CHAPTER 3
MATERIALS AND METHODS

3.1 Apparatus

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3.1.2 Lichen identification keys

3.1.3 Recording form

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3.1.11 Polyethylene diffusion tube

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3.1.13 Whatman no. 40 filter paper

3.1.14 Micropipettes, Pipettes, Forceps

3.1.15 Test tubes, Volumetric flasks

3.1.16 Cellulose acetate membrane filter 0.45 µm, Sartorius, Germany

3.1.17 Paper bag, Plastic zip lock bag

3.2 Chemicals

3.2.1 Triethanolamine, 99%, BDH, England

3.2.2 Sodium nitrite ($NaNO_2$), 99.0-100.5%, Merck, Germany

3.2.3 Sulfuric acid (H_2SO_4), 98.0%, BDH, England

3.2.4 Sodium carbonate (Na_2CO_3), 99.7%, Carlo Erba, Italy

3.2.5 Sodium bicarbonate ($NaHCO_3$), 99.5%, Merck, Germany

3.2.6 Sulfate stock standard (1000 µg/ml)

3.2.7 Deionised water, Milli-Q water

3.3 Instruments

3.3.1 Compound microscope, Olympus model CH-BI45-2, Japan

3.3.2 Stereo microscope, Olympus model SZ 3060, Japan

3.3.3 Ion Chromatography, Metrohm Ion Analysis model 732-733, Switzerland

3.3.4 pH meter, Scientific Instrument model IQ150, USA

3.3.5 Ultrasonic Cleaner, Cole-Parmer model 8891, USA

3.3.6 Analytical balance, Mettler Toledo model AB304-S, Switzerland

3.3.7 Oven, Scientific model 9000, USA

3.3.8 Ultraviolet Lamp, Vilber Lourmat model VL-6.LC, France

3.4 Description of study area

Lampang city is situated in the northern part of Thailand, at 269 m above sea level. The upper part of the province is comprised of dense forest. The middle part of the province comprises a plain supporting important agricultural resources for the province. The lower part of the province consists of dry dipterocarp forests and grasslands.

This study was performed in the center part of Amphoe Mueang Lampang, which is a developed area. The location of the study area is at approximately 18°13′ N to 18°20′ N latitude and 99°26′ E to 99°34′ E longitude, with an elevation of 269 m. The study area, which located in Lampang city (Figure 3.1), covered about 92 km^2 of the plain area on the banks of the Wang river. The study area hosts about 102,712 habitants with the population density of 3,072 people/km^2 (Na Ma, 2004). The land use within the study area is shown in Figure 3.2, and the locations of commercial and industrial zones are shown in Figure 3.3.

3.4.1 General climatic condition

Since Lampang is situated in a low lying plain surrounded by high mountains, there is a relatively high daytime temperature during the hot season and the low temperature during the cold season. The temperature ranges between 10.5 - 41.6 ˚C. The climate of the study area is influenced by the two major types of monsoons, the northeast and the southwest monsoon. The cold season, which occurs from mid October to mid February, is influenced by the northeast monsoon, which brings cold and dry air from the anticyclone in China mainland. This is followed by the hot season, which lasts from mid February until mid May. The transitional period from the northeast to southwest monsoons causes the warmer weather in this season. The southwest monsoon brings warm, moist air from the Indian Ocean and the onset of the wet season, which starts in mid May and ends in mid October (Thai Meteorological Department, 2005). The average annual rainfall is 75.13 mm (Lampang Meteorological Station, 2005).

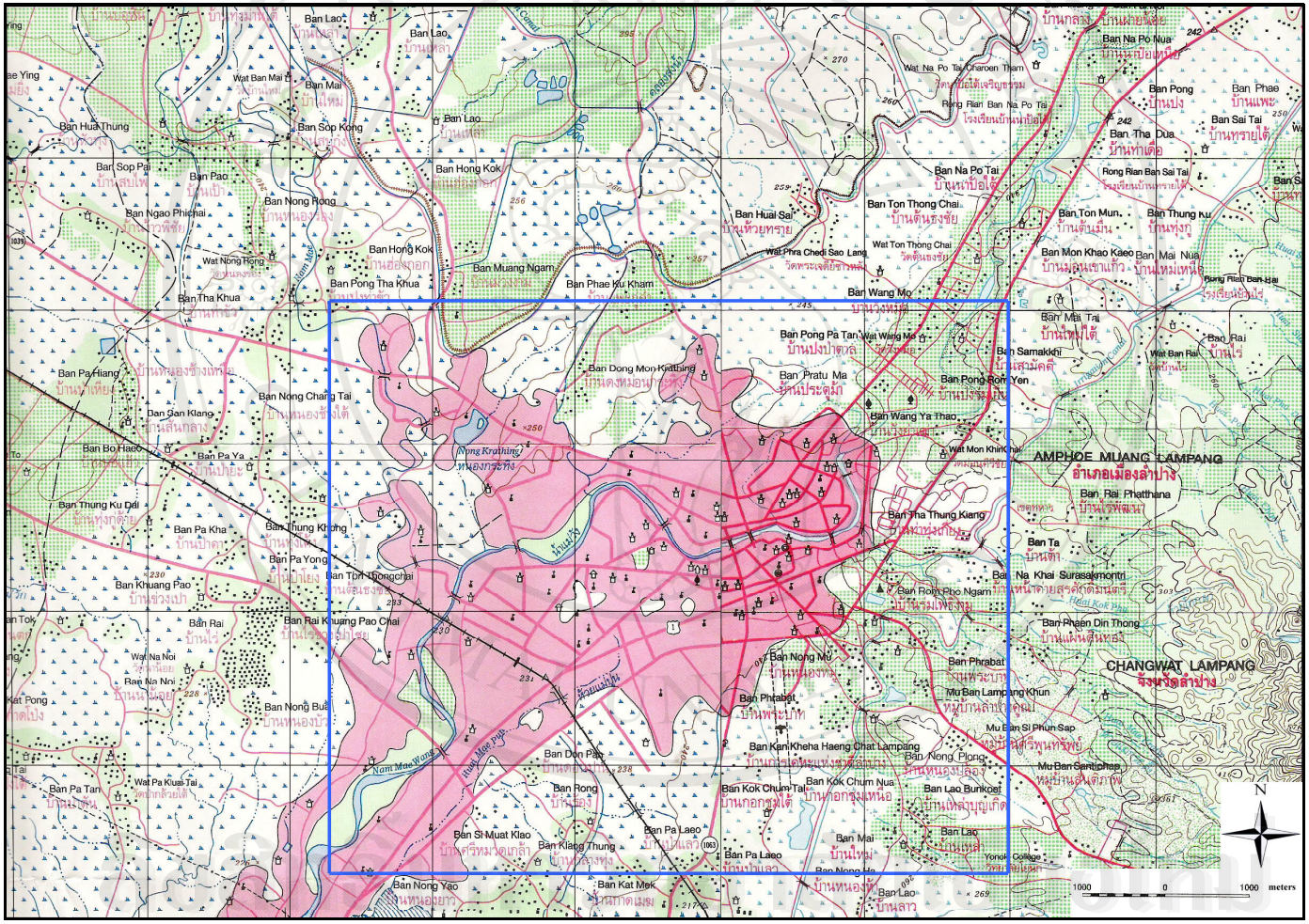

Figure 3.1 The study area (▬) in Lampang city, the red zone indicates the built up area (Royal Thai Survey Department, 1997)

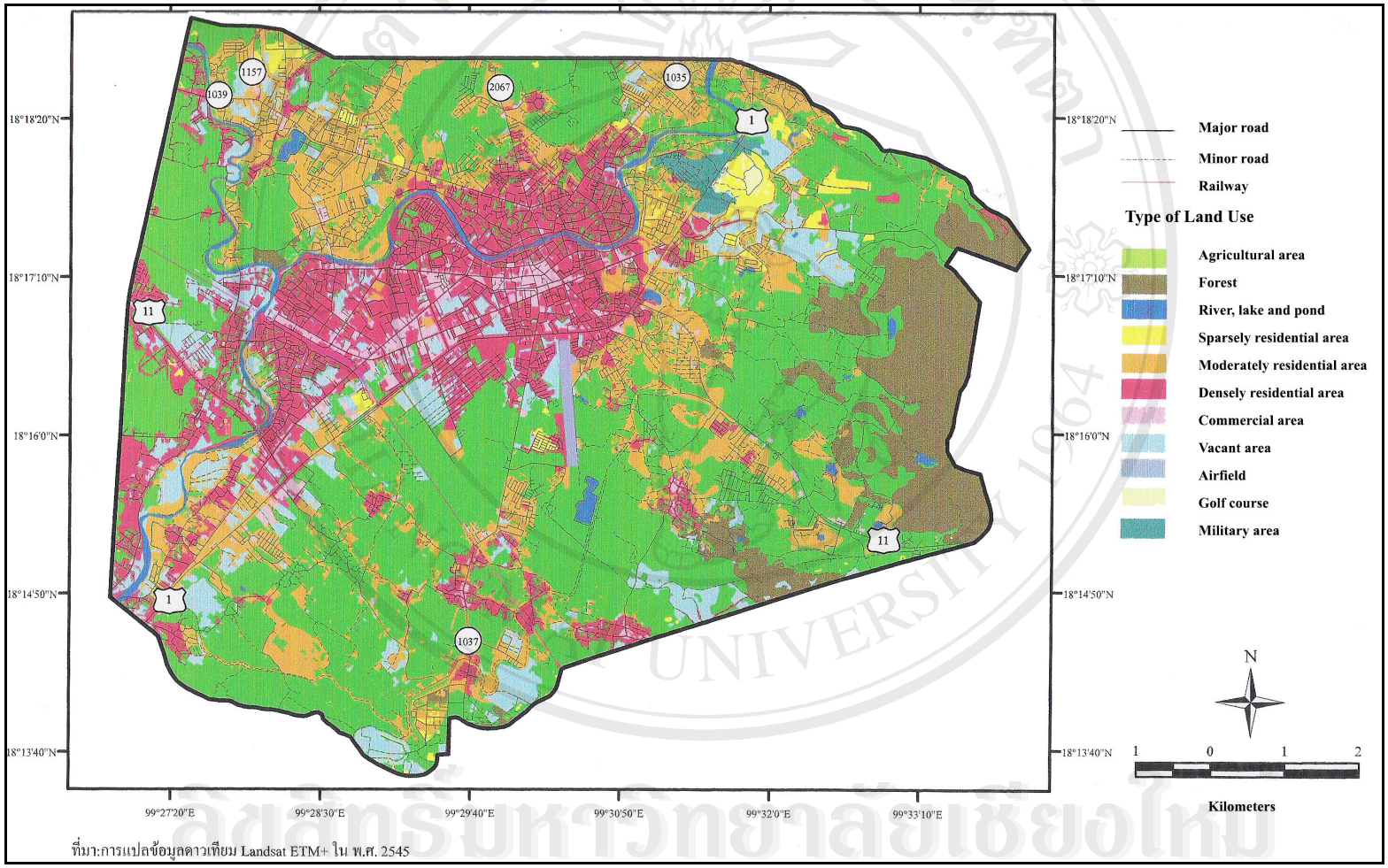

Figure 3.2 Land use type within Lampang urban area in 2002 (Na Ma, 2004)

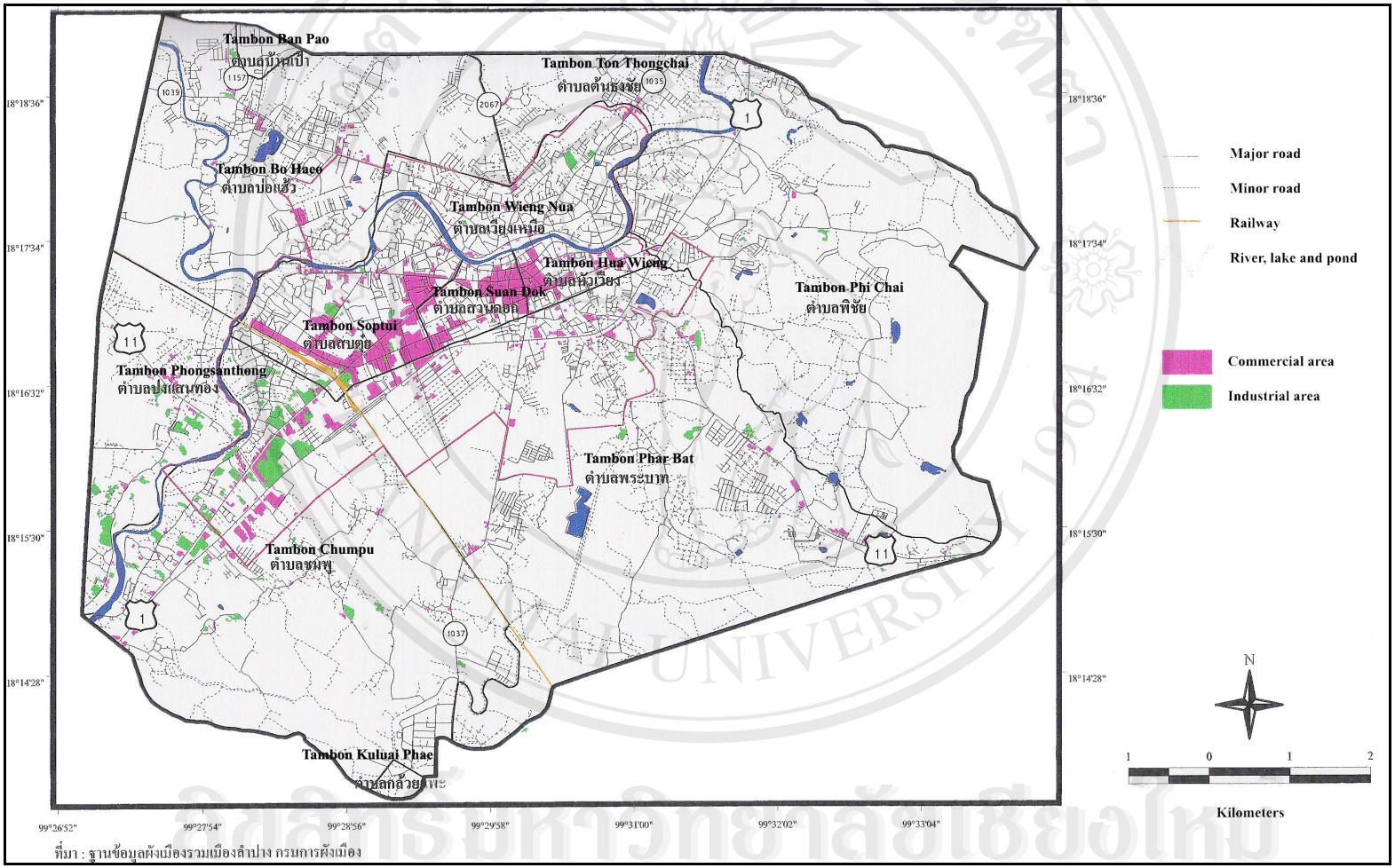

Figure 3.3 Commercial and industrial area within Lampang urban area (Na Ma, 2004)

The annual temperature, annual relative humidity, annual rainfall of Amphoe Mueang Lampang from in 2004 are shown in Figure 3.4, Figure 3.5 and Figure 3.6, respectively. The annual prevailing wind direction in year 2004 is shown in Figure 3.7

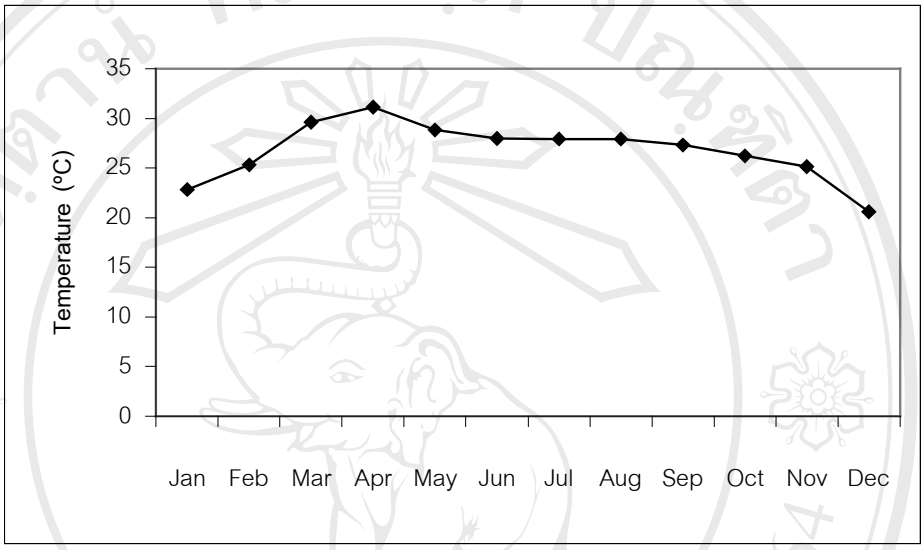

Figure 3.4 The annual temperature of Amphoe Mueang Lampang in 2004 (Lampang Meteorological Station, 2005)

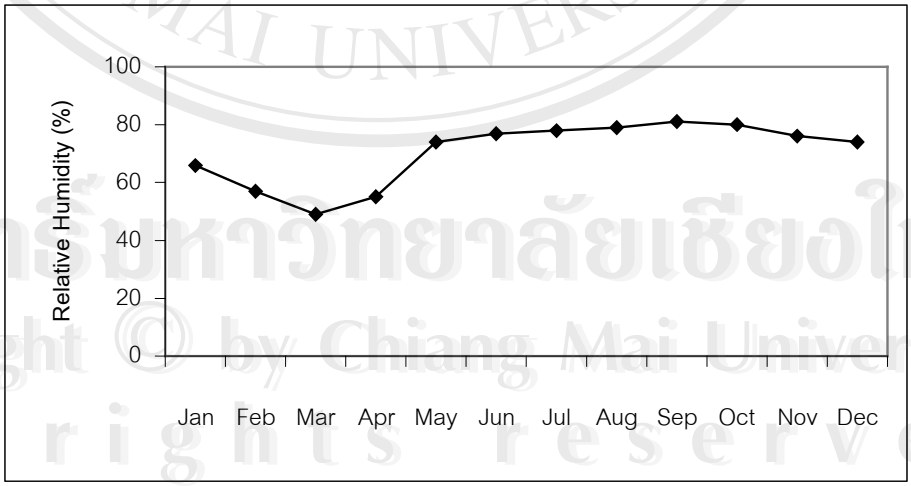

Figure 3.5 The annual relative humidity of Amphoe Mueang Lampang in 2004 (Lampang Meteorological Station, 2005)

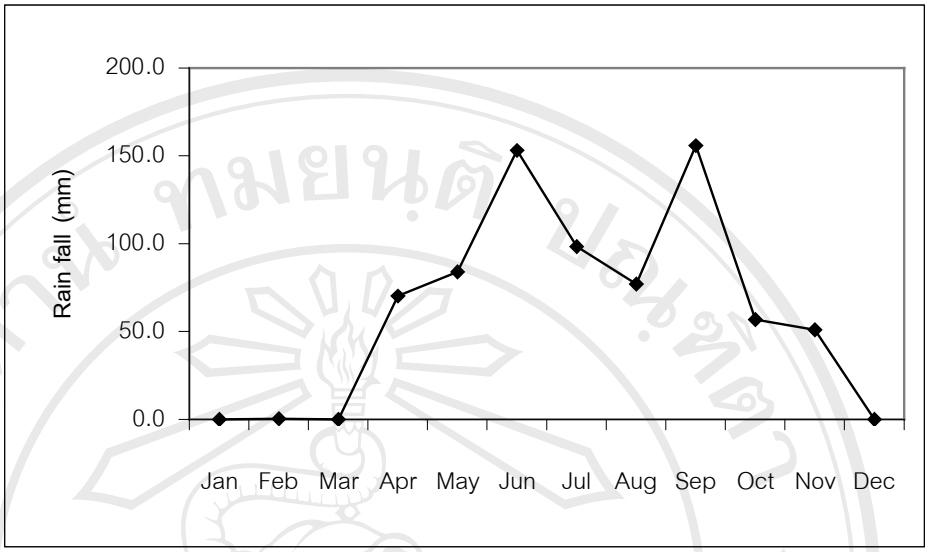

Figure 3.6 The annual rainfall of Amphoe Mueang Lampang in 2004 (Lampang Meteorological Station, 2005)

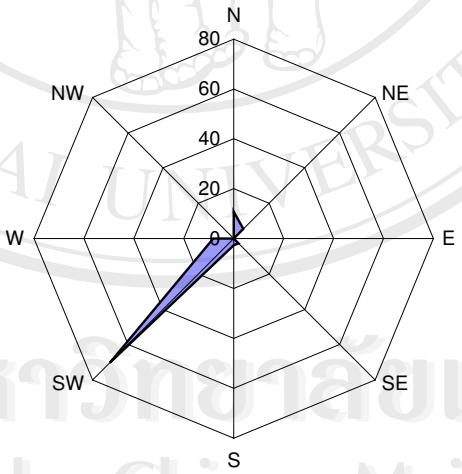

Figure 3.7 Annual prevailing wind distributions in study area (Lampang meteorological Station, 2005)

3.5 Mapping of lichen diversity

3.5.1 Sampling procedure

The method for mapping lichen diversity was based on the new standard protocol by Asta *et al.* (2002).

The study area, Amphoe Mueang Lampang was divided into thirty-nine sampling units of sampling area 1 ×1 km^2. Due to the differences in vegetation, the mango tree (*Mangifera indica* L.) was chosen as a suitable tree for lichen investigation, since it was a very common tree species in study area and the bark pH was not too low or too high compared to other common tree species, such as Longan (*Dimocarpus longan* Lour.), Litchi (*Litchi chinensis* Sonn.) and Umbrella trees (*Terminalia catappa* L.) (Saipunkeaw, 1994).

In each sampling area, six mango trees were sampled (Figure 3.8). Selected trees must be freestanding, with the girth more than 40 cm at 150 m above ground level. The inclination should not exceed 10˚ degree from the vertical. Trees that were injured by fungicides, liming, grazing animals and human activities were not selected.

The sampling grid consisted of four vertical ladders and each ladder was divided into five 10×10 cm quadrats. The ladder was attached vertically to the sampled tree trunk corresponding with the 4 cardinal points (North, East, South and West) at 150 cm above the ground, as shown in Figure 3.9, At least three vertical ladders should be placed on a given trunk. To avoid parts of tree trunks that that were not suitable for sampling (e.g. wound, knots, etc), the ladder was relocated by maximum shift of 20˚ in a clockwise direction. All lichen species present within each tree were recorded using a recording form (Appendix A). For each cardinal point, lichen species occurring inside the ladder were identified and the number of squares (1-5) in which they occurred was recorded as their frequencies.

Only well known and easily recognized lichens species were determined in the field but for those that were not identified, chips of bark containing the lichens were removed from the trunk by a pocketknife. They were identified later in the laboratory using lichen identification keys (Swinscow and Krog, 1988; Wolseley and Aguirre-Hudson, 1997; Sipman, 2003).

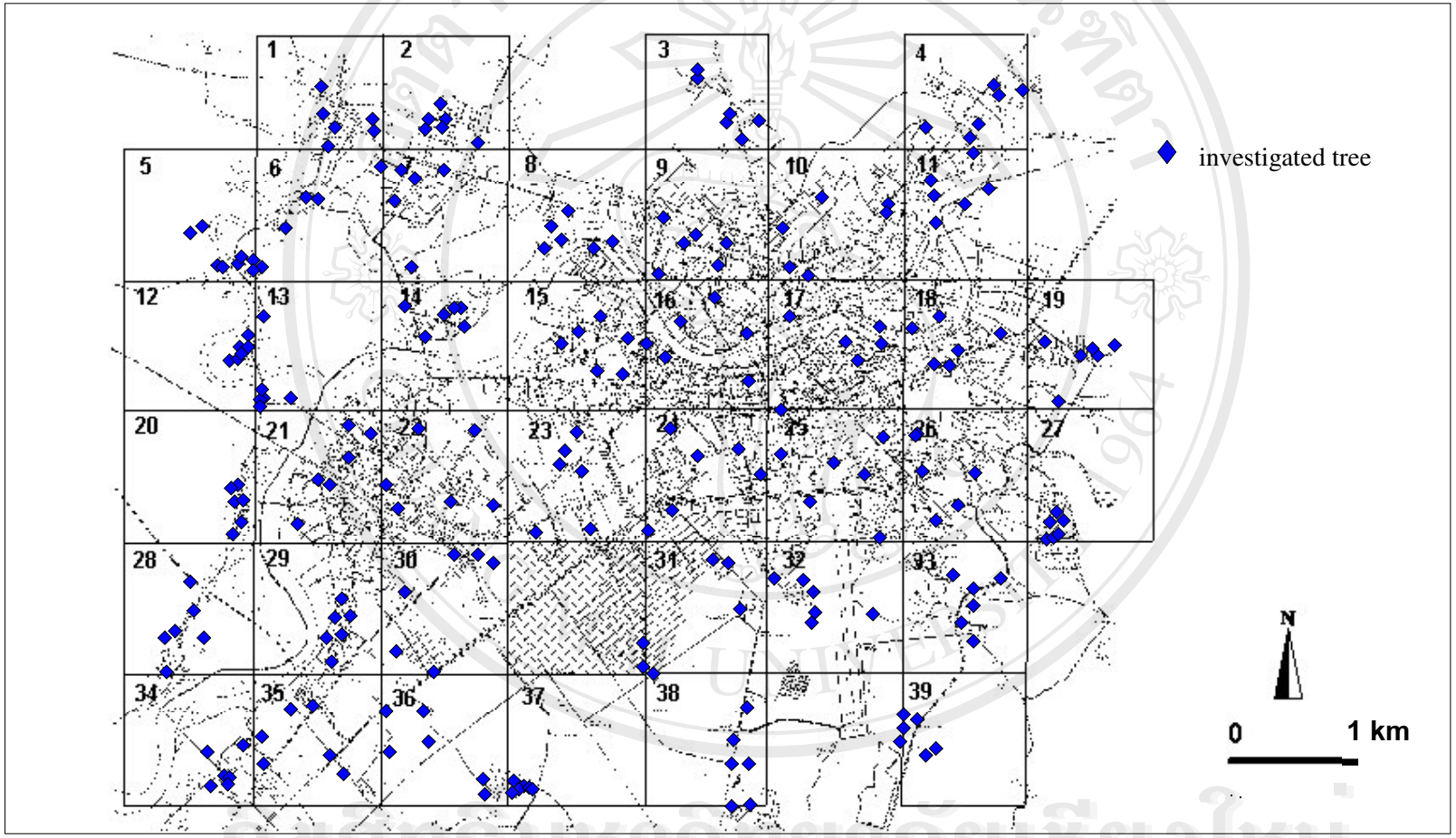

Figure 3.8 Sampling units and the location of the investigated Mango tree

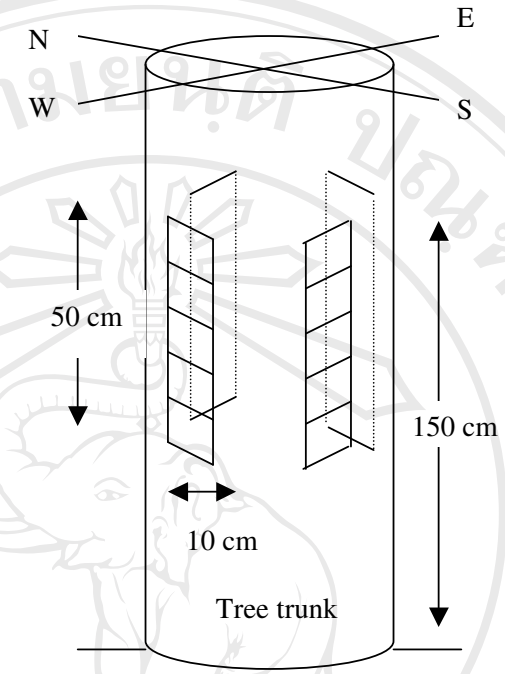

Figure 3.9 Sampling grid, consists of four ladder, with five 10 ×10 cm contiguous quadrats, attached at four cardinal points (Asta *et al.*, 2002)

3.5.2 Calculation of lichen diversity values (LDVs)

After all lichens species were determined, the frequencies of lichen species and lichen diversity values were analyzed.

Lichen diversity values (LDVs) of the sampling unit were a statistical estimator of environmental condition in that unit. To calculate LDVs of a sampling unit, first the frequencies of all lichen species found on each tree (i) within the unit were summed. Thus, there were four Sums of Frequencies (SF) for each tree. All examples of the calculation are shown in Appendix B.

$$\text{Tree (i)};\ SF_iN,\ SF_iE,\ SF_iS,\ SF_iW \qquad (3.1)$$

Next, for each cardinal point, the arithmetic mean of the Sums of Frequencies (MSF) for sampling unit j were calculated

$$MSFN_j = (SF1N_j + SF2N_j + SF3N_j + SF4N_j + \ldots + SFnN_j) / n \qquad (3.2)$$

Where;

MSF	mean of the sums of frequencies of all the sampled trees of sampling unit j
SF	sum of frequencies of all lichen species found at one cardinal point of tree i
n	number of trees sampled in sampling unit j
N,E,W,S	north, east, south, west

The Lichen Diversity Value of a sampling unit j (LDV_j) was the sum of the MSFs of each cardinal point.

$$LDV_j = (MSFN_j + MSFE_j + MSFS_j + MSFW_j) \qquad (3.3)$$

3.5.3 Determination of lichen diversity classes (LDC)

LDVs were grouped into classes sufficiently wide to reflect statistically and environmentally significant differences among sampling units for interpretion and presentation results. The widths of each of the lichen diversity classes were determined by the median of the standard errors (SE) of all the sampling units. To calculate the SE of the LDVs, the standard deviation of the sums of frequencies were determined. This was done by separately determining the SD for each cardinal point in each sampling unit and then averaging them.

$$\text{SD of each cardinal point} = \sqrt{\frac{\sum(SF_{ij} - MSF_i)^2}{n_i - 1}} \quad (3.4)$$

Where;

SF_{ij} sum of frequencies of cardinal point i, tree j

n_i number of trees in cardinal point i

MSF_i mean of sum of frequencies in cardinal point i

$$\text{Average SD of sampling unit j} = SDN_j + SDE_j + SDS_j + SDW_j \quad (3.5)$$

Thus, the SE of the LDVs was calculated according to the following formula;

$$\text{SE of sampling unit j} = \text{Average } SD_j \,/\, \sqrt{(n_j - 1)} \quad (3.6)$$

Where;

SD_j standard deviation of sums of frequencies in unit j

n_j number of trees sampled in unit j

The width of the LDV classes was then calculated as the following,

$$\text{Width of the LDV classes} = 3 \times \text{Median of SE} \quad (3.7)$$

3.5.4 Scale to interpret lichen diversity

Since the guideline by Asta *et al*. (2002) lacked a local interpretation scale in Thailand, the method for creating the interpretation scales referred to the VDI guideline (VDI, 1995) which is used in temperate areas. The LDVs of sampling unit j were assigned to lichen diversity classes according to the following scheme (Table 3.1)

Table 3.1 Determining the lichen diversity class of the investigated area

Lower class limit		Upper class limit
0	< LDVj≤	1 × class width
1 × class width	< LDVj≤	2 × class width
	etc.	

The obtained lichen diversity class was compared to the standard exposure scale (Figure 3.10) to obtain the suitable verbal expression and color code for the new interpretation scale of the investigated area. The thresholds of the standard exposure scale, which referred to VDI (1995), were the values of atmospheric pollution of 0-12.5-25-37.5-50. The verbal expressions and color codes were assigned to these numerical values.

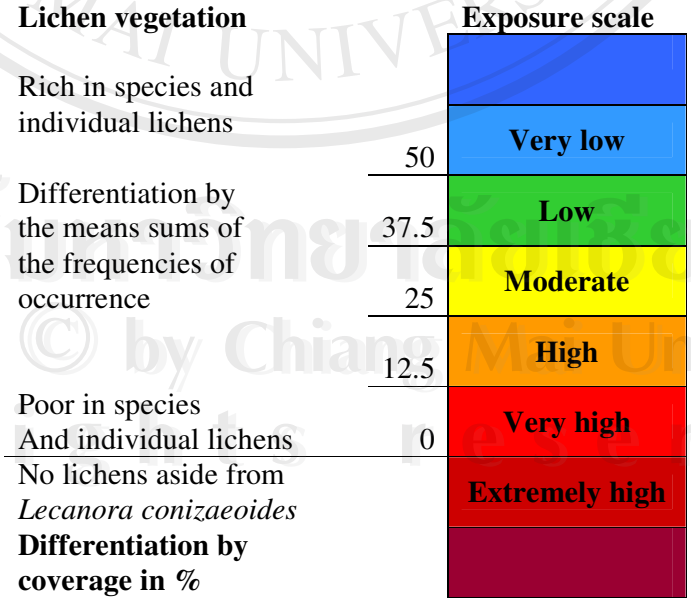

Figure 3.10 Standard exposure scale (VDI, 1995)

3.5.5 Mapping

LDVs of each sampling unit was assigned to the new interpretation scale. If the LDV classes fell into two exposure categories then this was indicated by combining the verbal expression of both categories, for example "moderate to low exposure" and by using hatched color codes (e.g. green hatching on yellow background). If several air quality classes fell into one exposure category, they were separated by black dots. The density of the dots decreased toward the range of better air quality (VDI, 1995). The sampling units were then colored according to the respective class. An air quality map was constructed by using MapInfo Professional 7.0 and Vertical Mapper 2.1 software. The results are shown in a map indicating zones with different lichen diversity.

3.6 Analysis of Bark pH

Pieces of bark 2-3 mm thick without lichens were removed around respective trees trunk at 1.50 m above the ground using a pocketknife. Chips of bark were collected in plastic bags and stored in a freezer until the time of analysis. The bark samples were dried at 80˚ C for 24 hours and then grounded. Samples of 2 g of bark were soaked with 10 ml distilled water. After 24 hours, pH was determined directly in the solution by pH meter (Staxäng, 1969).

3.7 Passive Sampling

3.7.1 Description of sampling site

To determine the NO_2 and SO_2 concentrations of each air pollution zone in the study area, the sampling sites for installing the diffusion tubes were decided after the air quality map was created. The locations of sampling sites were presented later in Chapter 4.

3.7.2 Climatic condition during the exposure period

The daily temperature and relative humidity during the exposure period, February 3 – 17, 2005 are shown in Figure 3.11 and Figure 3.12, respectively (Pollution Control Department, 2005). The average rainfall was 14.8 mm (Lampang Meteorological Station, 2005).

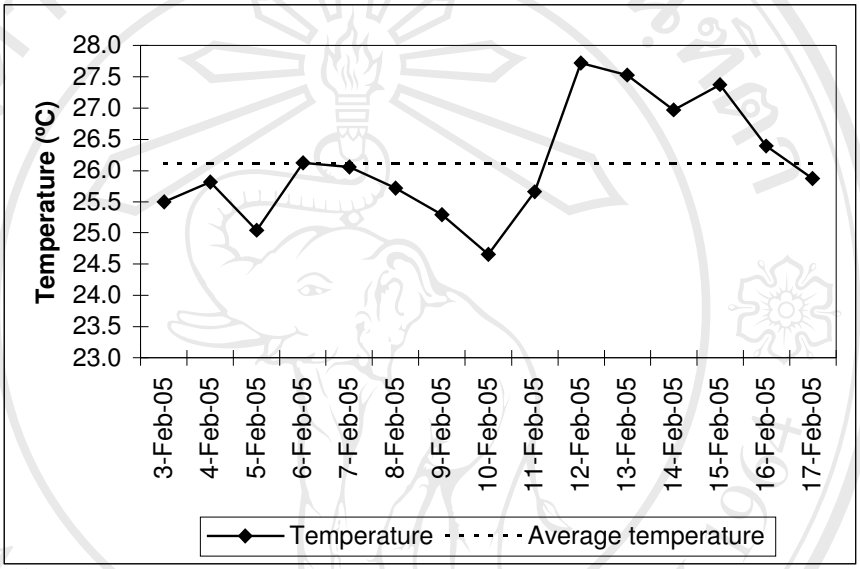

Figure 3.11 Daily temperature during the exposure period

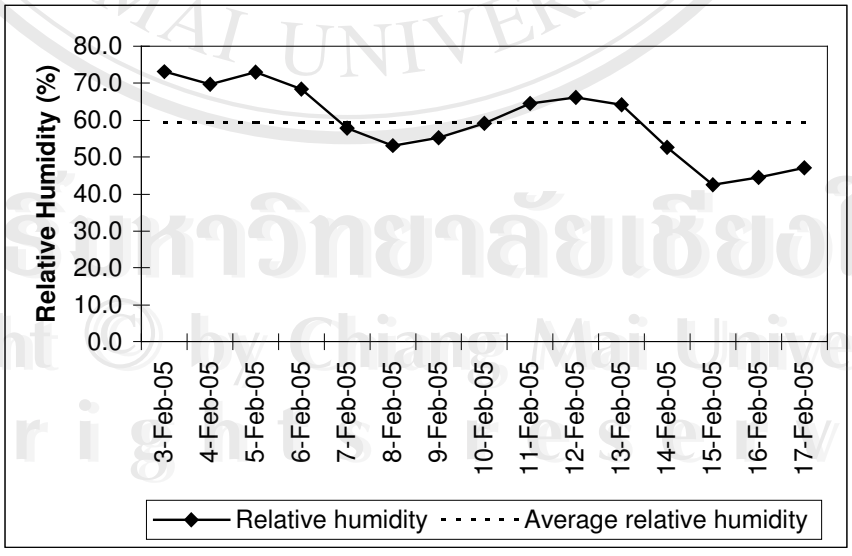

Figure 3.12 Daily relative humidity during the exposure period

3.7.3 Preparation of diffusion tube

Polyethylene tubes with a length of 5.4 cm and an internal diameter of 1.3 cm were cleaned and sonicated for one hour, then rinsed with milli-Q water. Filter paper, Whatman no. 40 was cut in a circle with the diameter being equal to the inner diameter of the tube. The filter paper was sonicated for one hour and then soaked overnight with milli-Q water. After being air-dried at 103 ºC for one hour, filter paper was placed inside the bottom of the tubes (Shakya, 2004)

3.7.4 Exposure of diffusion tube

On the exposure day, absorbent 50 µl of 20% TEA in milli-Q water was added directly onto the filter paper (Khaodee, in press). The diffusion tubes were vertically fixed, with the open end facing upward, inside the shields to protect them from wind, sunlight and rain. The protective shield containing three replications of tubes was hung at 1.50 m above the ground level. The tubes were exposed for two weeks from February 3 – 17, 2005.

After two weeks of exposure, the tubes were collected and closed with caps immediately, then sealed with parafilm. The tubes were placed in plastic zip lock bags and stored in a refrigerator until the time of analysis. The times of installation and collection were noted to calculate the exposure time.

For the laboratory blank, the diffusion tubes were prepared with the same procedure. Three replication tubes were fixed under the protective shield in the laboratory at room temperature without opening the caps.

3.7.5 Extraction of sample

Before extraction, the outer body of the tube was cleaned with deionised water and 4 ml of milli-Q water was added directly in the tube. The tube was capped and sonicated for 15 min to extract the nitrite and sulfate ions that were absorbed by TEA, in the form of solution. The sample solution was then filtered through cellulose acetate membrane 0.45 µm by the help of syringe (Khaodee, in press).

3.7.6 Analysis of nitrite (NO_2^-) and sulfate (SO_4^{2-}) by ion chromatography

Preparation of eluent

Stock standard of 180 mM Na_2CO_3 / 170 mM $NaHCO_3$ was prepared by dissolving 1.9078 g of Na_2CO_3 and 1.4282 g of $NaHCO_3$ in milli-Q water and diluting to 100 ml. The eluent of 1.8 mM Na_2CO_3 / 1.7 mM $NaHCO_3$ was then prepared by pipetting 10 ml of standard stock solution and diluting to 1 liter with milli-Q water. The eluent was filtered through a 0.45 µm cellulose acetate membrane to remove micro-particle, then degassed in an ultrasonic bath for 15 min to remove dissolved gasses (Shakya, 2004; Khaodee, in press).

Preparation of standards

The nitrite standard stock solution 1000 mg/l was prepared by dissolving 1.50 g of $NaNO_2$, which was previously dried overnight at 105 °C, and diluting to 1 liter with milli-Q water.

To prepare the nitrite primary standard 100 ppm, 10 ml of nitrite standard stock was pipetted and diluted in 100 ml volumetric flasks with milli-Q water. The sulfate primary standard was already prepared in the laboratory.

The working standards with combined NO_2^- and SO_4^{2-} was then prepared in the following concentrations; 0.2, 0.4, 0.6, 0.8 and 1 ppm. Therefore, in 10 ml volumetric flasks, a volume of 20 µl, 40 µl, 80 µl and 100 µl for each nitrite and sulfate primary standard was pipetted and diluted up to the mark with milli-Q water (Shakya, 2004; Khaodee, in press).

Analysis nitrite (NO_2^-) and sulfate (SO_4^{2-})

NO_2 and SO_2 were determined as nitrite and sulfate ions with analysis of extract by ion chromatography. Analytical conditions for this system were as follows (Khaodee, in press),

- eluent 1.8 mM Na_2CO_3 / 1.7 mM $NaHCO_3$
- flow rate 1.0 ml min^{-1}
- sample volume 20 µl

After setting the analytical condition, the base line was run until it remained constant. The working standards were injected to determine the calibration curve. Then the sample was injected. The concentrations of NO_2 and SO_2 in µg m^{-3} are calculated by applying the equation 2.5, 2.6 and 2.7.