# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Sampling sites

Investigation on *Pediastrum* spp. in standing freshwater was carried out during June 2011- May 2012. In total, 204 water samples from 68 sampling sites including lakes, dams, reservoirs, pond and ditch from 48 provinces were collected in this study (Figures 3 and 4). There were 8 sampling sites in the northern region, 18 in the central region, 20 in the north-eastern region, 6 in the eastern region, 6 in the western region and 10 in the southern region. The ecological data of each sampling site were recorded i.e. latitude and longitude and utilization of sampling site such as forest, agriculture and community. The list of sampling sites together with some general data are given in Table 2.

**Table 2 Location and some characteristics of sampling sites**

| Sampling site | Code | Size | Ordinations | |
|---|---|---|---|---|
| 1. Chiang Rai | | | | |
| 1.1 Canal (roadside) | CHR1 | M | 99°49'27"E | 19°54"28"N |
| 2. Chiang Mai | | | | |
| 2.1 Pond (Wat Umong) | CHM1 | S | 100°07'55"E | 15°42"11"N |
| 2.2 Pond (700$^{th}$ Anniversary Chiang Mai Sports Complex) | CHM2 | S | 100°07'55"E | 15°42"11"N |
| 2.3 Chiang Mai Moat | CHM3 | L | 98˚58′ 770″E | 18˚46′ 900″N |
| 3. Phayao | | | | |
| 3.1 Ang Leung reservoir | PHY1 | M | 99°53'45"E | 19°01"37"N |
| 3.2 Pond (in Phayao University) | PHY2 | S | 99°53'31"E | 19°01"58"N |
| 4. Phrae | | | | |
| 4.1 Pond (roadside) | PHR1 | S | 100°00'57"E | 17°48"03"N |

Size of reservoir was characterized from its capacity
S= capacity of reservoir<1 million $m^3$ and/or surface area of reservoir < $1km^2$
M= capacity of reservoir>1 million $m^3$ and<100 million $m^3$ and/or surface area of reservoir > $1km^2$ and <15 $km^2$
L= capacity of reservoir ≥100 million $m^3$ and/or surface area of reservoir >15 $km^2$

**Table 2 (Continued)**

| Sampling site | Code | Size | Ordinations | |
|---|---|---|---|---|
| 5. Uttaradit | | | | |
| 5.1 Pond (roadside) | UTD1 | S | 9°57'25"E | 17°34"44"N |
| 6. Sukhothai | | | | |
| 6.1 Pond (Wat Traphang Tong) | SKT1 | L | 99°42'32"E | 17°01"06"N |
| 7. Phitsanulok | | | | |
| 8.1 Pond (near Latina Hotel) | PSL1 | S | 100°16'33"E | 16°45"23"N |
| 8. Nakhon Sawan | | | | |
| 8.1 Nong Somboon | NKS1 | L | 100°07'55"E | 15°42"11"N |
| 8.2 Pond (in University of Central Thailand) | NKS2 | S | 100°06'50"E | 15°41"54"N |
| 9. Phichit | | | | |
| 9.1 Fish pond | PHC1 | S | 100°24'2.5"E | 16°10"58"N |
| 10. Phetchabun | | | | |
| 10.1 Huai Pa Dang Reservoir | PCB1 | L | 101°05'14"E | 16°26"48.6"N |
| 10.2 Pond (in Provincial Waterworks Authority) | PCB2 | S | 101°8'53.7"E | 16°26"9.7"N |
| 11.Saraburi | | | | |
| 11.1 Canal Pure (near restaurant) | SRB1 | L | 100°55'50.6"E | 14°31"40"N |
| 12. Pathum Thani | | | | |
| 12.1 Pond (Country Place Resort Club) | PTT1 | S | 100°45'00.4"E | 14°02"43.5"N |
| 13. Samut Prakan | | | | |
| 13.1 Canal (roadside) | SMP1 | L | 100°36'35.5"E | 13°35"17.9"N |
| 14. Phra Nakhon Si Ayutthaya | | | | |
| 14.1 Pond (in Red Cross Office) | PNS1 | S | 100°34'3.2"E | 14°21"1.5"N |
| 15. Sing Buri | | | | |
| 15.1 Pond (in restaurant) | SBR1 | S | 100°24'26.3"E | 14°51"37.6'N |
| 16. Ang Thong | | | | |
| 16.1 Canal (roadside) | ANT1 | M | 100°27'12.9"E | 14°29"33.7"N |
| 17. Suphan Buri | | | | |
| 17.1 Pond (in front of the Uthong National Museum Suphanburi) | SPB1 | M | 99°53'30"E | 14°22'16"N |
| 18. Uthai Thani | | | | |
| 18.1 Pond (Karung City Police Station) | UTT1 | S | 99°41'49"E | 15°15'31"N |
| 18.2 Bueng Reusi | UTT2 | M | 100°7'46"E | 15°30'2"N |
| 19 Nakhon Pathom | | | | |
| 19.1 Concrete pond (roadside) | NPT1 | S | 99°58'40"E | 13°47'52"N |
| 19.2 Lad Po Marshes | NPT2 | S | 99°58'40"E | 13°47'52"N |
| 20. Loei | | | | |
| 20.1 Pond (in Phurua Hospital) | LOE1 | S | 99°49'37"E | 18°28"27"N |
| 21. Udon Thani | | | | |
| 21.1 Nong Prajak Park | UDT1 | M | 102°46'47"E | 17°25'11"N |
| 22. Nong Khai | | | | |
| 22.1 Pond (roadside) | NKI1 | S | 103°04'41"E | 18°01'07"N |
| 23. Nakhon Phanom | | | | |
| 23.1 Pond (roadside) | NPN1 | S | 104°43'425"E | 17°23'25"N |
| 23.2 Pond (in Nakhon Phanom University) | NPN2 | S | 104°43'08"E | 17°23'50"N |

**Table 2 (Continued)**

| Sampling site | Code | Size | Ordinations | |
|---|---|---|---|---|
| 24. Sakon Nakhon | | | | |
| 24.1 Municipal Oxidation Pond | SKN1 | S | 104°10'09"E | 17°09'48"N |
| 25. Mukdahan | | | | |
| 25.1 Pond (in Chaloem Phra Kiat Kanchana Phisek Park) | MDH1 | S | 104°43'16"E | 16°32'32"N |
| 26. Yasothon | | | | |
| 26.1 Canal (in Shithummaram Temple) | YST1 | S | 104°08'27"E | 15°47'06"N |
| 27. Roi Et | | | | |
| 27.1 Pond (roadside) | RET1 | M | 104°08'27"E | 15°47'06"N |
| 27.2 Phalanchai Lake | RET2 | M | 103°38'55"E | 16°03'28"N |
| 28. Kalasin | | | | |
| 28.1 Pond (in Kud Nam Kin Park) | KLS1 | M | 103°30'00"E | 16°25'20"N |
| 29. Khon Kaen | | | | |
| 29.1 Pond (roadside) | KKN1 | L | 102°50'58"E | 16°26'45"N |
| 29.2 Concrete pond (roadside) | KKN2 | M | 102°50'25"E | 16°26'48"N |
| 30. Nakhon Ratchasima | | | | |
| 30.1 Pond (roadside) | NRS1 | S | 102°15'17"E | 14°58'47"N |
| 30.2 Canal (roadside) | NRS2 | M | 102°06'08"E | 14°58'34"N |
| 31. Surin | | | | |
| 31.1 Pond (roadside) | SUR1 | S | 103°32'11"E | 14°52'40"N |
| 31.2 Fish pond (in temple) | SUR2 | S | 103°32'27"E | 14°52'41"N |
| 32. Si Sa Ket | | | | |
| 32.1 Canal (in Somdech Phra Srinagarindra Park) | SSK1 | M | 104°18'24"E | 15°06'10"N |
| 32.2 Pond (Sisaket Mareenon Can Park) | SSK2 | S | 104°19'09"E | 15°06'16"N |
| 33. Ubon Ratchathani | | | | |
| 33.1 Pond (Ubon Ratchathani Cultural Center) | UBR1 | S | 104°50'44"E | 15°14'47"N |
| 34. Chachoengsao | | | | |
| 34.1 Pond (in Somdech Phra Srinagarindra Park) | CCS1 | S | 101°04'05"E | 13°41"17"N |
| 35. Chon Buri | | | | |
| 35.1 Pond (in Burapha University) | CBR1 | S | 105°55'36"E | 13°16"36"N |
| 36. Rayong | | | | |
| 36.1Pond ( in Phraphuttha Angkhirot Dhamma Hall) | RYN1 | S | 101°16'37"E | 12°40"30"N |
| 36.2 Klong (roadside) | RYN2 | M | 101°16'38"E | 12°40"29"N |
| 37. Chanthaburi | | | | |
| 37.1 ( in King Taksin the Great Park) | CTB1 | L | 102°06'11"E | 12°36"23"N |
| 38. Sa Kaeo | | | | |
| 38.1 Pond (in Sa Kaeo Hospital) | SKO1 | S | 102°04'20"E | 13°48"59"N |
| 39. Tak | | | | |
| 39.1 Pond (in Tak Municipal Wastewater Treatment) | TAK1 | M | 99°7'51"E | 16°51'35"N |

**Table 2 (Continued)**

| Sampling site | Code | Size | Ordinations | |
|---|---|---|---|---|
| 40. Kanchanaburi | | | | |
| 40.1 Pond (Chaloem Prakiarti Rama 9 Park) | KCN1 | M | 99°45'1"E | 13°57'13"N |
| 40.2 Pond (in Phanom Thuan Provincial Waterworks Authority) | KCN2 | M | 99°41'16"E | 14°7'21"N |
| 43.3 Fish pond | KCN3 | S | 99°43'26"E | 14°9'57"N |
| 41. Ratchaburi | | | | |
| 41.1 Concrete pond (in Jompratad Health Promoting Hospital) | RBR1 | S | 99°52'51"E | 13°24'34"N |
| 42. Phetchaburi | | | | |
| 42.1 Pond (roadside) | PBR1 | S | 99°55'4"E | 12°52"7"N |
| 43. Chumphon | | | | |
| 43.1 Pond (in Chumphon Khet Udomsakdi Hospital) | CHP1 | S | 99°11'11"E | 10°29"56"N |
| 44. Surat Thani | | | | |
| 44.1 Pond (in temple) | SRT1 | S | 99°11'05"E | 9°23"06"N |
| 44.2 Pond (in Rama 9 Public park) | SRT2 | M | 99°11'05"E | 9°08"19"N |
| 44.3 Pond (in Ban Sadet Subdistrict Administrative Organization) | SRT3 | M | 99°08'03"E | 9°00"19"N |
| 45. Nakhon Si Thammarat | | S | | |
| 45.1 Pond (in Somdech Phra Srinagarindra Park) | NST1 | L | 99°57'14"E | 8°27"17"N |
| 45.2 Pond (roadside) | NST2 | S | 99°55'21"E | 8°30"46"N |
| 45.3 Pond (in Nakhon Si Thammarat Airport) | NST3 | S | 99°56'24"E | 8°32"31"N |
| 46. Phatthalung | | | | |
| 46.1 Pond (roadside) | PTL1 | S | 100°08'53"E | 7°38"35"N |
| 47. Songkhla | | | | |
| 47.1Pond Hatyai City Municipality Park | SKA1 | M | 100°30'17"E | 7°02"33"N |
| 48. Satun | | | | |
| 48.1 Pond (roadside) | STN1 | S | 100°02'59"E | 6°37"59"N |

## 3.2 Investigation of *Pediastrum* spp.

3.2.1 Collection of *Pediastrum* spp.

*Pediastrum* spp. were collected by filtering 10 liters of water from each sampling site with 10 µm pore size plankton net. The samples were preserved by adding 0.7 ml of lugol solution to 100 ml of samples (Wetzel, 2001) and the fresh sample were kept in a cool box for isolation and cultivation.

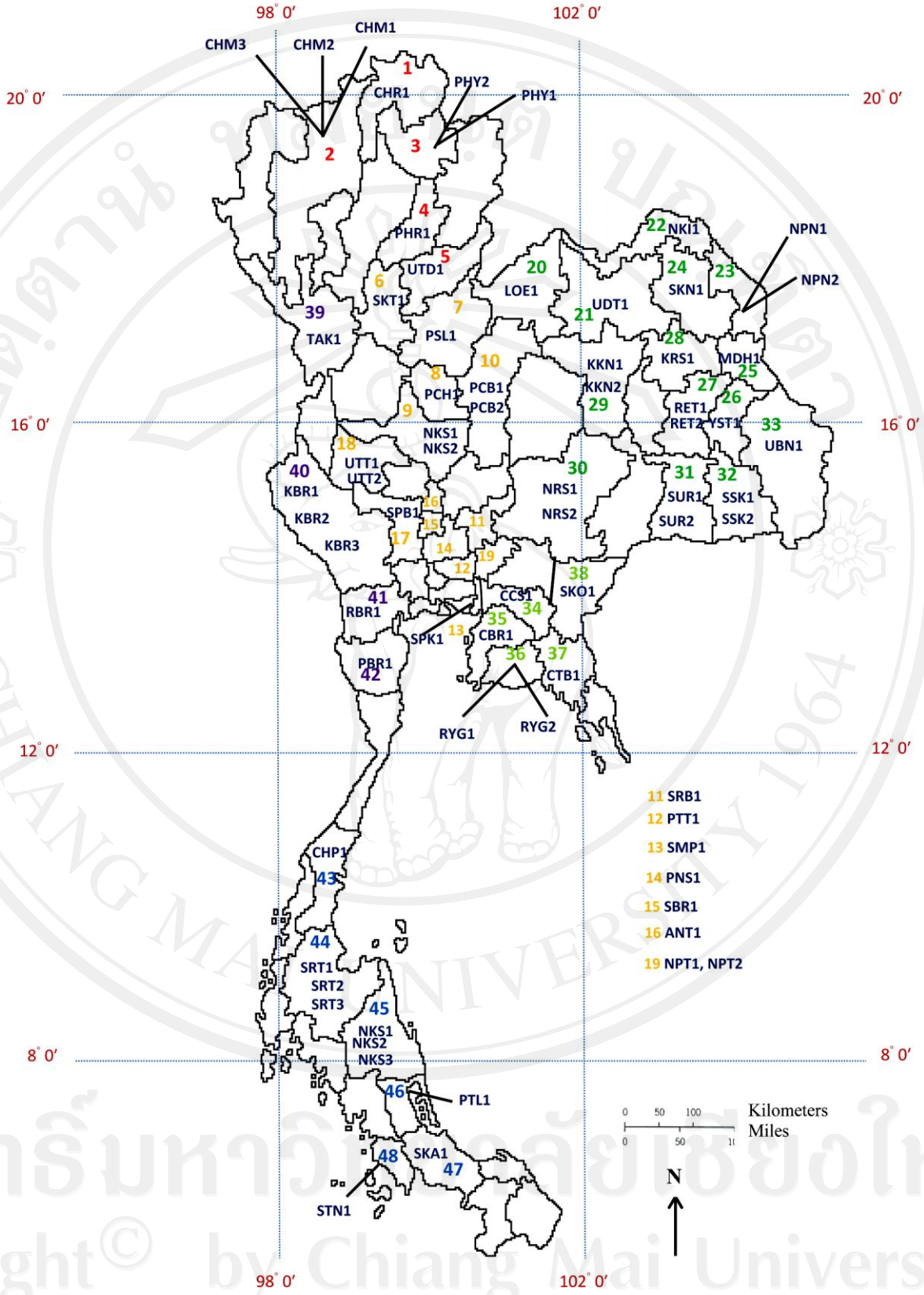


**Figure 3** Map of Thailand showed 68 sampling sites in 48 provinces in some freshwater resources

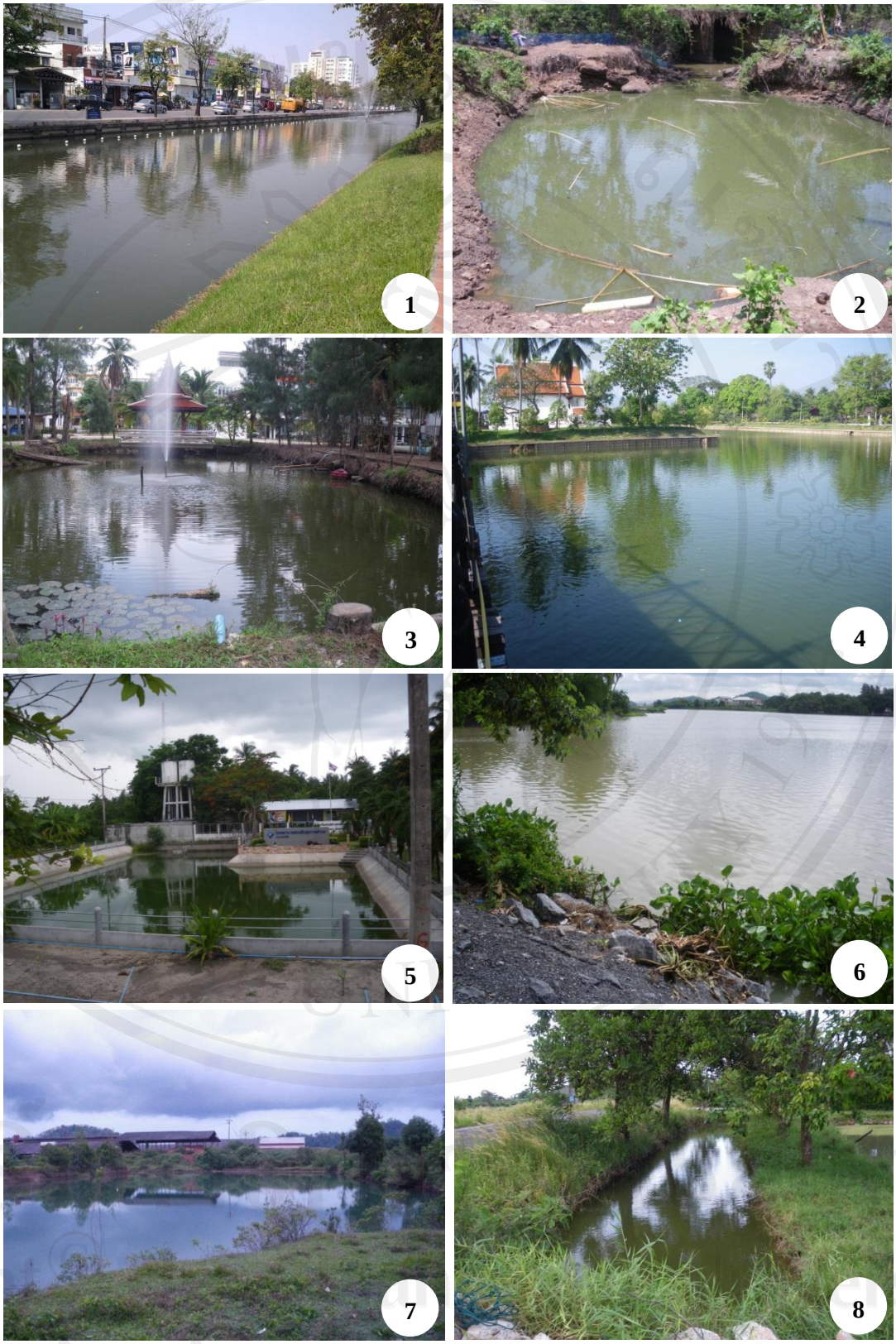


**Figure 4** Sampling sites in some freshwater resources of Thailand;
1. Chiang Mai Moat (CHM3) 2. Pond roadside (UTD1) 3. Pond in University of Central Thailand (NKS2) 4. Pond in Wat Traphang Tong (SKT1) 5. Concrete pond in Jompratad Health Promoting Hospital (RBR1)6. Klong Pure (SRB1) 7. Pond roadside (STN1) 8. Pond roadside (PTL1)

3.2.2 Identification and counting of *Pediastrum* spp.

The *Pediastrum* spp. samples were observed under 40X and 100X light microscope. The specimens were photographed using an Olympus Normaski microscope and reproduced by line drawing. Scanning electron microscope (SEM) study was also carried out. The samples were first rinsed with distilled water, laid on cover glasses and air dried at 30-40 ˚C and then affixed to aluminium stubs with carbon tape. Finally, the stubs were coated with gold and photographed by SEM according to the method of Wetzel (2001) Kowalska and Wolowski (2010).

Species identification was conducted according to Meneghini (1840), Presscott (1970), Huber-Pestalozzi (1983), Croasdale *et al*. (1994), Chang and Mi (1997), Komarek and Jankovska (2001), John *et al*. (2011) and Kowalska and Wolowski (2010 ).

The cells were counted by whole count method under light microscope (Rojo *et al*., 2009) (Appendix A)

**3.3 Water sampling procedure**

Water samples from each sampling site were collected in polyethylene bottles for nutrient and some physico-chemical analysis. The bottles were kept in a cool box (5-7$^{o}$C) for analysis in the laboratory. The samples were analyzed as follows:

**3.3.1 Physio-chemical parameters at the sampling sites**

3.3.1.1 Water and air temperature by a thermometer

3.3.1.2 Light intensity measurement by lux meter (Tecpel 530).

3.3.1.3 Transparency measurement of Secchi depth by Secchi disc

3.3.1.4 Conductivity measurement by a conductivity meter (electrode kit of TRANS Company).

3.3.1.5 pH measurement by a pH meter (electrode kit of TRANS Company)

3.3.1.6 Dissolved oxygen (DO) analysis by azide modification method (Eaton *et al*., 2005)

**3.3.2 Physio-chemical parameters in the laboratory**

3.3.2.1 Turbidity analysis by portable data logging spectrophotometer (HACH DR/2010)

3.3.2.2 Alkalinity analysis by the phenolpthalein methyl orange indicator method (Eaton *et al*., 2005)

3.3.2.3 Biochemical oxygen demand (BOD) by azide modification method (Eaton *et al*., 2005)

3.3.2.4 Chlorophyll *a* analysis (Saijo, 1975; Wintermans and De Mots, 1965)

The nutrient analysis was conducted according to the methods described by Eaton *et al*. (2005)

3.3.2.5 Nitrate nitrogen analysis by cadmium reduction method

3.3.2.6 Ammonium nitrogen analysis by nesslerization method

3.3.2.7 Soluble reactive phosphorus (SRP) analysis by ascorbic acid method

### 3.4 Cultivation of *Pediastrum* spp.

#### 3.4.1 Isolation

Colonies of *Pediastrum* spp. in the water samples were studied under a microscope and single colony were isolated with a glass micropipette. Each colony was washed at least five times with sterile medium and cultivated in the 12 multi well cell culture plate containing Jaworski's medium (JM). The alga was then purified by streak plate method on JM. Sub-culture was carried out until monoculture was obtained and transferred to broth medium for using as stock culture. The culture was incubated at 25 ºC under continuous illumination (Lee *et al*., 2009).

#### 3.4.2 Cultivation

*Pediastrum* spp. were cultivated in 3 media: Jaworski's medium, algal broth and bold basal medium for comparison (Appendix D). Then, the algae were cultivated at 4 levels of pH: 6.5, 7.0, 7.5 and 8.0 and finally incubated at 25 ºC and room temperature under continuous illumination. The experiment was carried out in 500 ml erlenmeyer flasks containing 300 ml of medium. Cell density was determined spectrophotometrically at a wavelength of 665 nm and cell counts by whole counts method (Rojo *et al*., 2009). When the growth reached the stationary phase, the cells were harvested by centrifugation and the biomass productivity (P) was calculated as maximum productivity (mg/L/d), according to the equation (Tang *et al*, 2011).

$$P = 1000. \frac{(X1 - X0)}{(t1 - t0)}.$$

X0 is the initial biomass (g/L) at time t0 (d)

X1 is the final biomass (g/L) at any time t1(d)

After the optimal condition for growth was identified, these algae were cultivated and scaled up to 20 L media carrying capacity bottles (Polycarbonate Carboys) for analysis of nutritional value (Lee *et al*., 2009).

**3.5 Analysis of nutritional value**

At the end of cultivation, the biomass of the dominant species of *Pediastrum* were selected and collected for nutritional analysis which was conducted according to the methods described by AOAC (1990).

3.5.1 Protein analysis by micro kjeldahl method

3.5.2 Fatty acid analysis by acid hydrolysis method

3.5.3 Fiber analysis by acid detergent method

3.5.4 Ash analysis by burned at 450 °c 1 hour

3.5.5 Carbohydrate analysis by calculating from

Carbohydrate (%) = 100 – (protein + fat + moisture + ash)

**3.6 Molecular examination**

Genomic DNA was extracted from living cell using the Genomic DNA Extraction Kit (Geneaid) and CTAB method. The 26SrDNA and rbcL rDNA gene were amplified by PCR using specific primer pairs. The PCR conditions for the rbcL and 26S rDNA reactions were 35 cycles at 94 ˚C, 3 min for predenature, denature at 94 ˚C for 30 sec, annealing at 55 ˚C for 30 sec, an extension step at 72 ˚C for 1 min 30 sec and with a final extension step at 72 ˚C for 7 min. The QiaQuick Nucleotide Purification Kit (Qiagen Inc.) was used to clean the PCR products, and cycle sequencing reactions of the purified PCR product were done with the ABIPRISM-Big

DyeTM Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) and analyzed using DNA sequencing software (McManus and Lewis, 2011).

**3.7 Phylogenetic tree**

The Basic Local Alignment Search Tool (BLAST) at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to find sequences similar to those of *Pediastrum* spp. Phylogenetic and molecular evolutionary analyses of the obtained sequences were conducted using the MEGA 5 computer program (Tamura et al. 2011). Alignments were checked manually. A Maximum Likelihood (ML) tree was constructed using software with the best fits model (Tamura three-parameter using a discrete Gamma distribution (_G) with five rate categories and by assuming that a certain fraction of sites is evolutionarily invariable (_I)) based on the lowest Bayesian Information Criterion (BIC) score and the substitution nucleotide matrix parameters calculated by the software. One thousand bootstraps were generated.

**3.8 Data evaluation**

The trophic status and water quality was classified according to the method of Peerapornpisal *et al*. (2007) based on Wetzel (2001) and Lorraine and Vollenweider (1981) by using selected parameters i.e. dissolved oxygen, biochemical oxygen demand, conductivity, ammonium nitrogen, nitrate nitrogen and soluble reactive phosphorus and chlorophyll *a* (Appendix B and C). The multivariate statistical package (MVSP) for windows was used. Canonical correspondence analysis (CCA) was to find the *Pediastrum* spp. and the relationship between water

quality and *Pediastrum* spp. Growth of *Pediastrum* taxa in each experiment was analyzed as mean $\pm$ S.D. Statistical comparison between groups was analyzed using SPSS for Windows$^{TM}$ version 14.0.