incubated for 5 hours at 37°C for culture to reached exponential phase (Czajkowska *et al*., 2004). The pure culture was added into 300 mL nutrient broth (10% pure culture v/v nutrient broth) and incubated at 37°C for 1½ hours. The inoculum concentration of 10^5-10^6 CFU/mL was determined by optical density at 650 nm and by calibration curve developed (Ling *et al*., 2003).

3.42 Die-off rate experiment

The die-off rate of *E. coli* was conducted using sediment from three different stations which are Station 3, 4 and 5. Fifty gram of sediment was placed in a 250 mL conical flask and 25.5 mL of sterile distilled water was added to saturate the sediment (Ling *et al*., 2005). The sediment was inoculated with 5 ml of *E. coli* inoculum and mixed. The beakers were covered with aluminium foil to exclude light and incubated separately at 25°C and 30°C. The experiment was performed in duplicate. Sampling was done every two days for two weeks until the *E. coli* colonies were undetectable. One gram of sediment samples were diluted in sterile saline (NaCl 0.8%) before spreading on EMB agar plate. The plate was incubated at 37°C for 24 hours before enumeration.

3.5 Sediment analysis

3.51 Particle size analysis (PSA)

Particle size analysis of the sediment was carried out using pipette method (Gee and Bauder, 1986). The purpose of this method is to determine the percentage by weight of sand, silt and clay from each sample. Hexametaphosphate (HMP) was added to the sediment in order to remove organic matters which cause the soil to aggregate. After the dispersal of the soil particles, the remaining of the sediment was sieved to separate the sand fraction. For the separation of sand fraction, the suspension was poured through a 53 µm sieve into a 1 L sedimentation cylinder. A 20 cm diameter sieve was placed in a large funnel held by a stand above the cylinder and sand was washed thoroughly on the sieve and the washing was collected. The sand was transferred to a tared beaker, dried at 105 ºC and weigh. After that, the sand was transferred to the nest of sieve arranged from top to bottom with decreasing size in following order: 1000, 500, 250, 106, 53 µm

and pan. The sieve was shaked on a sieve shaker for 3 minutes and each sand fraction and the residual silt and clay that passed through the 53 µm was weighed. For the determination of silt and clay fraction, the cylinder containing mixture of silt and clay suspension in was placed in a water bath and 10 mL of HMP solution was added. After letting the suspension to equilibrate, the suspension was stirred thoroughly and the time at completion of stirring and temperature of water bath was recorded. After the appropriate intervals, the pipette was placed into cylinder at appropriate depth and withdraws a 25 mL sample in about 12 second. The sample was discharged into beaker, dry at 105 ºC and weighed.

3.52 Organic matter content analysis

Organic matter content was determined by Loss-On-Ignition (Nelson and Sommers, 1996). The beakers were heated in furnace at 400 ºC for 2 hour, cooled down and the tare weigh was determined to 0.1 mg. Then, 1 to 3 g of air dried soil ground was added to a tared beaker and heat at 105 ºC for 24 hour. The beaker was cooled in desiccators over $CaCl_2$ and weight of beaker plus sample was determined to 0.1 mg. The weight of oven dried sample was obtained by subtraction. The samples were ignited in furnace at 400 ºC for 16 hour. The beaker was cooled in desiccators over $CaCl_2$ and weight of beaker plus ignited sample was determined to 0.1 mg. The LOI content of the sample was calculated as

$$\text{LOI, \%} = \frac{\text{Weight}_{105} - \text{Weight}_{400} \quad \text{x } 100}{\text{Weight}_{105}}$$