CHAPTER 2

MATERIALS AND METHODS

1. Study area and sampling sites

Nakhon Si Thammarat province is one of the 76 provinces in Thailand. It is the second largest province in the south after Suratthani province. It has a total land area of 9,942 km^2 with a population of over 1.5 million as reported in 1996. Neighboring provinces are (from south clockwise) Songkhla, Pathalung, Trang, Krabi, and Suratthani. The province is administratively divided into 23 districts (amphoe), which are further sub-divided into 163 sub-districts (tambon).

Ron Phibun district is located approximately 800 km south of Bangkok on the shore of the gulf of Thailand on the east side of the Malay Peninsula. It is one of the 23 districts in Nakhon Si Thammarat province, and composed of 6 sub-districts, i.e, Hin Tok, Sao Thong, Ron Phibun, Khuan Chum, Khuan Phang and Khuan Koey. It has an area of 504.76 km^2. The Ron Phibun sub-district is situated between longitude 99° 46′ - 99° 54′ E and latitude 8° 04′ - 8° 15′ N. It consists of 16 villages and located in the center of a tin mining area of Primary Tin-Wolfram-Arsenic (Sn-W-As) deposits and secondary placer tin deposits that were exploited 100 years ago. In the western part of the district, the Ron Na-Suang Chan mountain subrange is a wide mountanious area.

This research project was conducted at Ron Phibun district, Nakhon Si Thammarat province. Samples were collected between July 2004 and June 2005 at four locations in dredging ponds at Ron Phibun and Hin Tok sub-districts and two locations in dug ponds used by the local community at Sao Thong and Khuan Koey sub-districts. At each sampling site, a GPS was used to collect positioning data in order to allow accurate mapping of our sites. The map of the study is shown in Figure 2. These locations were selected based upon the following criteria: 1) The latest survey's findings 2) The arsenic contamination tested areas 3) Recommended areas by research papers such as Bunnag (2000), JICA (2000), Williams *et al*. (1996).

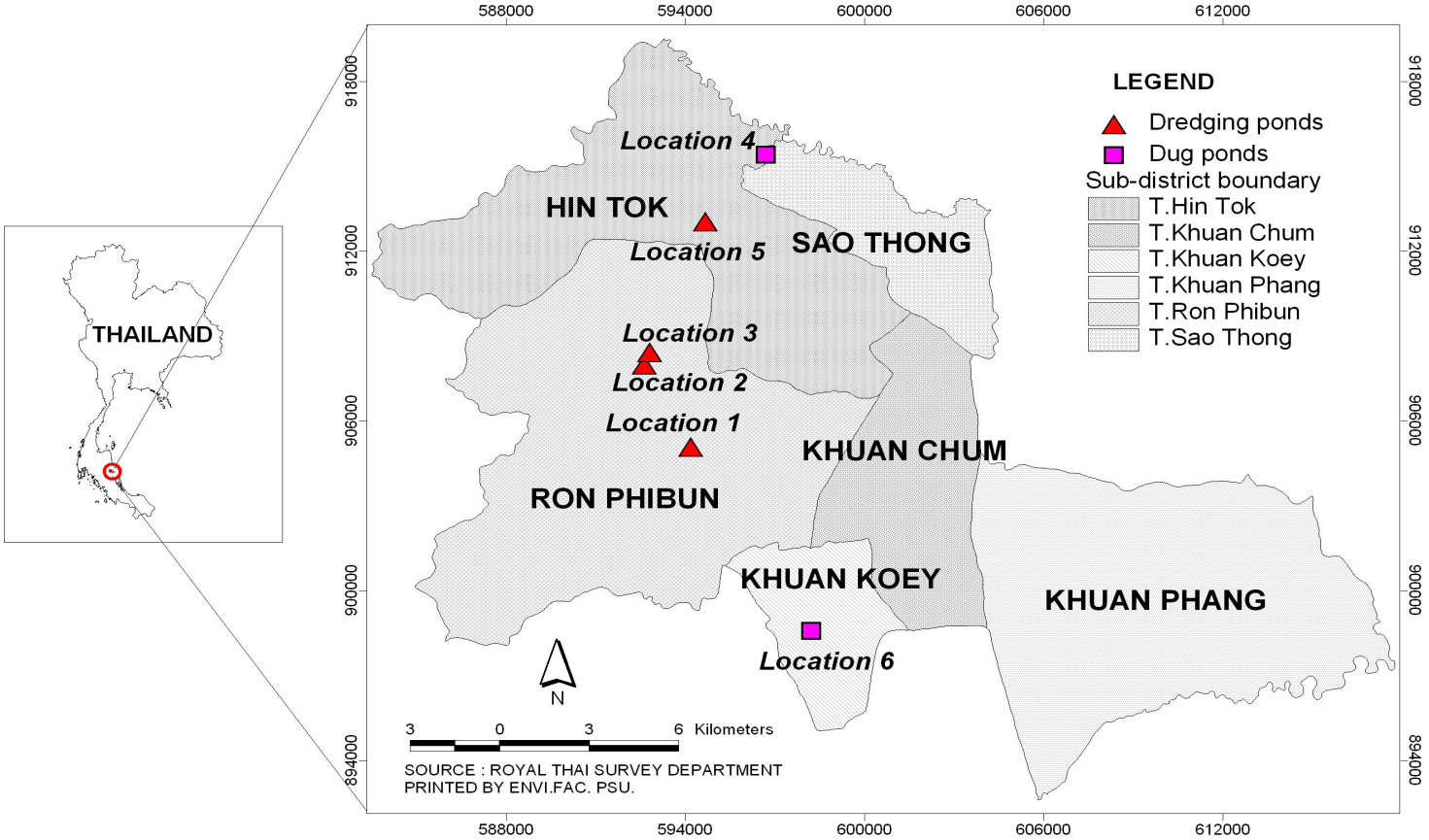

Figure 2. The 6 sampling locations along the Ron Phibun district of Nakhon Si Thammarat province.

Dredging ponds

Old mining sites are scattered on the eastern slope of the mountain encircling the Ronphibun basin. The ore deposit is of vein type and the size of each vein varies from a few centimeters to 30 cm. in width, but seldom exceeds 1 m and the horizontal length is more or less 100 cm. Several veins are concentrated in an area of a few hundred square meters from any one mining site. Around ten mining sites exist in this area. A description of selected ponds in the abandoned tin mining areas is shown in Table 1.

Table 1. Description of the sampling locations along the Ron Phibun district (dredging ponds)

Sites	Ordination	Locations	Site characters
1	47P0594173UTM0905041 (Figure 4a)	Moo 12, Ron Phibun sub-district	This site is relatively shallow and is the smallest dredging pond in this tin mining area. The substratum consists of brown silt. Communities of submerged aquatic plants are also observed in most of the water surface area. The dominant species is *Hydrilla verticillata* (LF) Royle. They grow rapidly in the dry season. At this time, they may interfere with water depth.
2	47P0592610UTM0907997 (Figure 4b)	Moo 3, Ron Phibun sub-district	This site is used for aquaculture ponds. The anthropogenic activities have an effect on water quality and may cause limits or changes in phytoplankton communities. Around the site is also planted many kinds of fruit

			such as rambutan, banana and rubber trees.
3	47P0592799UTM0908476 (Figure 4c)	Moo 3, Ron Phibun sub-district	This site is a big pond. Some part of this site has blooms of *Hydrilla verticillata* (LF) Royle. The water is relatively turbid. The substratum consists of brown silt. This site is also used for multiple purposes such as fishing, aquaculture, and agricultural irrigation.
5	47P0594657UTM0913222 (Figure 4e)	Moo 2, Hin Tok sub-district	This site is the biggest dredging pond in this tin mining area. This water is relatively turbid and brown in colour. In the surface photic zone, the sediments can change seasonally. It is possible that a high deposition rate of sediment in this area is the principle limiting factor for phytoplankton production. The substratum consists of gravel, sand and brown silt.

Dug ponds

There are many available ponds which are all performed by human. The gravel and rocks were added. Additional fencing was constructed to prevent access. The ponds were filled to capacity with water or by that recieved by precipitation. Ponds can serve as a source of irrigation water or emergency water source in the event of fire; provide recreational opportunities such as wildlife watching, skating and fishing; or can dramatically enhance the natural environment, attracting and benefitting wildlife. The water temperature of ponds is fairly even from top to bottom and changes with the outside air temperature. There is little wave action in the water body, and the pond bottom is usually mud-covered. The amount of dissolved oxygen in the pond may vary greatly during a day. Two dug ponds were selected for sampling. A description of the dug ponds is shown in Table 2.

Table 2. Description of the sampling locations along the Ronphibun district (dug ponds)

Sites	Ordination	Locations	Site characters
4	47P0596692UTM0915634 (Figure 4d)	Moo 6, Sao Thong sub-district	This site is artificially dug pond for community use. The water is clear and slow flowing and has an approximate depth of 3 m. An absence of aquatic macrophytes is also the common characteristic of this site. This site was enclosed by agricultural areas.
6	47P0598203UTM0898358 (Figure 4f)	Moo 1, Khuan Koey sub-district	This site is built for irrigationand domestic supply. It is smaller in size and protected by concrete. There is no appearance of any macrophyte species.

2. Climate and hydrology

Ron Phibun's climate is tropical with high temperature and humidity and dominated by monsoons. During each year there are two seasons, nine months rainy (May to November) and three months summer (Febuary to April). In addition, rainy season is affected by tropical monsoon, and can be divided into two seasonal periods. Wind direction is predominantly to the southwest from May to October (light rainy period) and northeast from November to January (heavy rainy period). Monthly average temperature varies from 25.8 $^{\circ}$C to 28.5 $^{\circ}$C. Ron Phibun has a high average rainfall around 2,381.8 mm/yr (Nakhon Si Thammarat Provisional Administration, http://www.nakhonsithammarat.go.th/air.php). The information on precipitation during 1999 to 2003 is shown in Figure 3.

Both surface and groundwater drainage systems are water sources in Ron Phibun. Surface drainage systems are orientated flowing predominantly west-east, with headwaters in the Ron Na-Suang Chan mountains. Groundwater drainage systems include two types of aquifer 1) a shallow aquifer with a depth of less than 10 m consists of unconsolidated alluvial gravel, sand, and clay, typically yielding 20-50 m^3/h and 2) a deeper carbonate-rich aquifer at a depth of more than 15 m. This aquifer generally yields 10-20 m^3/h with an easterly or southeasterly hydraulic gradient. Hydraulic interaction between the two aquifers is strictly limited due to an intervening clay bed which acts as an efficient aquiclude. The principal bedrock mining areas of the Ron Phibun district occupy the headwaters of the Huai Ron Na River, which flows southeast ward from the granite massif through areas of alluvial mining to the north of Ron Phibun town. The principal alluvial mining areas of the district are drained by the Klong Sak, Klong Rak Mai, and Klong Nam Khun systems. Its all surface drainage is slow flowing and extensively canalized in the east of the main Nakhon Si Thammarat highway (Williams *et al.*, 1996).

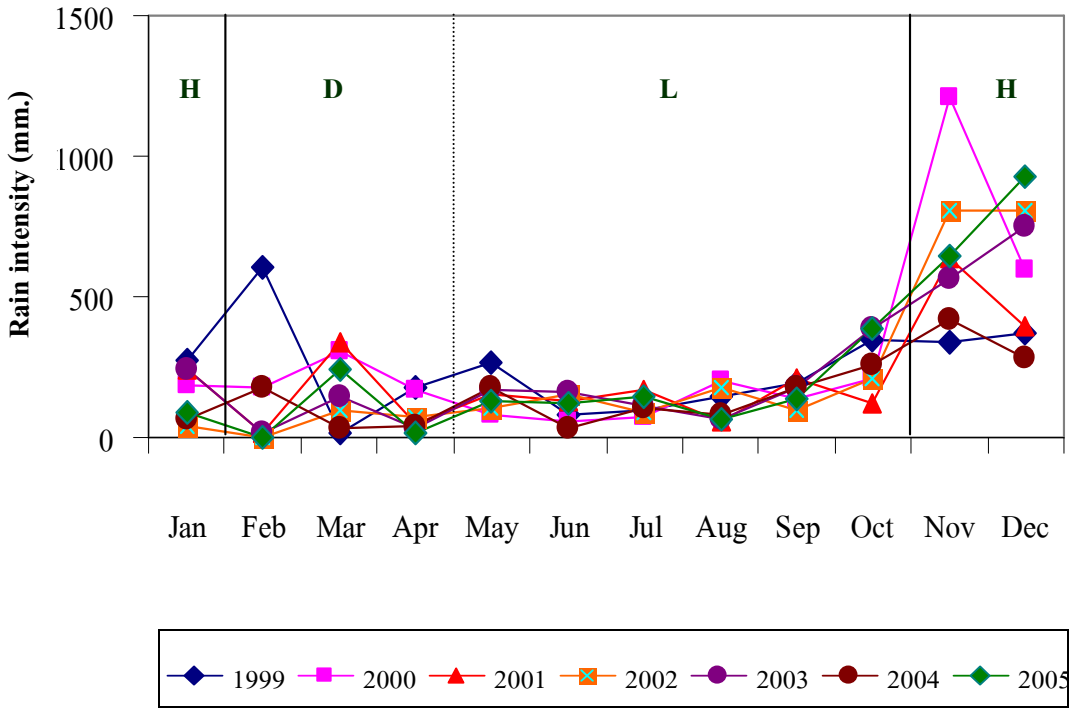

Figure 3. Temporal pattern of rain intensity from 1999 to 2005:

L = light rainy period, H = heavy rainy period, D = dry period.

Source: Meteorological Department (2007)

a) location 1

b) location 2

c) location 3

d) location 4

e) location 5

f) location 6

Figure 4. The dredging ponds and dug ponds along the Ron Phibun district

3. Sampling methods and analysis

1. Phytoplankton sampling

Samples for phytoplankton were collected at 3 points and at a depth to 30 cm. Each time thirty-five liters of water sample was filtered through a 20 μm plankton net; the retained material was preserved with buffered formalin to a final concentration of 5-10% formaldehyde for further analysis in the Plankton Research Unit Laboratory.

2. Water sampling

One liter of surface water sample was collected using polyethylene bottle at the same points as for phytoplankton sampling. All samples were stored in ice containers during their transport to the laboratory. Chlorophyll *a*, Total Suspended Solids (TSS), Biochemical Oxygen Demand (BOD_5), nitrite-nitrogen, nitrate-nitrogen, ammonia-nitrogen, dissolved phosphorus and Dissolved Oxygen (DO) concentrations were analysed as soon as possible. Moreover, total arsenic was determined by Hydride Generation Atomic Absorption Spectrophotometry (HG-AAS).

3. Phytoplankton analysis

Phytoplankton classification was conducted by the methods of Croasdale and Flint (1986a), Croasdale and Flint (1986b), Croasdale and Flint (1986c), John *et al*. (2002), Komarek and Anagnostidis (1999), Peerapornpisal (2005), Whitford and Schumacher (1973), Wongrat (2001). For determination of the abundance of phytoplankton, a micropipette was used to add the phytoplankton samples into a Sedgwick-Rafter chamber and specimens were counted with an Olympus CH-2 compound microscope (UNESCO, 1978). The Sedgwick-Rafter counting cell has no lines on the slide for measurement and is rectangular (50x20 mm), 1 mm deep with an area of 1,000 mm^2 and holds 1 mL of water. Count the target cells in the entire counting slide. Replication of counts of one mL samples is recommended for the statistical

treatments. When the counting cell in Sedgwick-Rafter is complete, the phytoplankton density is calculated for each genus (NIO, 2004) by use the following formula:

$$N = \frac{nV_2}{V_1} \times 1{,}000$$

Where N = total number of phytoplankton cells per liter of water filtered

n = average number of phytoplankton cells in 1 ml of plankton sample

V_2 = volume of plankton concentrate (mL)

V_1 = volume of total water filtered (L)

Furthermore, some phytoplankton genera were filamentous or colonial forms such as *Anabaena* or *Microcystis*, while in others, small unicellular genera of *Chlorella* are generally also found in natural waters. These phytoplankton taxon are not easily observed and counted by Sedgwick-Rafter chamber due to their small in size. Therefore, drop count method was also used for the cell counts in this study (NIO, 2004). The common glass slide mounted with a drop of concentrated phytoplankton sample and covered with cover slip is placed under the microscope provided with a mechanical stage. The phytoplankton are then counted from the microscopic field. In this way all the plankton present in entire microscopic field are counted. The total number of cells then calculated by summing the phytoplankton numbers of all the microscopic fields. If this total number is of one drop of the concentrated phytoplankton, then total number is in 1 mL of the phytoplankton concentration has to be calculated before calculating this, number of drops which from 1 mL has to be counted by adding the drops which form 1 mL has to be counted by adding the drops of water into the graduated centrifuge tube. If one drop of concentrated phytoplankton contains some known number then cells present in 1 mL can be calculated. For example, if 16 drops forms 1 mL, and suppose 50 phytoplankton cells are counted in one drop. Then the plankton in 1 mL are calculated as follows:

Phytoplankton in 1 mL concentrate = 16 x 50 = 80 cells

Phytoplankton per litre = 800 x 1,000 cells

= 800,000 cells

4. Water samples analysis

Chlorophyll *a* concentration was extracted by 90% acetone and then determined by Spectrophotometer following the methodology in APHA, AWWA and WEF (1998; Appendix AI).

Total Suspended Solids (TSS) were determined by gravimetric method (APHA, AWWA and WEF 1998; Appendix AII).

Dissolved Oxygen (DO), oxygen was determined by Winkler method (APHA, AWWA and WEF, 1998; Appendix AIII).

Biochemical Oxygen Demand (BOD_5), oxygen was determined by Winkler method (APHA, AWWA and WEF, 1998; Appendix AIV).

Dissolved phosphorus was determined by ascorbic acid method (APHA, AWWA and WEF, 1998; Appendix AV).

Nitrogen : NO_2^--N was determined by colorimetric method (APHA, AWWA and WEF, 1998; Appendix AVI).

NO_3^--N was determined by colorimetric method after cadmium reduction (APHA, AWWA and WEF, 1998; Appendix AVII).

NH_3-N was determined by phenate method (APHA, AWWA and WEF, 1998; Appendix AVIII).

During sampling, selected environmental factors were determined at all sampling locations. For physical factors, pH was determined by YSI model 60/10 FT, conductivity and water temperature were determined by YSI model 30/10 FT and light intensity was determined by Lux meter in the field at the time of sampling.

5. General procedure for the determining total arsenic and hydride generating conditions.

For estimation of arsenic compounds, 50 mL water samples were filtered through a 0.45 μm cellulose membrane filter. After filtration the water samples was immediately acidified by the addition of 0.05 mL of concentrated hydrochloric (conc. HCl), to provide a pH lower than 2. The analytical technique used for determining total arsenic concentrations was hydride generation atomic absorption spectrophotometry (HG-AAS). The samples are first reduced to As^{3+} prior to analysis. To 1 mL of sample was added 1 mL 6 M HCl and 1 mL 5% (w/v) KI and 5% (w/v) ascorbic acid. Ten percentages HCl was then added to bring the solution to 10 mL. Reduction of As(V) to As(III) occurred within 1 hour at room temperature. As (III) was converted to arsine (AsH_3) by 0.5 % (w/v) sodium borohydride ($NaBH_4$) in 0.05 % (w/v) sodium hydroxide (NaOH). The arsine gas was purged with argon gas to heated quartz cell and atomized. The atomic absorption spectrometer operated at 193.7 nm was equipped with a heated quartz cell. The flow rates in the arsine generation system were as follows; 10% HCl 9 cm^3/min flow rate, 0.5% (w/v) $NaBH_4$ 5 cm^3/min flow rate, argon 50 cm^3/min flow rate. The limit of detection of total arsenic is 0.1 μg/L. This method is modified from manufactured procedure (PEI, 1999).

4. Statistical analysis

The computer statistical package, SPSS for Windows version 12.0 was used to perform the box plot to identify the differences in total arsenic concentrations at each sampling location.

The multivariate statistical programme (MVSP version 3.0, Kovach Computing Services, UK) performs several types of eigenanalysis ordination and was used to carry out diversity indices, cluster analysis and Canonical Correspondence Analysis (CCA) (Kovach, 1998). A diversity analysis comprised the species richness, evenness and Shannon-Weiner diversity indices (log_{10}-based). Ordination analyses included a cluster analysis and CCA. Cluster Analysis was used to establish any similarities of the abundance of phytoplankton species and environmental variables. It was carried out in order to group the sampling locations. Percentage

similarity was applied to the abundance data and to obtain the clusters by the Unweighted Pair Group Method Algorithm (UPGMA). The cluster method chosen was the average lingkage. In addition, Canonical Correspondence Analysis (CCA), a direct gradient analysis technique, was used to elucidate the relationship between biological assemblages of species and their environment. Rare taxa create a large number of zero values and noise in data sets, and this in turn can cause increased distortion of ordinations. To reduce the amount of noise, rare taxa (those with an occurrence of < 0.1% of total phytoplankton number) were removed from the data set. CCA assumes the data have a multivariate normal distribution and Palmer (1993) recommended transforming environmental data into log values. Since there is no way to test for a multivariate normal distribution, the CCA was run with both log transformed and untransformed environmental data. The untransformed environmental data did not alter the results from this study in any way, so the results presented in this research are based on transformed data. The data for cluster analysis and CCA were standardized by a log(x+1) transformation to meet the basic requirement of the statistical test, except for pH the values that had skewed distributions.