

CHAPTER III METHODOLOGY

3.1 Sampling site selection

The Chao Phraya, Bang Pakong and Mae Klong Estuaries were targeted as sampling sites of this study because their sediments originated from different primary rocks.

3.2 Sediment collection and preservation

Three sediment samples were collected nearly at the same salinity (22-24 ppt) from the Chao Phraya, Mae Klong and Bang Pakong Estuaries by Petersen grab sampler in September 1999. Sampling sites are shown in Figure 3.1 and Table 3.1, respectively. After collection, sediments were stored frozen in plastic bags until freeze-dried.

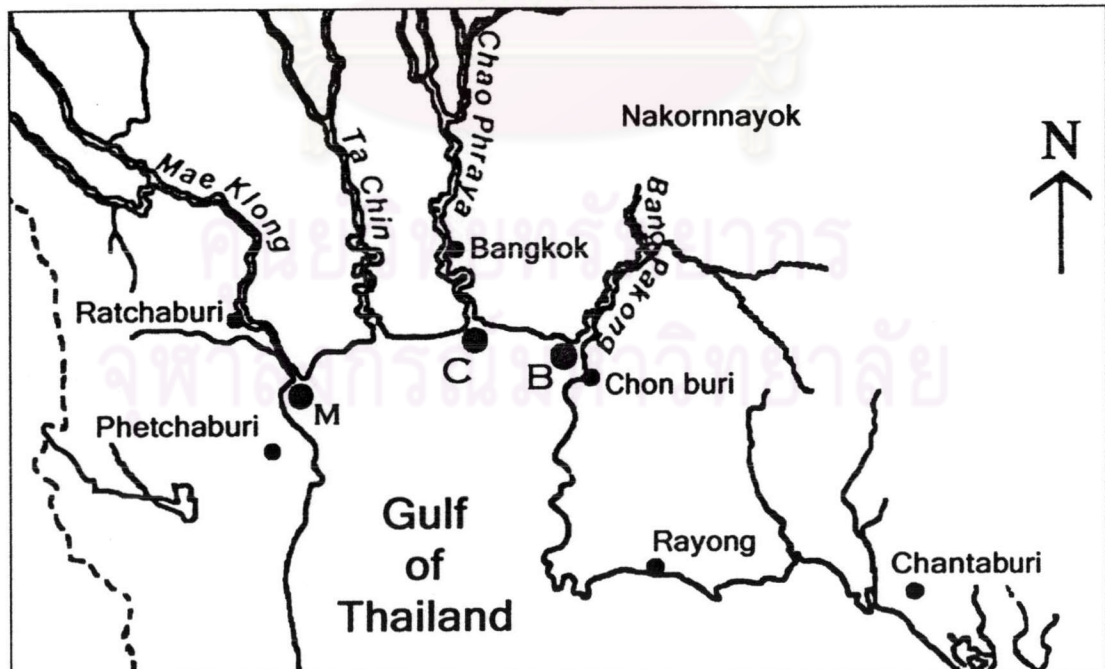


Figure 3.1 The sediment sampling site

Table 3.1 Sediment sampling position

Sediment sampling site	Latitude	Longitude
Chao Phraya estuary (C)	13 ° 30 ' 49" N	100 ° 35 ' 30" E
Mae Klong estuary (M)	13 ° 20' 00" N	100 ° 1' 00" E
Bang Pakong estuary (B)	13 ° 26' 46" N	100 ° 55' 42" E

3.3 Physico-chemical properties of bottom water

Physical parameters of estuarine bottom water at sampling site were measured in the field (Table 3.2). Some bottom water was collected and preserved before analyzing reactive phosphate concentration in laboratory by the procedure of Strickland and Parsons (1972).

Table 3.2 Physical and chemical parameters of bottom water measured at the sampling sites

Physical parameters	Instruments
Salinity	YSI meter model 30
DO	YSI meter model 55
Temperature	YSI meter model 30
pH	Accumet® pH meter BASIC AB15

3.4 Preparation of sediment samples

Wet sediments were freeze-dried, gently crushed in an agate mortar, well-mixed and subsampled by Coning and Quartering method (Walton, 1978) before analysis.

3.5 Sediment analysis

Subsampled sediments were analyzed for their grain size distribution and dry sieved through a 125 µm stainless steel test sieve (Encotte®) before further analysis as outlined in Figure 3.2. At least three replicates of <125 µm sediment samples (Figure 3.3) were conducted in each method. In this chapter, only the determination of total, inorganic and organic phosphorus and the employed sequential extraction methods for phosphorus speciation are described. Determination of major elements

and total phosphorus, oxidizable organic matter and carbonate contents in sediment samples are presented in Appendices A, B, C and D.

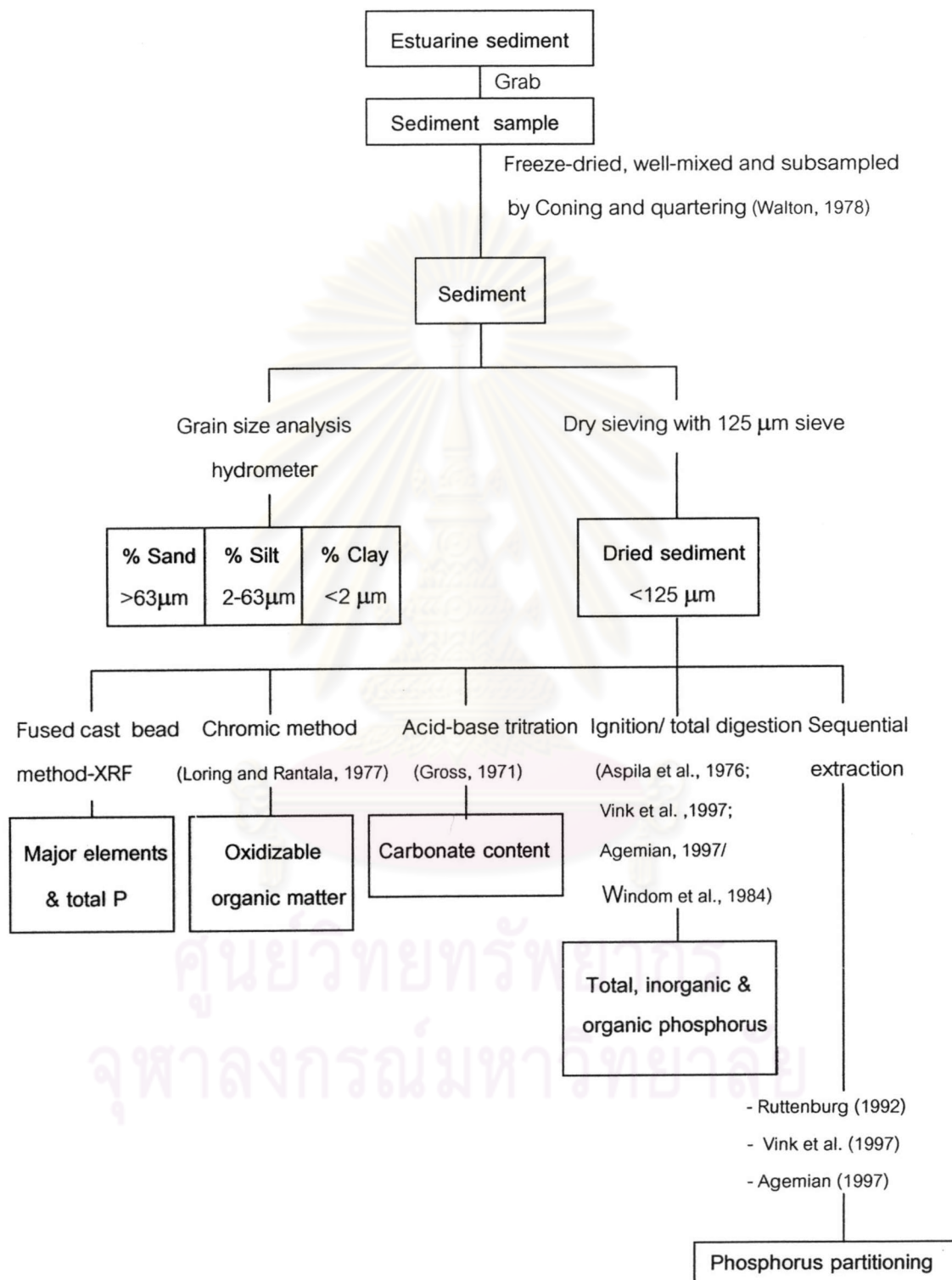


Figure 3.2 Schematic sequences for analytical procedures employed.



Figure 3.3 < 125 μm sediment samples from the Mae Klong (M), Chao Phraya (C) and Bang Pakong (B) Estuaries

3.6 Preparation of labwares and reagents for phosphate analysis

To reduce contamination in phosphate analysis, all glasswares and other plasticwares were soaked in 10 % (v/v) HCl overnight, rinsed three times with double distilled-deionized water, dried in clean place and kept in clean plastic bags until use (Koroleff, 1976). All reagents were prepared from analytical grade chemicals that contain less than 0.0005% phosphorus. Double distilled deionized water was used throughout this work.

3.7 Determination of total, inorganic and organic phosphorus in sediments

3.7.1 Dry combustion and acid digestion method (Aspila et al., 1976)

For determination of the concentration of total phosphorus, dry sediment was heated at 550 $^{\circ}\text{C}$ for 2 h. After heating, the residue was digested for 16 h with 1 N HCl. The orthophosphate concentration in acid digestion is a measure of total

phosphorus content in sediment. The inorganic phosphorus content in sediment was determined by measuring the orthophosphate released when the unheated sediment was digested by 1 N HCl. The concentration of organic phosphorus was then calculated from the difference between total and inorganic phosphorus.

The determination of soluble phosphate in the dilute acid extract was conducted by the method of Koroleff (1976) as described in section 3.9.2.

Apparatus

- room temperature waterbath shaker
- spectrophotometer (Milton Roy® Spectronic 401)
- crucible
- muffle furnace (THERMOLYNE, SYBRON Corporation)
- centrifuge (Centurion Scientific® 1040 series)
- pH meter (Accumet® BASIC AB15)

Reagents

- 1 N HCl; dilute 89 ml of concentrated HCl to 1 L using deionized water

Procedure

0.25 g of sediment (at least three replicates) is accurately weighed and transferred into a 30 ml crucible. The uncovered crucibles are placed into a warm muffle furnace and heated at 550 °C for 2 h (constant temperature). Allow to cool to room temperature and finally transfer into 100 ml polypropylene bottles. Add 25 ml of 1 N HCl to the bottles, shake the mixtures for 14-18 h (overnight) on a shaker bath (left the water temperature at ambient room temperature). Transfer the extract solution into 10 ml centrifuge tubes and centrifuge at 2000 rpm for approximately 5 min. Dilute ten times or more and analyze for total phosphorus concentration by phosphomolybdate complex method (Koroleff, 1976). Standard calibration curves for phosphate are prepared

by using 1 N HCl. Calculate concentration of total phosphorus in sediment samples by using the equation from standard calibration curve in the unit of $\mu\text{g/g}$ sediment.

3.7.2 Modified Aspila method (Vink et al., 1997)

Vink et al. (1997) modified Aspila method by increasing concentration of hydrochloric acid from 1 N to 6 N in the acid digestion step and neutralized the acid extracted solution with 0.5 M NaHCO_3 before analyzing soluble phosphate by the procedure of Strickland and Parsons (1972) as described in section 3.9.1.

Apparatus

The same as described in Aspila et al. (1976)'s method.

Reagents

- 6 N HCl; dilute 515 ml of concentrated HCl to 1 L using deionized water.
- 0.5 M NaHCO_3 ; dissolve 21 g of NaHCO_3 by deionized water and then make volume to 1 L.

Procedure

The same as described in the method of Aspila et al. (1976). The acid extract solution is then neutralized with 0.5 M NaHCO_3 before analyzing soluble phosphate by the procedure of Strickland and Parsons (1972) as outlined in section 3.9.1.

3.7.3 Dry combustion and acid digestion method (Agemian, 1997)

Description is almost the same as that of Aspila et al. (1976). Except the sediment extract was analysed for soluble phosphate according to Strickland and Parsons (1972) as shown in section 3.9.1 instead of Koroleff's.

3.7.4 Total digestion method (total phosphorus analysis)

Three replicates of sediment were digested with a mixture of HF, HClO₄ and HNO₃ in a 100 ml teflon beaker at 180 – 200 °C according to Windom et al. (1994)'s method. After digestion, sediment extract is neutralized, diluted and analyzed for soluble phosphate either by Strickland and Parsons (1972)'s method or Koroleff (1976)'s method as described in sections 3.9.1 and 3.9.2.

Apparatus

- Hotplate
- Hotplate thermometer
- 100 ml teflon beaker with teflon cover plate
- balance
- Fume hood

Reagents

- HF 47%, HClO₄ 65%, HNO₃ 65%

Procedure

Transfer 0.25 g of sediment into a 100 ml teflon beaker. Under fume hood, add 5 ml of HF and 1 ml of HClO₄, place the beaker on a hot plate and heat at 180-200 °C until white fume develop, then remove the beaker. Add 2 ml of HNO₃ and put the beaker back on the hot plate. Heat the beaker until the solution inside is nearly dried, then add another 2 ml HNO₃ and continue the digestion. If brown color is still persist, add 0.5 ml of HClO₄ and then repeat digestion with 2 ml HNO₃ until the solution is clear. Remove the beaker from the hot plate, allow it to cool, and then make up the volume to 25 ml with deionized water. Digested extract is neutralized and diluted before analyzing soluble phosphate according to Strickland and Parsons (1972)'s or Koroleff (1976)'s methods as outlined in section 3.9.1 and 3.9.2, respectively.

3.8 Analysis of Phosphorus speciation by the selected sequential extraction methods

3.8.1 Ruttenberg (1992)'s method

Three replicates of each sediment sample were sequentially extracted for their phosphorus speciation according to SEDEX scheme in Figure 3.4. The solid to extractant ratio is 0.25 g: 25 ml of extractant except in step II-A, where residue was extracted with 22.5 ml of CDB. The extraction was maintained by agitation on shaker and incubated at 25 °C. At the end of each extraction step, the mixture was centrifuged at 3500 rpm for 15 min, supernatants were carefully removed by pasteur pipette and then analyzed for soluble phosphate concentration by phosphomolybdic blue method (Koroleff, 1976) as described in section 3.9.2. Except in step II, supernatants were analyzed for phosphorus following the isobutanol procedure of Watanabe and Oilsen (1962) after first reacting it with 1%(v/v) FeCl_3 (Lucotte and Anglejan, 1985) to obviate the interference of citrate with reduction of the molybdate complex. Separate standards and reagent blanks were made up for all steps in the same dilution as extracted solution so that their solution matrix matched that of samples. Turbidity blanks of extracted solution were also checked to correct absorbance of unfiltered sediment extracts.

Apparatus

- Shaker
- Spectrophotometer(Milton Roy®Spectronic 401)
- Centrifuge(Centurion Scientific® 1040 series)
- 50 ml polyethylene centrifuge tube with a lid
- Separatory funnel 100 ml
- Volumetric flask 25 ml
- Crucible
- Muffle furnace(THERMOLYNE, SYBRON Corporation)
- pH meter (Accumet® BASIC AB15)
- 100-1000 μl (Eppendorf®), 1000-5000 μl (Gilson®) micropipettes

Reagents

- 1 M MgCl_2 : dissolve 203.33 g of MgCl_2 (f.w. = 203.30) in deionized water and make volume to 1 L. Adjust pH to 8 before use.
- 0.3 M Na_3 -citrate: dissolve 44.13 g of $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ (f.w.=294.10) in deionized water, then make volume to 500 ml. Keep solution in Polyethylene bottle.
- 1.0 M NaHCO_3 : dissolve 42.01 g of NaHCO_3 (f.w. = 84.01) in deionized water, then make volume to 500 ml. Keep this solution in Polyethylene bottle.
- Citrate-bicarbonate-dithionite (CDB reagent): dissolve 1.125 g of Na-dithionite in 45 ml of citrate bicarbonate solution and adjust pH to 7.6 before use.
- 1 M Sodium acetate (NaOAc) buffer pH 4: dissolve 68.04 g of NaOAc (f.w.=136.08) in deionized water, then make volume to 500 ml and adjust pH to 4 with glacial acetic acid.
- 1 M HCl: the same as described in Aspila method in section 3.7.1.
- 1% (v/v) FeCl_3 : dissolve FeCl_3 7.44 g in 10 ml of deionized water and dilute 100 times before use.

Procedure

Weigh 0.25 g of $<125 \mu\text{m}$ sediment and transfer into a 50 ml centrifuge tube with a cap and then sequentially extract as following:

Step I Exchangeable or loosely sorbed P

1. Shake sediment in the centrifuge tube for 2 h with 25 ml of 1 M MgCl_2 (pH 8) on a shaker bath.
2. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant. Repeat the extraction with a second aliquot of 1 M MgCl_2 for additional 2 h.
3. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant. Then shake the residue with 25 ml deionized water for 2 h.
4. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant. Then shake the residue with 25 ml deionized water for 2 h.
5. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant.
6. All supernatants from 2 to 5 were colorimetrically analysed for soluble phosphate by the procedure of Koroleff (1976). The sum of soluble phosphate in all aliquots is equal to loosely bound P.

Step II Easily reducible ferric bound P or Fe-P

1. Shake the residue from step I for 8 h with 22.5 ml of CDB reagent
2. After 8 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of 1 M MgCl_2 .
3. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of deionized water.
4. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant.

5. Each supernatant is made to the volume of 50 ml, then add 0.5 ml of 1 % (v/v) FeCl_3 . Determine the content of Fe-P by isobutanol method (Watanabe and Oilsen, 1962; Lucotte and Anglejan, 1985) as described in section 3.9.3.

Step III Authigenic apatite plus CaCO_3 bound P plus biogenic apatite

1. Shake the residue from step II for 6 h with 25 ml of 1 M sodium acetate buffer to pH 4.
2. After 6 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of 1 M MgCl_2 .
3. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of 1 M MgCl_2 .
4. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of deionized water.
5. After 2 h, centrifuge at 3500 rpm for 15 min. Carefully remove the supernatant.
6. Each supernatant is analysed for soluble phosphate concentration by the procedure of Koroleff (1976). The sum of soluble phosphate in all aliquots is equal to authigenic apatite plus CaCO_3 bound P plus biogenic apatite.

Step IV Detrital apatite plus other inorganic P

1. Shake the residue from step III with 25 ml of 1 M HCl for 16 h.
2. After 16 h, centrifuge at 3500 rpm for 15 min and carefully remove the supernatant.
3. Supernatant from this step was analysed for soluble phosphate in detrital apatite plus other inorganic P fraction by the procedure of Koroleff (1976).

Step V Organic P

1. Transfer the residue from step IV into a 30 ml crucible. Dry the residue in an oven at 50 °C and then ash at 550 °C for 2 h.
2. After the residue is cooled in a dessicator, extract with 25 ml of 1 M HCl for 16 h.
3. After 16 h, extracted solution is carefully removed by a pasteur pipette and transferred into a pyrex test tube. Centrifuge at 3500 rpm for 15 min. Remove the supernatant and analyzed for soluble phosphate in organic P fraction using the method of Koroleff (1976).

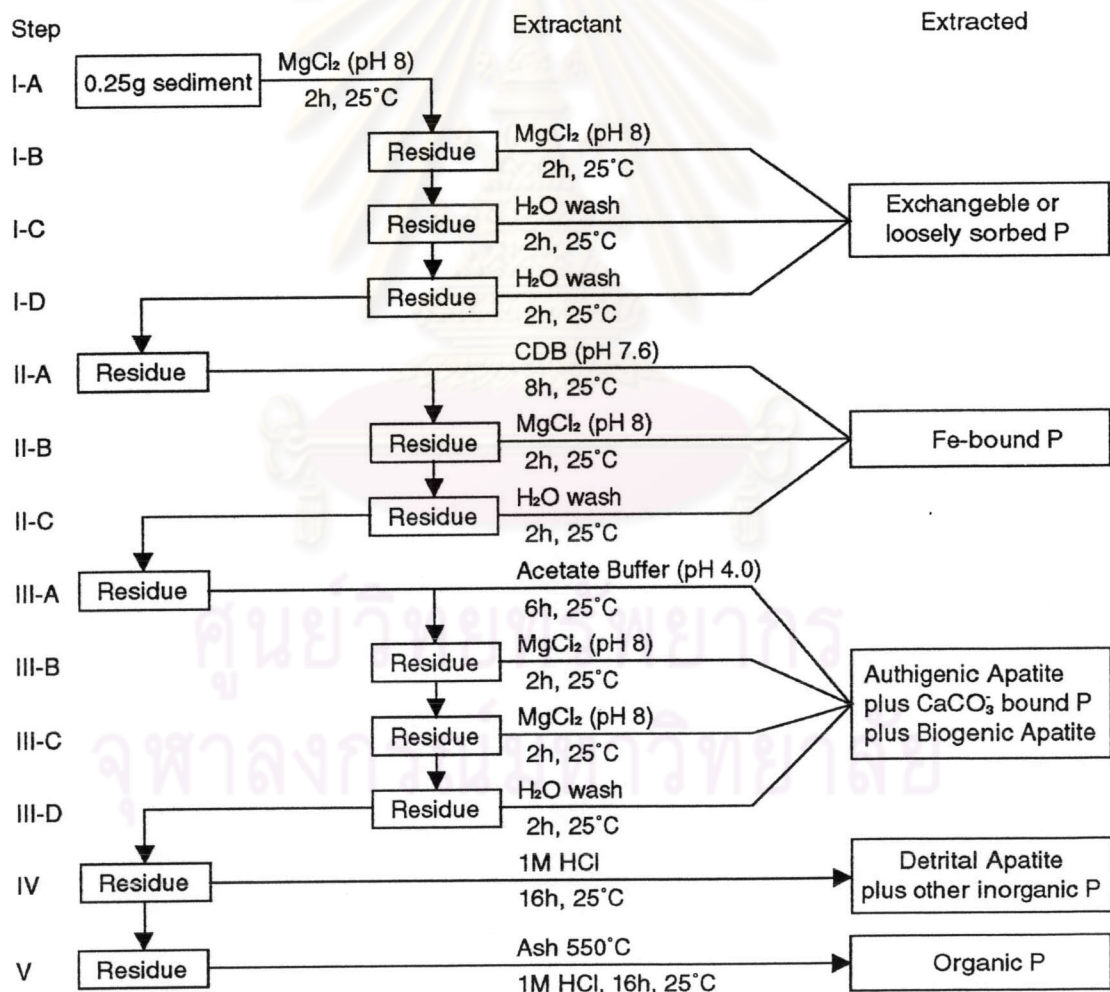


Figure 3.4 SEDEX scheme for phosphorus partitioning in sediments (Ruttenberg, 1992)

3.8.2 Vink et al. (1997)'s method

Three replicates of each sediment sample were sequentially extracted for their phosphorus speciation according to the extraction scheme in Figure 3.5. The solid to extractant ratio is 0.25 g: 25 ml of extractant except in step II where the residue was extracted with 10 ml of 1 % (w/v) sodium dodecyl sulfate (SDS) six times. The extractions were performed by agitation on a shaker at 25 °C. After centrifuging at 4000 rpm for 10 min, the supernatant is carefully removed by a pasteur pipette and then analyzed for soluble phosphorus by Strickland and Parsons (1972)'s method as described in section 3.9.1. Except in step III, supernatant is analyzed for soluble phosphate in Fe-P fraction according to the isobutanol extraction procedure of Watanabe and Oilsen (1962) after first reacting it with 1% (v/v) FeCl_3 (Lucotte and D' Anglejan, 1985) to obviate the interference of citrate with reduction of the molybdate complex (as described in section 3.9.3). Separate standards and reagent blanks were done for all steps in the same dilution as extracted solution so that their solution matrix matched that of samples. Turbidity blanks of extracted solution were also checked to correct absorbance of unfiltered sediment extracts.

Apparatus

Membrane filter (Milipore®) and Swinnex® inline filter holder, the other apparatus described in section 3.8.1.

Reagents

- 1 M MgCl_2 , CDB reagent, 1M NaOAc and 1 M HCl are the same as described in section 3.8.1.
- 1% (w/v) SDS, HCO_3^- buffer : dissolve 10 g of sodium dodecyl sulfate in 1000 ml of deionized water and buffer to pH 8.57 with 0.2 M NaHCO_3 .
- 6 N HCl : dilute 515 ml of conc. HCl with 300 ml deionized water in a 1000 ml volumetric flask. After cooling, make up to the volume.

- 0.5 M NaHCO₃: dissolve 21 g of Na HCO₃ and dilute to 500 ml with deionized water.

Procedure

Weigh 0.25 g of <125 μm sediment and transfer into a 50 ml centrifuge tube with a cap and then sequentially extract as following:

Step I Exchangeable or Loosely sorbed P

1. Shake sediment in the centrifuge tube for 2 h with 25 ml of 1 M MgCl₂ (pH 8) on a shaker bath.
2. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant. Repeat the extraction with a second aliquot of 1 M MgCl₂ for additional 2 h.
3. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant. Then shake the residue with 25 ml deionized water for 2 h.
4. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant. Then shake the residue with 25 ml deionized water for 2 h.
5. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant.
6. All supernatants from 2 to 5 are colorimetrically analysed for soluble phosphate by the procedure of Strickland and Parsons (1972). The sum of soluble phosphate in all aliquots is equal to loosely bound P.

Step II Organic P

1. The residue from step I is extracted six times with 10 ml of 1% (w/v) SDS, pH 8.57 in a water bath at 80°C for 2 h and rinse twice with 10 ml of 0.2 M NaCl and deionized water.
2. A 20 ml aliquot of the combined SDS + rinse solution is dried at 100 °C in a crucible.

3. The dry aliquot was then combusted at 600 °C for 4 h and extracted with 6 N HCl for 16-24 h.
4. The extracted solution is filtered, neutralized with 0.5 M NaHCO₃ and analyzed for soluble phosphate in organic phosphorus fraction by the procedure of Strickland and Parsons (1972).

Step III Easily reducible ferric bound P

1. Shake the residue from step II for 8 h with 22.5 ml of CDB reagent.
2. After 8 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of 1 M MgCl₂.
3. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of deionized water.
4. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant.
5. All supernatants are made to the volume of 50 ml, then added 0.5 ml of 1 %(v/v) FeCl₃ and determination of Fe-P content by isobutanol method (Watanabe and Oilsen, 1962; Lucotte and Anglejan, 1985) as described in section 3.9.3.

Step IV Authigenic apatite plus CaCO₃ bound P plus biogenic apatite

1. Shake the residue from step III for 6 h with 25 ml of 1 M Sodium acetate buffered to pH 4.
2. After 6 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of 1 M MgCl₂.
3. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of 1 M MgCl₂.
4. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant. Shake the residue for 2 h with 25 ml of deionized water.
5. After 2 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant.

6. Each supernatant is analysed for soluble phosphate concentration by the procedure of Strickland and Parsons(1972). The sum of soluble phosphate in all aliquots is equal to authigenic apatite plus CaCO_3 bound P plus biogenic apatite.

Step V Detrital apatite plus other inorganic P

1. Shake the residue from step IV with 25 ml of 1 M HCl for 16 h.
2. After 16 h, centrifuge at 4000 rpm for 10 min. Carefully remove the supernatant.
3. Supernatant from this step is analysed for soluble phosphate in detrital apatite plus other inorganic P fraction by the procedure of Strickland and Parsons (1972).

Step VI Residual P

1. The residue from step IV is transferred into 30 ml crucible. Dry the residue in an oven at 50 °C and then ash at 550 °C for 2 h.
2. After the residue was cooled in dessicator, residue was extracted with 25 ml of 1 M HCl for 16 h.
3. After 16 h, extracted solution was carefully removed by pasteur pipette and transferred into Pyrex® test tube. After centrifuging at 3500 rpm for 15 min, supernatant is diluted and analyzed for soluble phosphate in organic P fraction by the procedure of Strickland and Parsons (1972).

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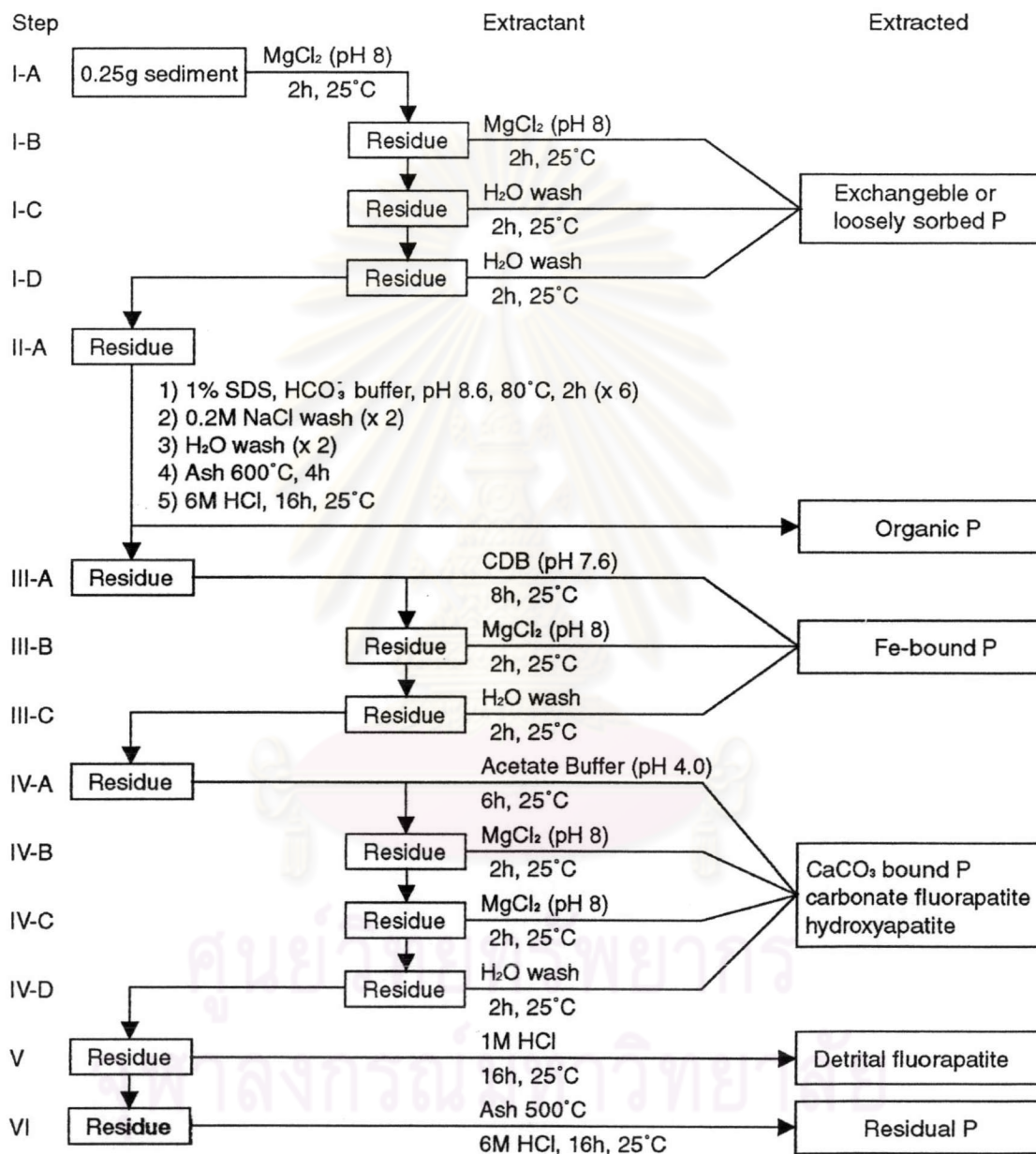


Figure 3.5 Sequential extraction scheme for phosphorus partitioning in sediments

(Vink et al., 1997)

3.8.3 Agemian (1997)'s method

Three replicates of each sediment sample were analyzed for phosphate species following the extraction scheme of Agemian (1997) as outlined in Figure 3.6. Sediment to extractant ratio is 0.50 g : 25 ml. The extraction was maintained by agitation on a shaker at 25 °C, except in step V the residue was extracted at 85 °C. After centrifuging at 3500 rpm for 15 min, supernatants were carefully removed and analyzed for soluble phosphorus by the method of Strickland and Parsons (1972) as described in section 3.9.1 except step II, supernatants were analyzed by the isobutanol method (Watanabe and Oilsen, 1962) as described in section 3.9.3, but without addition of FeCl_3 . Separate standards and reagent blanks were done for all steps in the same dilution as extract solution so that their solution matrix matched that of sediment extract. Turbidity blanks of extracted solution were also measured to correct absorbance of unfiltered sediment extracts.

Apparatus

100 ml volumetric flasks and the other apparatus as described in section 3.8.1.

Reagents

- 0.5 M NaCl : dissolve 14.61 g of NaCl and dilute to 500 ml by deionized water.
- 0.11 M $\text{Na}_2\text{S}_2\text{O}_4$: dissolve 10.56 g of $\text{Na}_2\text{S}_2\text{O}_4$ and dilute to 500 ml with deionized water.
- 0.11 M NaHCO_3 : dissolve 4.621 g of NaHCO_3 and dilute to 500 ml with deionized water.
- Bicarbonate ditionite (BD reagent): mix 0.11 M $\text{Na}_2\text{S}_2\text{O}_4$ and 0.11 M NaHCO_3 buffered to a pH of 7.0.
- 0.1 M NaOH: dissolve 2 g of NaOH and dilute to 500 ml with deionized water.
- 0.5 M HCl: dilute 45 ml of conc. HCl with deionized water. After cooling make volume to 1000 ml.

- 1 M NaOH: dissolve 20 g of NaOH and dilute to 500 ml with deionized water.
- 1 M H₂SO₄: dilute 56 ml of conc. H₂SO₄ (sp.gr.1.84) with deionized water.
After cooling, make volume to 1000 ml.

Procedure

Weigh 0.5 g of sediment sample into a 25 ml centrifuge tube with a cap

Step I Loosely bound or exchangeable phosphorus

1. Shake the sediment in the centrifuge tube for 1 h with 25 ml of 0.5 M NaCl.
2. Centrifuge for 15 min at 3500 rpm and decant the supernatant into a 100 ml volumetric flask.
3. Repeat the extraction with a second aliquot of 0.5 M NaCl, centrifuge and combine the second supernatant with the previous extracted solution.
4. Acidify extracted solution in the volumetric flask by adding 1 ml of 1 M H₂SO₄ and make up to volume.
5. Analyze soluble phosphate in the extracted solution by the procedure of Strickland and Parsons (1972).

Step II Iron and aluminum-bound phosphorus

1. Extract the sample left in centrifuge tube from the previous extraction step by shaking with 25 ml of BD reagent for 1 h.
2. Centrifuge for 5 min at 3500 rpm and transfer the supernatant to a 100 ml volumetric flask.
3. Repeat with a second 25 ml of BD reagent. Centrifuge and add the supernatant to previous extracted solution in the volumetric flask.
4. Shake the sediment in the centrifuge tube with 25 ml of 0.5 M NaCl, centrifuge and add the supernatant to the previous extracted solution in the volumetric flask.

5. Acidified solution in the volumetric flask with 4 ml of 1 M H_2SO_4 and make up to volume.
6. Analyze soluble phosphate in the extracted solution by the procedure of Strickland and Parsons (1972).

Step III Polyphosphates

1. Extract sample left in the centrifuge tube from the previous extraction step by shaking with 25 ml of 0.1 M NaOH for 18 h.
2. Centrifuge for 15 min at 3500 rpm and transfer the supernatant to a 100 ml volumetric flask.
3. Shake the sediment in the centrifuge tube with 25 ml of 0.5 M NaCl, centrifuge, and add the supernatant to the previous extracted solution in the volumetric flask.
4. Acidify solution in the volumetric flask with 3 ml of 1 M H_2SO_4 and make up to volume.
5. Analyze soluble phosphate in the extracted solution by the procedure of Strickland and Parsons (1972).

Step IV Calcium-bound phosphorus

1. Extract the sample left in centrifuge tube from the previous extraction step by shaking with 25 ml of 0.5 M HCl for 1 h.
2. Centrifuge for 15 min at 3500 rpm and transfer the supernatant to a 100 ml volumetric flask.
3. Shake the sediment in the centrifuge tube with 25 ml of 0.5 M NaCl, centrifuge and add the supernatant to previous extracted solution in the volumetric flask. Make up to the volume.
4. Analyze soluble phosphate in the extracted solution by the procedure of Strickland and Parsons (1972).

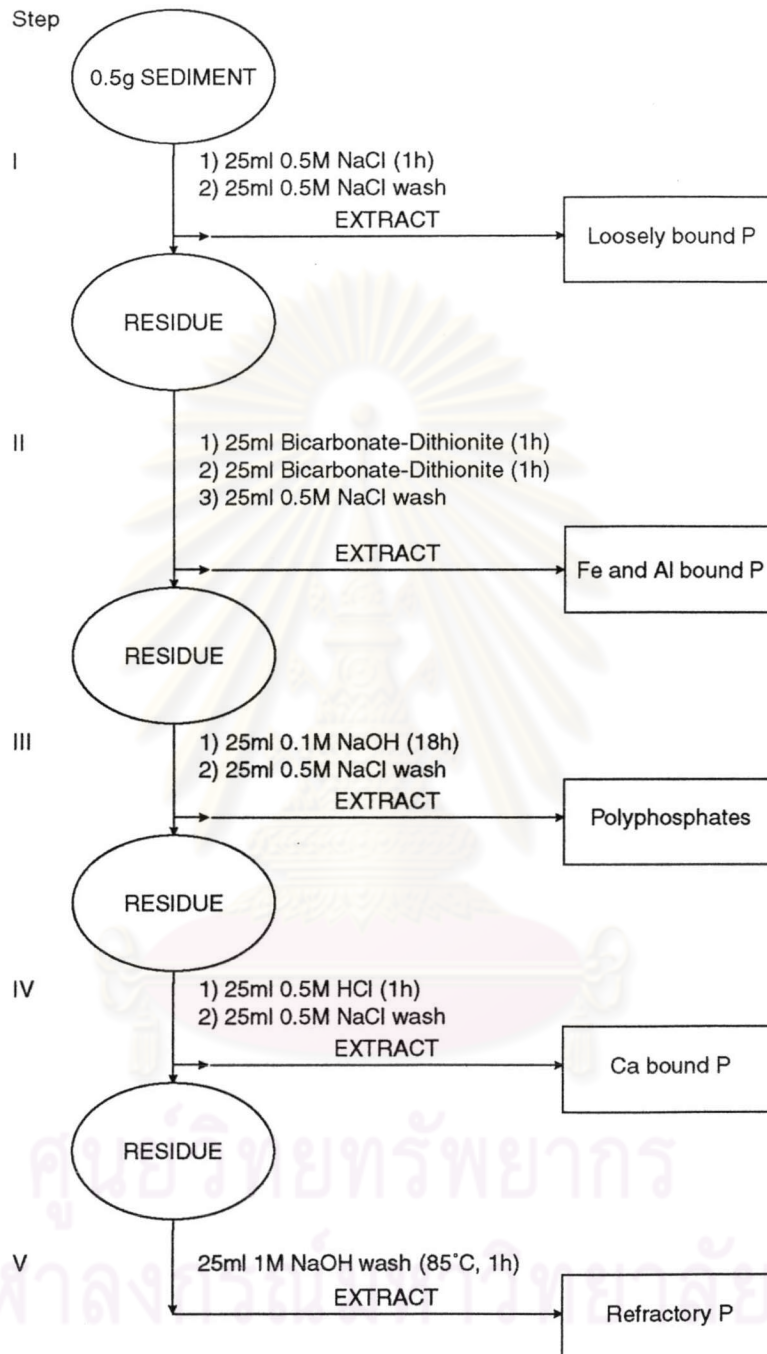


Figure 3.6 Sequential extraction scheme for phosphorus partitioning in sediments

(Agemian, 1997)

Step V Residual or refractory phosphorus

1. Extract the sample left in centrifuge tube from the previous extraction step by shaking with 25 ml of 1 M NaOH at 85°C for 1 h.
2. Cool and centrifuge for 15 min at 3500 rpm and transfer the supernatant to a 100 ml volumetric flask.
3. Acidify with 3 ml of 1 M H₂SO₄ and make up to the volume.
4. Analyze for soluble phosphate in the extract solution by the procedure of Strickland and Parsons (1972).

3.9 Soluble phosphate analysis

Soluble phosphate in all sediment extracts, except CDB- and BD- extracts, were colorimetrically analyzed by either the phosphomolybdate blue complex method of Strickland and Parsons (1972) or Koroleff (1976). These methods are based on the same principle prepared by Murphy and Riley (1962). Ammonium molybdate and antimony potassium tartrate rapidly react in an acid medium with phosphate ions in dilute solution to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The developed color is measured manually by a spectrometer within the optimum time limit.

CDB- and BD- extracts were analyzed for soluble phosphate by isobutanol method according to Watanabe and Oilsen (1962). This method is based on the separation of the phosphomolybdate complex from the color solution and possibly interfering ions before reduction with stannous chloride.

3.9.1 Strickland and Parsons (1972)'s method

Strickland and Parsons (1972) used the similar procedure as described by Murphy and Riley (1962) for determination of reactive phosphorus in sea water in their practical handbook of seawater analysis. The capability of this method ranges from 0.03 to 5 μM .

Apparatus

- Spectronic 401 (Milton Roy®)
- pH meter (Accumet® BASIC AB15)
- Micropipette (100-1000 μ l Eppendorf®, 1000-5000 μ l Gilson®)
- Beakers and test tubes (Pyrex®)
- 2 quartz cuvettes

Reagents

- Ammonium molybdate solution: dissolve 15 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ in 500 ml of deionized water. Store in a plastic bottle. Keep the reagent out of direct sunlight.
- Sulphuric acid solution: add 140 ml of concentrated sulphuric acid (sp.gr. 1.82) to 900 deionized water. Allow the solution to cool and store it in a glass bottle.
- Ascorbic acid solution: dissolve 27 g of ascorbic acid in 500 ml of deionized water. Store solution in a plastic bottle and frozen in a freezer. Defroze before use and refreeze at once.
- Potassium antimonyl tartrate solution: dissolve 0.34 g of potassium antimonyl-tartrate in 250 ml of deionized water, warming if necessary. Store in a glass or plastic bottle.
- Mixed reagent: mix together 10 ml of ammonium molybdate, 25 ml sulphuric acid, 10 ml ascorbic acid and 5 ml potassium antimonyl-tartrate solutions. Prepare this reagent for immediate use. Do not store more than 6 h.

- Standard phosphate stock solution: dissolve 0.816 g of anhydrous KH_2PO_4 in 1000 ml of deionized water. Store in a dark bottle with 1 ml of chloroform. The solution is stable for many months. This standard contains 6 $\mu\text{mol P/ml}$.
- Standard phosphate working solution: dilute standard phosphate stock solution 100 times with deionized water and then prepare working phosphate standards by adding 0.25 ml, 0.50 ml, 0.75ml, and 1.0 ml of diluted phosphate standard to a series of 25 ml volumetric flasks and make up to the volume with the extractant that has the same dilution as sediment extract. These working standards are freshly mixed daily.

Procedure

1 ml of mixed reagent is added to 10 ml of diluted extract solution and mixed at once. After 15 min and within 2 h, measure the absorbance of developed color in a 1 cm quartz cuvette at a wavelength of 885 nm. Cell to cell blanks are measured to check the stability of spectrophotometer. Reagent and turbidity blanks are conducted for correcting the absorbance of dilute extracted solution.

Corrected absorbance of solution is calculated as following:

$$A_{\text{corrected}} = A_{\text{sample}} - A_{\text{RB}} - A_{\text{TB}}$$

whereas:

$A_{\text{corrected}}$	=	corrected absorbance of sample
A_{sample}	=	absorbance of sample
A_{RB}	=	absorbance of reagent blank
A_{TB}	=	absorbance of turbidity blank

Note: A_{sample} , A_{RB} and A_{TB} were subtracted by the absorbance of cell to cell blank before calculation.

Calibration

A series of working standards prepared by extractant in the same dilution as sediment extract and reagent blank of extractant were measured their absorbances at a wavelength of 885 nm. After correcting values by using equation above, these absorbances were used to construct a standard calibration curve for phosphorus in the same matrix of extractant. Linear regression analysis was applied to calculate the standard calibration curve equation and the relative coefficient (r^2) of the relationship between concentration and absorbance of phosphate standards. Relative coefficient of accepted standard calibration curve is not less than 0.9995.

Calculation of phosphorus concentration

Concentration of phosphorus in extracted solution was calculated from the linear equation of standard calibration curve by using corrected absorbance ($A_{\text{corrected}}$) of extracted solution. After that, the calculated concentration was multiplied by degrees of dilution.

3.9.2 Koroleff (1976)' s method

This method is a modification of the Murphy and Riley procedure as outlined above. The single solution is split into two solutions: the first solution contains sulphuric acid, ammonium molybdate and antimony ions and the second one is acidified ascorbic acid solution. In addition, the final concentration of acid and molybdate are decreased from 0.2 M to 0.1 M and from 0.1% to 0.045%, respectively. This lower final acidity is also used in Strickland and Parsons (1972). By using two solutions, the reagent is more stable and the compensation for turbidity is easier. Capability range of this method is 0.01–28 μM . To obtain a rapid color development and to depress the interference of silicate, it is fundamental importance that the final reaction pH is less than 1.

Apparatus

The apparatus used in this method are similar to those described by Strickland and Parsons (1972) section 3.9.1

Reagents

- 4.5 M sulphuric acid: carefully add 250 ml of concentrated acid (sp.gr. =1.84) to 750 ml deionized water. Allow to cool and dilute to 1000 ml. Store in a polyethylene bottle.
- Acidified ascorbic acid: Dissolve 10 g ascorbic acid in 50 ml of deionized water, then add 50 ml of 4.5 M sulphuric acid. Store in an amber lab glass bottle in a refrigerator, whereby the reagent is stable for at least a week and can be used as long as it remain colorless.
- Mixed reagent: dissolve 12.5 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$, in 125 ml of deionized water. Also dissolve 0.5 g potassium antimony tartrate, $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$, (with or without $\frac{1}{2}\text{H}_2\text{O}$), in 20 ml of deionized water. Add the molybdate solution to 350 ml of 4.5 M sulphuric acid, stir continuously. Add the tartrate solution and mix well. Store in a lab glass bottle. This mixed reagent is stable for several months.
- Phosphate standard stock solution: KH_2PO_4 is dried at 110°C then cooled in a dessicator. Exactly 136.1 mg is dissolved in deionized water to which 0.2 ml of 4.5 M sulphuric acid has been added. Finally dilute to 100 ml. Store cold in a glass bottle the solution is stable for months. This stock standard contains $10.0\ \mu\text{mol P/ml}$.
- Standard phosphate working solution: dilute standard phosphate stock solution 100 times with deionized water and then prepare working phosphate standards by adding 0.25 ml, 0.50 ml, 0.75ml, 1.0 ml of

diluted phosphate standard to a series of 25 ml volumetric flasks and make up to the volume with the extractant that has the same dilution as sediment extract. These working standards are freshly prepared.

Procedure

1 ml of mixed reagent is added to 50 ml of diluted extract solution and mixed at once. After 15 - 30 min, absorbance is measured at a wavelength of 880 nm by using a 1 cm quartz cuvette. Cell to cell blanks are measured to check the stability of spectrophotometer. Reagent and turbidity blanks are measured to correct the absorbance of dilute extracted solution. Calculation of corrected absorbance is the same as described in section 3.9.1.

Calibration

Standard calibration curve for phosphorus was conducted the same as described in section 3.9.1.

Calculation of phosphorus concentration

The same as described in section 3.9.1.

3.9.3. Watanabe and Oilsen (1962)'s method

CDB or BD extract was analyzed for soluble phosphate in Fe-P fraction by isobutanol method that is based on the separation of the phosphomolybdate complex from the color solution and possibly interfering ions before reduction with stannous chloride. Capability range of this method is from 2 $\mu\text{g P}$ to 22 $\mu\text{g P}$.

Special apparatus

- 100 ml separatory funnels (acid cleaned and ethanol rinsed)
- A teflon vessel for SnCl_2
- 100-1000 μl (Eppendorf®), 1000-5000 μl (Gilson®) micropipettes

Reagents

- 0.5 M H_2SO_4 : dilute 28 ml of conc. H_2SO_4 (sp.gr. 1.84) with deionized water in a 1000 ml volumetric flask. After cooling, make up to the volume. Store in a plastic bottle.
- 5 M H_2SO_4 : dilute 140 ml of conc. H_2SO_4 (sp.gr. 1.84) with deionized water in a 500 ml volumetric flask. After cooling, make up to the volume. Store in a plastic bottle.
- Molybdate reagent: dissolve 50 g of ammonium molybdate in 500 ml of deionized water and add 400 ml of 5 M H_2SO_4 . Dilute to 1 l and store in a polyethylene bottle.
- Stannous chloride (stock solution): dissolve 10 g of anhydrous SnCl_2 in 25 ml concentrated HCl. Store in a small teflon vessel (refrigerator).
- Stannous chloride (dilute solution): dilute 1 ml of SnCl_2 stock solution to 200 ml with 0.5 M H_2SO_4 just before use.
- Isobutanol: boiling range of 106 °C to 110 °C
- Absolute ethanol
- Standard phosphate stock solution: the same as described in section 3.9.2. Standard phosphate stock solution contains 10.0 $\mu\text{mol P/ml}$.
- Standard phosphate working solution: dilute standard phosphate stock 100 times with CDB or BD in the same matrix as sediment extract. This working standards is freshly mixed every time of analysis.

Procedure

1. Pipette 0.3-15 ml of an aliquot (BD extract or CDB extract treated with FeCl_3) into a 100 ml separatory funnel.
2. Add 5 ml of molybdate-sulphuric acid reagent and make up to the volume with 20 ml of deionized water.
3. Add 10 ml of Isobutanol and shake for 2 min.
4. After 15 min, discard the aqueous layer and wash by shaking once with 10 ml of 0.5 M H_2SO_4 . Stand for 15 min and then discard the aqueous layer.
5. Add diluted stannous chloride 15 ml, shake for 1 min. Stand for 15 min, then discard aqueous layer.
6. Transfer the blue isobutanol layer into a 25 ml volumetric flask. Rinse the funnel and make up to the volume with absolute ethanol.
7. Allow color to develop for 30 min. Color is stable for at least 4 hours.
8. Measure absorbance of the blue solution against a blank containing all the reagents with spectrophotometer at a wavelength of 660 or 720 nm. Corrected absorbance can be calculated by subtracting absorbance of reagent blank from absorbance of sample. Cell to cell blank is also conducted to check the stability of the spectrophotometer while determination.

Corrected absorbance of sample can be calculated as following:

$$A_{\text{corrected}} = A_{\text{sample}} - A_{\text{RB}}$$

whereas:

$A_{\text{corrected}}$	=	corrected absorbance of sample
A_{sample}	=	absorbance of sample
A_{RB}	=	absorbance of reagent blank

Note: A_{sample} , A_{RB} and A_{TB} were subtracted by the absorbance of cell to cell blank before calculation.

Calibration

Standard calibration curve was prepared by pipetting known concentration of phosphate in the range of 0 – 22 μ g (0-7 μ M) from standard working solution into a series of 100 ml separatory funnels and the blue color was developed as outlined in the procedure. Linear regression analysis was applied to calculate the standard calibration curve equation and the relative coefficient (r^2) of the relationship between concentration and absorbance of phosphate standards. Relative coefficient of accepted standard calibration curve is not less than 0.9995.

Calculation of Fe-P concentration in CDB or BD extract

Concentration of Fe-P in CDB or BD extract (C_{cal}) was calculated from the equation of standard calibration curve by using corrected absorbance ($A_{corrected}$) of extracted solution.

3.10 Calculation of phosphorus concentration in sediment

Total phosphorus and phosphorus species except Fe-P were calculated as following:

$$C = \frac{C_{cal} \times V \times 31}{1000 \times W}$$

whereas C = Phosphorus concentration in sediment (μ g/g sediment)

C_{cal} = Phosphorus concentration from standard calibration curve and multiplied by dilution (μ M)

V = Volume of extractant (ml)

W = Sediment weight (g)

Fe-P from CDB extraction was calculated as following:

$$C_{\text{Fe-P}} = \frac{\mu\text{mole of Fe-P} \times 50.5 \times V_{\text{extract}} \times 31}{V_{\text{analyze}} \times 20 \times W}$$

whereas: $C_{\text{Fe-P}}$ = Concentration of Fe-P in sediment ($\mu\text{g/g}$ sediment)

$\mu\text{mole of Fe-P}$ = $\mu\text{mole of Fe-P}$ calculated from standard calibration curve by using corrected absorbance and final volume of isobutanol blue solution

$$= \frac{C_{\text{cal}} \times 25 \mu\text{mole}}{1000}$$

V_{extract} = Volume of CDB extractant (ml)
= 22.5 ml (Ruttenberg, 1992)
= 25 ml (Vink et al., 1997)

V_{analyze} = Volume of analyzed aliquot (ml) = 0.3 - 15 ml

W = Sediment weight (g)

Fe-P from BD extract (Agemian, 1997) was calculated using

$$C_{\text{Fe-P}} = \frac{\mu\text{mole of Fe-P} \times 100 \times 31}{V_{\text{analyze}} \times W}$$

whereas: $C_{\text{Fe-P}}$ = Concentration of Fe-P in sediment ($\mu\text{g/g}$ sediment)

$\mu\text{mole of Fe-P}$ = $\mu\text{mole of Fe-P}$ calculated from standard calibration curve by using corrected absorbance and final volume of isobutanol blue solution

$$= \frac{C_{\text{cal}} \times 25 \mu\text{mole}}{1000}$$

V_{extract} = Volume of CDB extractant (ml)

V_{analyze} = Volume of analyzed aliquot (ml) = 1-2 ml

W = Sediment weight (g)