CHAPTER 2

MATERIALS AND METHODS

1. Nutrients

1.1 Study area

Phuket Marine Biological Center is located at Laem Pan Wa, the southern part of Phuket island where the present work took place. Because of the limited number of studies on nutrients and nutrient limitation on phytoplankton production in Thailand, Phuket coastal water was chosen to study the effect of nutrient on marine phytoplankton. Bioassays of limiting nutrients was carried out in the laboratory at PMBC.

1.2 Sampling method

Coastal water samples were taken from the pier at Phuket Marine Biological Center. The water here is of little influenced from water run off from land, sewage discharge and other harmful effects.

Water from 3 different depth (surface, 3 m., 6 m.) were sampled to determine the depth with maximum production. The results showed that mainly it was at 3 m. depth and occasionally at the surface. Therefore in order to avoid supersaturation phenomenon, and other sources of variation associated with surface water (Strickland & Parsons, 1968) only samples at 3 m. depth was used. When the water samples were taken, they would be poured into a dark plastic container.

1.3 Laboratory method

The Bioassay experiment started immediately upon return to laboratory. While carrying on the experiments, it was important to incubate the samples as quickly as possible after addition of the nutrients. A constant climate chamber is available in the laboratory of PMBC. It was set at temperature of $27 \pm 1\ ^{\circ}C$ and light 18000 lux, giving optimum conditions for photosynthesis of phytoplankton (Luarsinsup, 1978).

The method was standardized as follows; filling the water sample into BOD dark and light bottles, addition of reagents to be tested, incubation in the constant climate chamber for 4 hours, then analyse the primary production according to the Light and Dark Oxygen Method of Strickland & Parsons (1968).

Reagent grade chemicals were used to prepare stock solution of nutrient. The solution was at first prepared in the highest concentration and then diluted gradually into 4 different dilutions. (Table 1)

The response of phytoplankton to the added nutients was studied in the laboratory. Nutrients were immediately put into the BOD bottles (light & dark). Incubated for 4 hours in the constant climate chamber. It is very important not to leave any air bubbles in the bottle. If this happens, titration for oxygen will be in error. Therefore, care was taken to fill the stopper with distilled water in order to prevent air bubbles in the bottles.

Table 1 Six different kinds of chemical compounds used in the experiment on nutrient limitation. Only $PO_4^{\equiv}$ - P and NO_3^- - N were used in 4 dilutions separately and in combination.

Nutrient	Concentration (µg-at/l)			
$PO_4^{\equiv}$ - P	0.5	1.0	2.5	5.0
NO_3^- - N	5.0	10.0	25.0	50.0
NH_4^+ - N	-	5.0	-	-
Fe - F	-	10.0	-	-
EDTA	-	10.0	-	-
Si	-	1.0	-	-

The bottles were shaken every hour. Temperature and light intensity in the chamber were also checked at post-experiment period.

2. Coral water

2.1 Study area

The coral reef area for the present study was selected infront of Phuket Marine Biological Center. This study area was underneath the water even at the lowest low tide.

2.2 Sampling method

A big plastic bag, 20 x 25 cm., was used to cover

the coral head (Porites lutea). It is tied very carefully at the base of the head in order to prevent the outside sea water from mixing with coral water. A long transparent plastic tube lead from the top of the plastic bag was connected with a small pump at the surface of the water. This equipment is practical enough for sucking up the coral water (Figure 1).

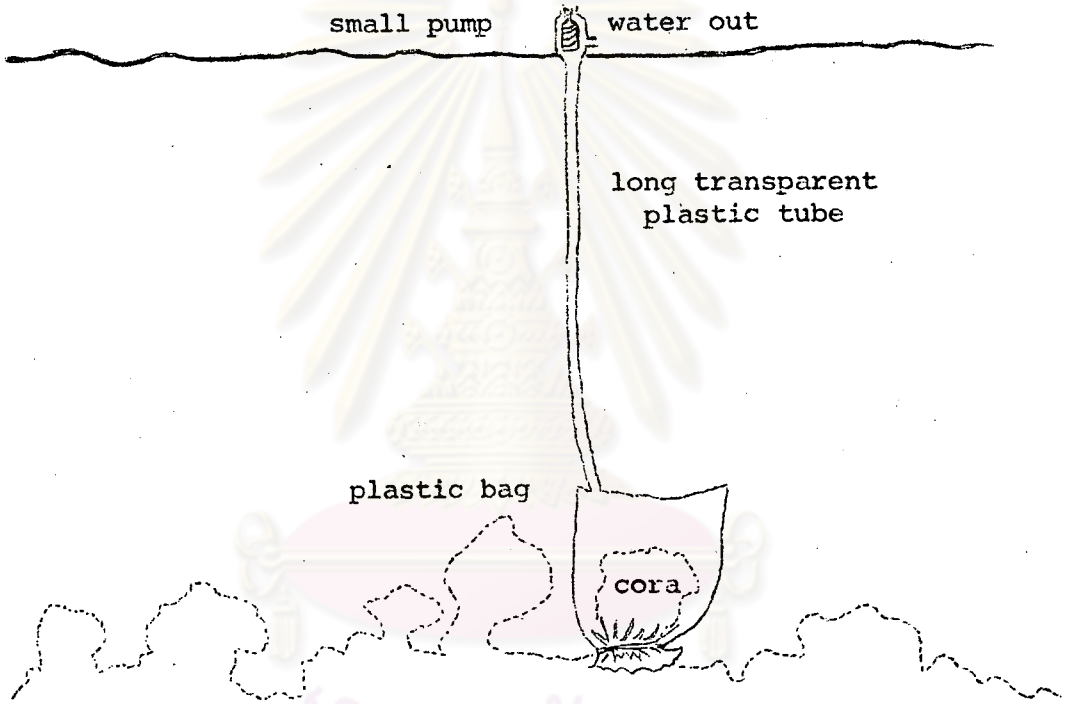

Figure 1 Practical method used in sampling coral water.

Initially, the difference of dissolved oxygen in the plastic bag was measured every 2 hours in order to determine the most productive period. Coral water was pumped up and mixed with sea water in various dilutions as shown in Table 2.

Table 2 Four different dilutions of the coral water and sea water mixtures in the experiment. Pure coral water and sea water were also sampled to determine the gross primary production in comparison with other mixtures.

Bottle No.	1	2	3	4	5	6
Sea water (ml.)	300	250	200	150	100	-
Coral water (ml.)	-	50	100	150	200	300

2.3 Laboratory method

The bioassay experiment was carried out immediately after control sea water and coral water were taken to the laboratory. For the determination of primary production, the dark and light bottles oxygen method was used (Strickland & Parsons, 1968). Determination of $PO_4^{\equiv}$, NO_3^- and NO_2^- were also carried out. The primary production of the ambient water around the plastic bag was also measured.

3. Mangrove Water

3.1 Study area

The study was carried out at Ao Yon Mangrove. It is a small mangrove forest surrounded by small hills with a shallow channel containing out-flowing mangrove water at low tide. The rough zonation of plants of plants is shown in figure 2.

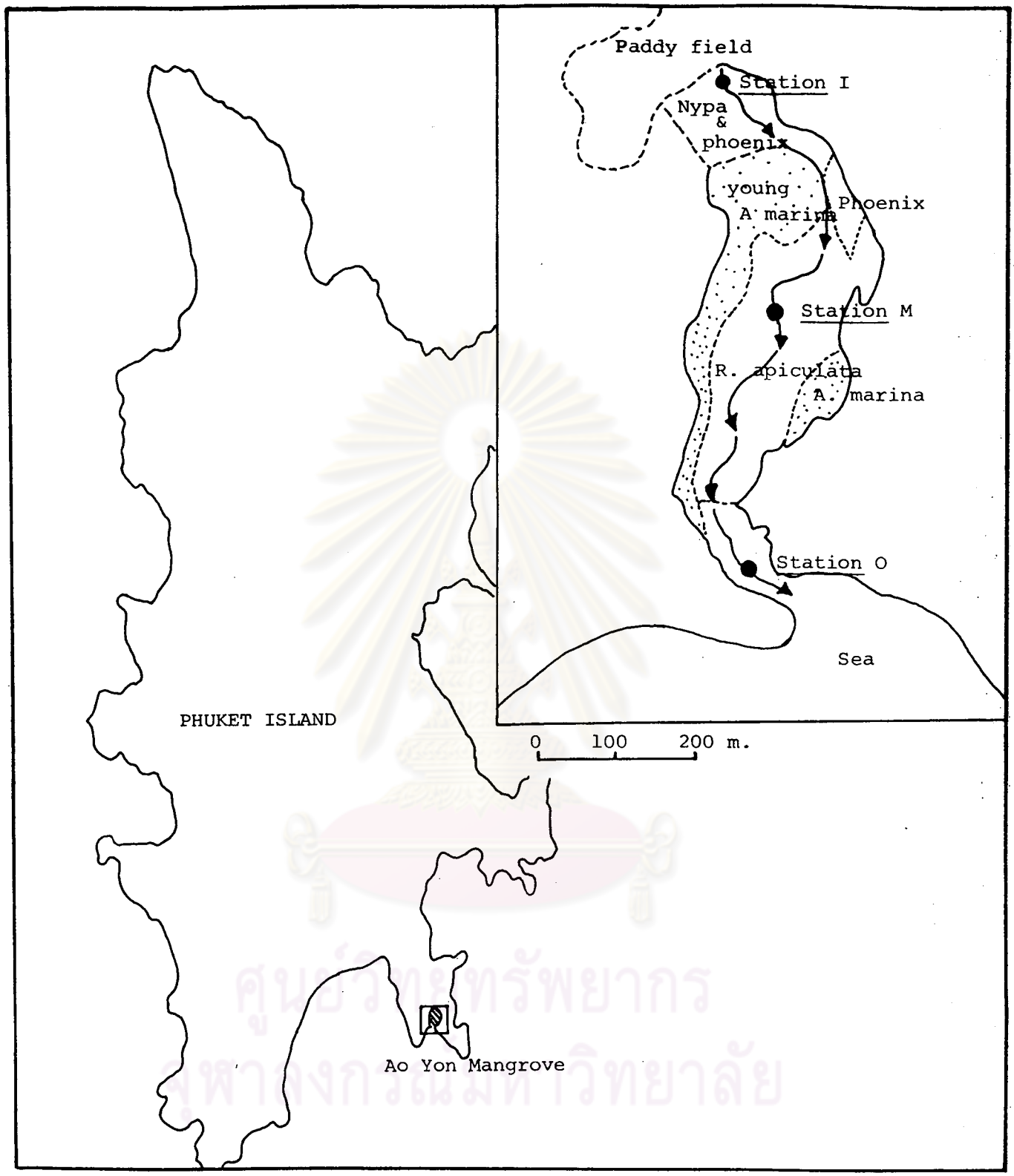

Figure 2 The position of Ao Yon mangrove in Phuket Island. Small inserted map shows the details of Ao Yon communities with mangrove channel running through (~). ● indicates the sampling stations.

Mangrove water samples were taken from 3 stations.

Station I - at the inner part of the mangrove.

Station M - at the middle part of the mangrove (the depth of water in the channel is not more than 30 cm. at lowest low tide)

Station O - at the outer part of the mangrove, opened to the sea.

3.2 Sampling method

The mangrove water was sampled at each station at four different levels of tide. Height of water predicted in decimeters above the lowest low water, according to tide tables. (Hydrographic Dept, 1981).

I	15 - 16 decimeters
II	18 - 18 decimeters
III	21 - 27 decimeters
IV	31 - 32 decimeters

3.3 Laboratory method

Mangrove waters and sea water samples were taken to the laboratory shortly after sampling and the bioassay experiments were carried out immediately by light and dark bottles'oxygen method according to Strickland and Parsons (1968). Mangrove water had to be filtered through zooplankton net (300 μm) to avoid big particles. Four dilutions of mangroves water and sea water were used as shown in Table 3.

Table 3 Four different dilutions of mangrove water and sea water mixtures. Pure sea water and mangrove water were also used to determine the gross primary production in comparison with other mixtures.

Botlle No.	1	2	3	4	5	6
Sea water (ml.)	300	250	200	150	100	0
Mangrove water (ml.)	0	50	100	150	200	300

4. Domestic sewage

4.1 Study area

At present, no sewage treatment plant exists in Phuket. Sewage from town area is drained by a natural stream running through the town, Klong Bang Yai, which drains a catchment area of 56 km^2. No heavy industries supply waste discharge throungh this Klong, except for some small industries. This Klong mostly recieves water from domestic & agricultural runoff, and partly from on land tin mines (Figure 3). Water from Klong Bang Yai runs directly out into Phuket Bay.

Sewage water samples were taken from 3 stations.

Station I: Near Nimit Theatre, it is at a new klong recently dug for helping Klong Bang Yai to drain the waste water. Mud crabs were present in numerous number along both sides of

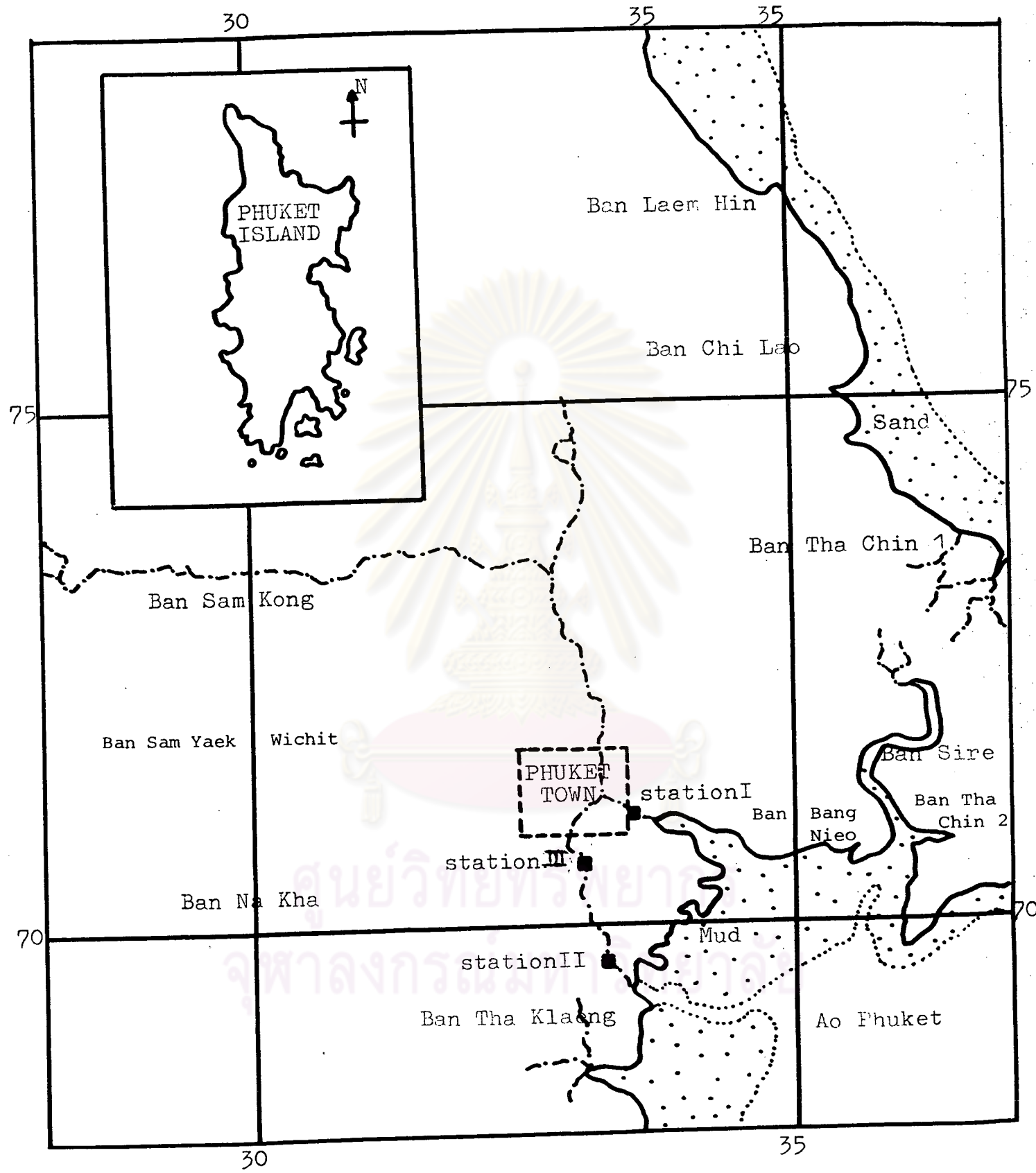

Figure 3 Sampling station in Phuket Island. ■ = sampling station for waste water experiment.

this klong. The water looked turbid. The odor was not so bad a smell as at station III.

Station II: At the opening of Klong Bang Yai. Where waste water will be drained out to the Phuket Bay. Many mud crabs were also present. Water flowed slowly from land to the bay at all times.

Station III: At the inner part of Klong Bang Yai. It is the area where the little klongs confluence and the combined domestic waste water runs down to the bay. This station had the most concentrated sewage, possibly containing many harmful, pathogenic bacteria. During and after the rain, the turbidity in this klong was very high because of the on land tin mine water which was drained out and combined with the domestic sewage. For this study, the effect of water from tin mines could be excluded from this station because of no rain during the sampling period and sampling was done at low tide. This station had the strong odor of H_2S. Mud crabs disappeared from this station. There was only black mud or soil at both sides of the klong. Sometimes the black mud had patches of blue green algae on the surface.

4.2 Sampling method

Waste water samples were taken at low tide and filled into the dark plastic gallon-size bottes to avoid the error from phytoplankton photosynthesis during the interval prior the laboratory treatment.

4.3 Laboratory method

Domestic waste water and sea water were taken to the laboratory and mixed together in different dilutions (Talbe 4). The bioassay experiments were carried out immediately by light and dark bottles oxygen method (thickland & Parsons, 1968). Determination of salinity, $PO_4^{\equiv}$ - P, NO_3^- - N and NO_2^- - N were also carried out.

Table 4 Four different dilutions of domestic waste water and sea water mixtures used in the experiment, Pure domestic waste water and sea water were also used to determine the gross primary production in comparison with other mixtures.

Bottle No.	1	2	3	4	5	6
Domestic Waste water (ml.)	300	275	250	200	150	-
Sea water (ml.)	-	25	50	100	150	300

5. Tin mine water

5.1 Study area

Phuket Tin Mine (Area I) and Chao Pa Tin Mine (Area II) were chosen in this experiment (Figure 4) due to their control in waste water discharge as regulated by the Ministry of Industry. Both tin mine areas operated by the gravel pump mining process (Figure 5).

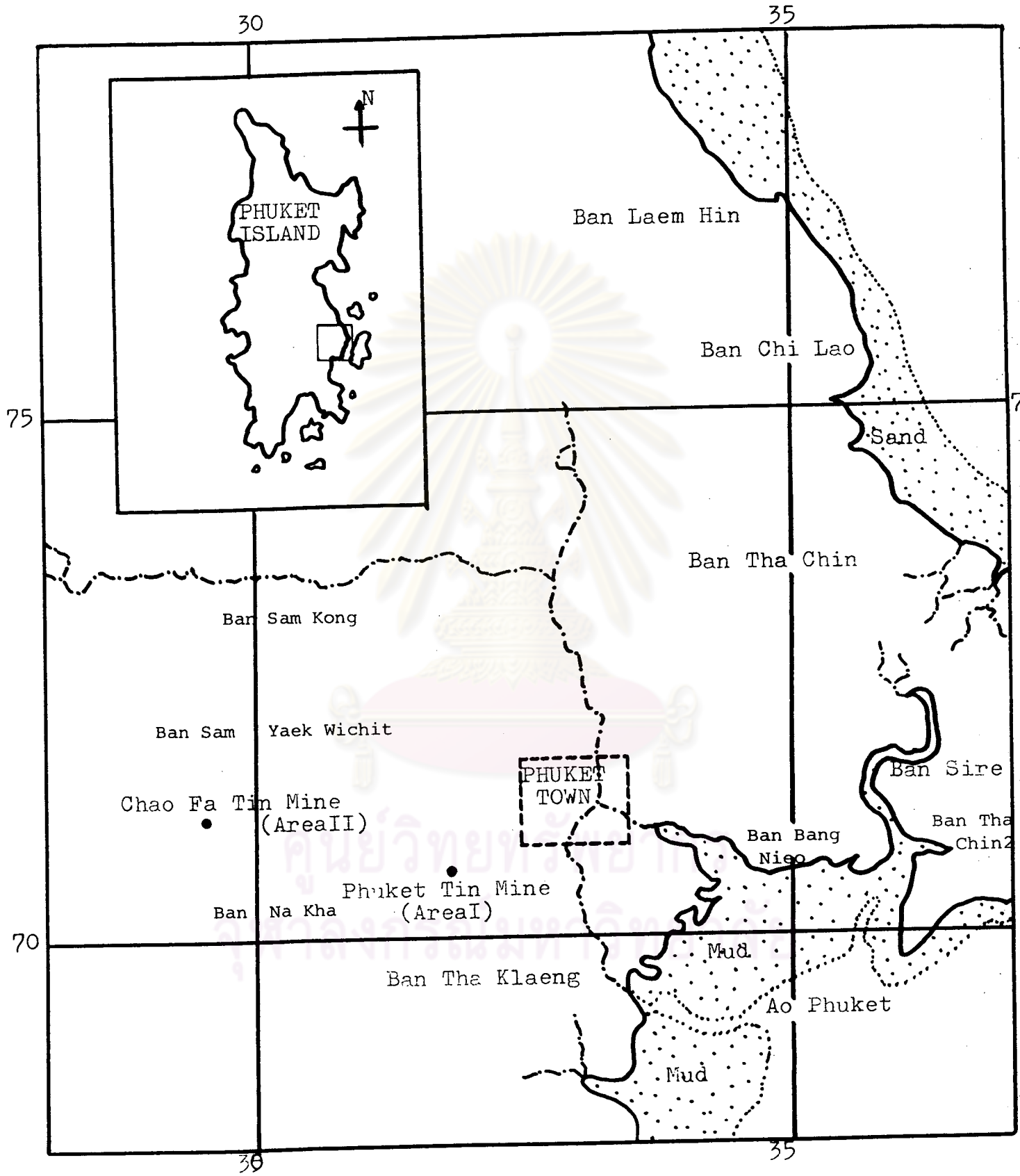

Figure 4 Sampling stations for tin mine experiment (●).

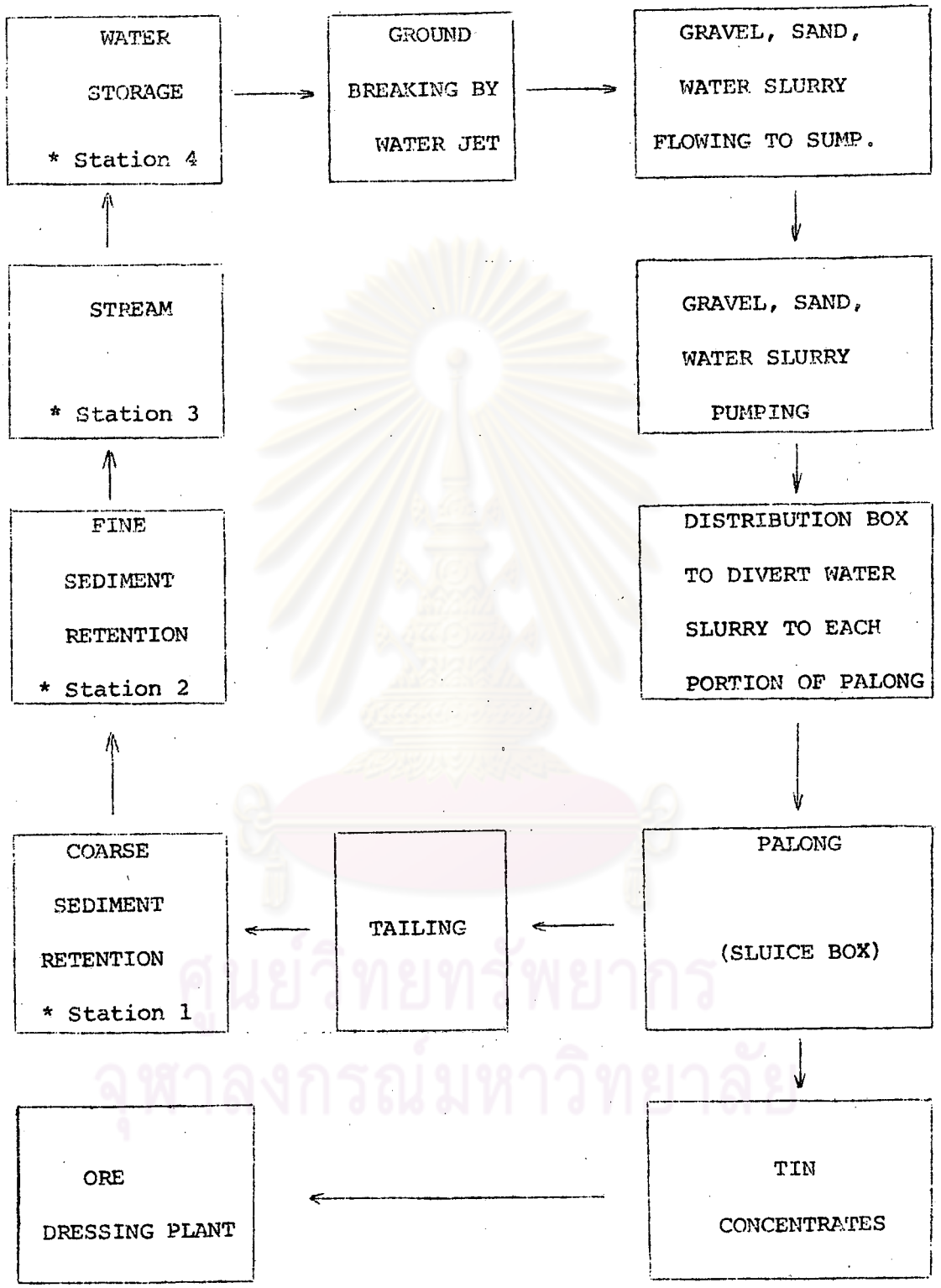

Figure 5 Schematic diagrame of Gravel Pump Mining Process. Station 1 - 4 are located according to the 4 compartments indicated in the figure.

5.2 Sampling method

At both mining areas selected, tin mine water from 4 stations were sampled ond put into big dark bottles separately.

5.3 Laboratory method

Tin mine water sampled from stations 1 and 2 from both mining areas were very turbid even the samples were allowed long time to precipitate. This condition will cause light obstruction for phytoplankton photosynthesis. Therefore, tin mine water samples from station 1 and 2 were not used to determined the gross primary production. Water sampled from station 3 and 4 were mixed with sea water in different dilutions (Table 5). The bioassay experiments were carried out immediately. Determination of salinity, pH, $PO_4^{\equiv}$ - P, NO_3^- - N and NO_2^- - N were also carried out.

Table 5 Three different dilutions of the tin mine water and sea water mixtures. Pure sea water was also used to determined the gross primary production with other mixtures. For every bottles, Fe^{+3} - F and EDTA were added at 10 µg-at/l. each.

Bottle No.	1	2	3	4
Tin mine water (ml.)	300	250	200	150
Sea water (ml.)	-	50	100	150

6. Calculation and determination methods

After the incubation period the samples were taken out and determined for dissolved oxgen by Winkler's method. Every steps of the procedure follow Strickland and Parsons (1968) :-

Thiosulphate (0.01 N) was used for titration of 50 ml. water samples after addition of Winkler reagent I & II (manganous sulphate and alkaline iodide) and acidification with sulphuric acid. The dissolved oxygen content was calculated from the formula

$$\text{mg - at } O_2/l = 0.1006 \times f \times v$$

where V = ml. thiosulphate used by titration

f = titration factor

$$= \frac{5.0}{v}$$ for the 0.01 N thiosulphate

v = ml. thiosulphate from calibration procedure

$$\text{mg}O_2/l = 16.00 \times \text{mg-at } O_2/l$$

The gross primary production (GPP) was calculated using the formula

$$GPP = \frac{605 \times f \times (V_{LB} - V_{DB})}{N \times PQ} \text{ mg C. } m^{-3} . \text{ hr}^{-1}$$

where V_{LB} = ml. thiosulphate consumed by the light bottle.

V_{DB} = ml. thiosulphate comsumed by the dark bottle.

N = hours of incubation

PQ = photosynthetic quotent = 1.2

$$GPP = \frac{605 \times f \times (V_{LB} - V_{DB}) \times 3}{PQ} \text{ mg C. } m^{-3} . \text{ day}^{-1}$$

Determination of available NO_3^-, NO_2^- and $PO_4^{\equiv}$ in water samples were carried out every time of sampling and also follow Strickland and Parsons (1968).

Outline of method:-

NO_3^- - The nitrate in water sample was reduced almost quantitatively to nitrite when passing the water sample through a reduction column (a column containing cadmium filings loosely coasted with metallic copper) after concentration ammonium chloride as added. The nitrite thus produced was determined by diazotizing with sulphanilamide and coupling with N - (1-naphthyl) - ethylene-diamine to form a highly coloured azo dye the extinction of which was measured by spectrophotometer using a wavelength of 543 nm.. Correct the observed extinciton by that of a reagent blank, then calculated nitrate present from the expression,

μg-at N/l = correct extinction -0.95 C.

C = concentration of nitrite present in the sample in μg-at N/l

NO_2^- - The nitrite in the water sample was allowed to react with sulphanilamide in an acid solution. Then the resulting diazo compound reacted with N-(1-naphthyl) - ethylenediamine and form a highly coloured azo dye, the extinction of which was measured by spectrophotometer using a wavelength of 543 nm. Calculated the nitrite concentration from the expression,

μg-at N/l = corrected extinction x F

where $F = \frac{2.00}{E_s - E_b}$

E_s = mean extinction of the four standards

E_b = mean extinction of the two blanks.

$PO_4^{\equiv}$ - The water sample was allowed to react with a composite reagent containing molybdic acid, ascorbic acid and trivalent antimony. The resulting complex heteropoly acid was reduced in situ to give a blue solution, the extinction of which was measured by spectrophotometer using a wavelength of 885 nm.. Correct the measured extinction by subtracting both the turbidity and reagent blanks. Calculated the phosphate concentration in µg-at P/l = correcte extinction x F

µg-at P/l = corrected extinction x F

where $F = \frac{3.00}{E_s - E_b}$

E_s = mean extinction of the four standards

E_b = mean extinction of the two blanks.

- Temperature, salinity and pH were obtained, using a mercury thermometer, refractometer and portable pH meter, respectively.

- The following precautions had been taken:-

- All the glassware used in the experiments needed to be cleaned carefully by cleaning solution after every few weeks.

- Titration must be carefully carried out with precision.

- Calculate and evaluate the results.

7. Determination of primary production by ^{14}C-uptake (Strickland & Parsons, 1968)

Out line of method:-

The radioactive carbonate, $^{14}CO_3^{-2}$, 2 ml. was added to sea water in a clear BOD bottle, leaving an air space of about 3 - 5 ml. at the top of the bottle. After photosynthesis by the endemic phytoplankton population had continued for 4 hours, filtered the entire contents of the BOD bottle onto a membrane filter. Then exposed to fumes of acid solution and formation before being stored to dry. The radioactivity from the carbon in phytoplankton was measured with a Geiger - Miiller counter. This uptake of radioactive carbonate, as a fraction of the whole, was assumed to measure the uptake of total carbonate, as a fraction of the whole, and hence the rate of photosynthesis could be evaluated. Calculated the primary production from,

$$\text{Radiocarbon-measured photosynthesis } (mgC/m^3/hr) = \frac{(R_s - R_b) \times W \times 1.05}{R \times N}$$

R_s = the normalized counting rate (in counts per minute) of the sample planchette.

R_b = the normalized counting rate of a blank.

R = the normalized counting rate to be expected from the entire activity of the ampule.

N = the number of hours during which the sample was exposed to light.

W = weight of carbonate carbon present in the water in mg C/m^3 that can be calculated by the relation,

$$W = 12{,}000 \times A \times F_T$$

where A = total carbonate alkalinity in milliequivalent per liter.

F_T = approximated to 0.95