CHAPTER 3
MATERIALS AND METHODS

3.1 Apparatus

3.1.1 pH Meter, Model 340, Delta, MERTER TOLEDO
3.1.2 Spectrophotometer, Envi science, Model DR2500
3.1.3 Elemental analyzer, Leco CHN-2000, Model FN24B3
3.1.4 Hot plate
3.1.5 Hot air oven
3.1.6 Filter paper No.5: Whatman, England
3.1.7 Filter paper GF/C: Whatman, England
3.1.8 Suction
3.1.9 Buchner funnel
3.1.10 Aspirator
3.1.11 Volumetric flask, test tube, pipette, cylinder, beaker, glass bottle, erlenmeyer flask, glass bead, laboratory bottle, glass funnel, and etc.

3.2 Materials and chemical reagents

3.2.1 *Crinum asiaticun* Linn, *Echinodorus cordifolius*, *Spathiphyllum Clevelandii*, *Rhizophora apiculata*, *Thalia dealbata* J. Fraser, *Heliconia psittacorum*, *Sonnertia ovata*, and *Sagittaria montevidensis*
3.2.2 *Pseudomonas putida* and *Flavobacterium oryzihabitans*
3.2.3 Distilled water
3.2.4 Reagent for test nitrate (5 Nitrate Reagent)
3.2.5 Nitric acid
3.2.6 Sulfuric acid
3.2.7 Sodium hydroxide
3.2.8 Phenolphthalein indicator aqueous solution
3.2.9 Anhydrous KH_2PO_4
3.2.10 Potassium antimonyl tartrate
3.2.11 Ammonium molybdate
3.2.12 Ascorbic acid
3.2.13 Extran MA-03 Phosphate Free
3.2.14 Phosphoric acid
3.2.15 Sulfanilamide
3.2.16 *N*-(1-naphthyl)-ethylenediamine dihydrochloride
3.2.17 Sodiumtetraboratedecahydrate
3.2.18 Boric acid
3.2.19 Copper sulfate
3.2.20 Methyl red indicator
3.2.21 Ethyl alcohol
3.2.22 Methylene blue
3.2.23 Domestic wastewater: obtained from wastewater treatment plant of Tungkru District, Bangkok, Thailand that was influent before treatment

3.3 Methods

3.3.1 Plant culture condition

Plants were selected at the same stage of growth and weight. Plants were cleaned with tap and distilled water to disperse soil particles that hold at plant stems and roots. Then plants were conditioned in tap water for a week before use in the experiment at the Remediation Laboratory of King Mongkut's University of Technology Thonburi (KMUTT) Bangkhuntien.

3.3.2 Screening plants for phosphorus removal

Crinum asiaticun Linn, *Echinodorus cordifolius*, *Spathiphyllum clevelandii*, *Rhizophora apiculata*, *Thalia dealbata* J. Fraser, and *Heliconia psittacorum* were screened for phosphorus removal by providing as control and treatment system. Plants were cultured in domestic wastewater that each pot used 1.5 L of wastewater and used 600 g of plants, grown in the greenhouse. In addition, *Sonnertia ovata*, *Sagittaria montevidensis*, and *Echinodorus cordifolius* were supplemental screened. Plants were cultured in domestic wastewater that each pot used 3 L of wastewater, 500 g of soil and used 600 g of plants, grown in the greenhouse. The water samples were collected in order to analyze concentration of total phosphate by the ascorbic acid colorimetric method after digest with sulfuric acid-nitric acid [45]. In addition, pH was determined by using pH meter (pH Meter, Model 340, Delta, MERTER TOLEDO).

3.3.3 Comparison of the abilities between *E. cordifolius* and *S. montevidensis* in phosphorus, nitrogen, and COD treatment from domestic wastewater under soil conditions

The experimental design was performed as two experimental groups and two control groups in the treatment system. The two experimental groups were *E. cordifolius* + soil + domestic wastewater and *S. montevidensis* + soil + domestic wastewater. The two control groups used only domestic wastewater and soil + domestic wastewater. All treatments were done in triplicate in separate pots.

E. cordifolius and *S. montevidensis* were compared during 5 cycles to determine their ability of phosphorus treatment in domestic wastewater. The plants were cultured in pots (12 inches of diameter); each pot used 300 g of soil and 3 liters of domestic wastewater. After phosphorus treatment, in each cycle, a new lot of domestic wastewater was replaced in the system. The water samples were collected in order to analyze the concentration of total phosphate by the ascorbic acid colorimetric method [46] in each cycle. COD was determined following conventional methods of analysis [45]. In addition, comparison of two plants in treatment of nitrogen, ammonia-nitrogen, and nitrate nitrogen were determined by distillation and titration, and the cadmium reduction method [45], respectively.

3.3.4 Enhancing phosphorus removal of plants by using sawdust bottom ash

In treatment pots were put 1% and 5% (w/w) sawdust bottom ash in 300 g of soil and used the same weight of plants (*E. cordifolius*) and used 2.5 L of wastewater. The water samples were collected in day 0, 1, 3, and 6 for analyzed total phosphate following standard methods [46]. Sawdust bottom ash is a waste from using a by-product from wood furniture industries as a fuel that low cost and contained calcium, magnesium, and potassium are composition [47]. Calcium in sawdust bottom ash has property for attach with phosphorus well [48]. Therefore, sawdust bottom ash was selected for phosphorus removal enhancing or finding suitable material that can adsorb with phosphorus.

Moreover, in treatment pots were put 1% (w/w) sawdust bottom ash in 300 g of soil and used the same weight of plants (*S. montevidensis*) and used 2.5 L of wastewater. The water samples were collected in day 0, 1, 4, 8, 12, 18, and 21 for analyzed total phosphate following standard methods [46].

3.3.5 Phosphorus removal from domestic wastewater by *E. cordifolius*

Phosphorus removal by *E. cordifolius* under soilless and soil conditions were studied for 4 cycles. After phosphorus removal in each cycle, new lot of domestic wastewater was replaced in the system. The water samples were collected for phosphate concentration analysis by the ascorbic acid colorimetric method after digest with sulfuric acid-nitric acid [46].

3.3.6 The relationship among plants, microorganisms and soil in phosphorus removal of cycle 4

The effects of plants, microorganisms and soil in phosphorus removal were studied by setting the experiments of soil + wastewater, plant + soil + wastewater, plant + wastewater, and sterile soil + wastewater. The percentage of plant uptake, soil adsorption and microorganisms uptake in the systems were determined by equations;

$$\% \text{ P removal} = \frac{(\text{Initial P conc.} - \text{Final P conc.})}{\text{Initial P conc.}} \times 100$$

% P adsorption by soil = % P adsorption of sterile soil in wastewater

% P uptake by microorganisms in soil = (% P adsorption of soil in wastewater) – (% P adsorption of sterile soil in wastewater)

% P uptake by microorganisms in wastewater = (% P uptake of wastewater) – (% P uptake of sterile wastewater)

% P uptake by plants = (% P uptake of plant in soil containing wastewater) – (% P adsorption of soil in wastewater)

3.3.7 Relation between photosynthesis and biomass

Photosynthesis of *E. cordifolius* both control (*E. cordifolius* + soil + tap water) and treatment systems (*E. cordifolius* + soil + wastewater) were determined by measurement of chlorophyll fluorescence with FMS-2 portable pulse-modulated fluorometer (Hansatech Instruments Ltd, King's Lynn, England) which was leafclip system [49]. *E. cordifolius* in control and treatment systems were harvested after treatment for 4 cycles in order to determine biomass. Harvested plants were washed with distilled water, fresh weight was recorded and oven dried (60-65 °C) until constant weight. Then plant samples were cooled in desiccators and measured dry weight [15].

3.3.8 Percentage of carbon, nitrogen, and phosphorus in plant after 4 treatment cycles

E. cordifolius in control (*E. cordifolius* + soil + tap water) and treatment systems (*E. cordifolius* + soil + wastewater) were harvested after treatment for 4 cycles. Plants were washed with distilled water, oven-dried (60-65 °C) and then ground to a fine powder for determination the percentage of carbon, nitrogen, and phosphorus in plant. Percentage of carbon was determined by burning plant samples at 550°C and calculated as follows [50];

$$\% \text{ Organic matter} = \frac{(\text{The weight of plant samples before burning} - \text{after burning})}{\text{The weight of plant samples before burning}} \times 100$$

$$\% \text{ Carbon} = \frac{\% \text{ Organic matter}}{1.8}$$

Percentage of nitrogen was analyzed by macro Kjeldahl method [51] which calculated as follow;

$$\% \text{ Nitrogen} = \frac{(A-B) \times N \times 0.014 \times 100}{\text{The weight of plant sample (g)}}$$

When: A = Volume of H_2SO_4 titrated for the sample (ml)
B = Volume of H_2SO_4 titrated for the blank (ml)
N = Normality of H_2SO_4 solution

Percentage of phosphorus was determined by digesting plant samples with sulfuric-nitric acid and followed by vanadomolybdophosphoric acid colorimetric method [51]. Then the percentage of phosphorus in the plant was calculated as follows:

$$\% \text{ Phosphorus} = \frac{\text{P concentration (mg L}^{-1}) \times 100}{\text{The weight of plant samples (mg)}}$$

3.3.9 Phosphorus and nitrogen treatment by *Echinodorus cordifolius* in domestic wastewater for 5 cycles

The plants were cultured in pots (12 inches in diameter); each pot used 600 g of plants, 300 g of soil and 3 liters of domestic wastewater which was obtained from the wastewater treatment plant of Tungkru District, Bangkok, Thailand. The experimental design was performed as experimental groups and control groups in the treatment system. The experimental groups used *E. cordifolius* + soil + domestic wastewater and the control groups used only domestic wastewater and soil + domestic wastewater. All treatments were done in triplicate in separate pots. Phosphorus and nitrogen treatment by *E. cordifolius* were studied after treatment for 5 cycles. The remaining phosphate, ammonia-nitrogen and nitrate-nitrogen concentrations in domestic wastewater were determined by the ascorbic acid colorimetric method, distillation and titration, and the cadmium reduction method [45], respectively.

3.3.10 The sustainability of the system in phosphorus treatment from domestic wastewater by *Echinodorus cordifolius* for 20 cycles

The system was comprised of only domestic wastewater, plant + soil + domestic wastewater, and soil + domestic wastewater. Phosphorus treatment in domestic wastewater by *E. cordifolius* was studied continuously for 20 cycles to determine the sustainability of the system. A new lot of domestic wastewater was replaced in the system after phosphate concentration in the previous test run had passed the standard criteria. The remaining phosphate concentration in the systems was determined by the ascorbic acid colorimetric method [46].

3.3.11 The relationship among plants, microorganisms, and soil in phosphorus treatment for 20 cycles

The relationship between plants, microorganisms, and soil in phosphorus treatment was investigated during the study of the sustainability in phosphorus treatment by *E. cordifolius* in domestic wastewater for 20 cycles. The systems were performed as plant + soil + domestic wastewater and soil + domestic wastewater. Only domestic wastewater and sterile domestic wastewater were used to study the effect of phosphorus uptake by microorganisms in domestic wastewater. In addition, sterile soil + sterile domestic wastewater was also studied in order to determine the effect of phosphorus

adsorption by soil. The percentage of phosphorus uptake by plants, phosphorus adsorption by soil, and phosphorus uptake by microorganisms of each test run in the system was determined by the following equations in section 3.3.6.

3.3.12 Percentage of carbon, nitrogen, and phosphorus in plants after phosphorus treatment for 20 cycles

E. cordifolius was harvested after phosphorus treatment for 20 test runs from the systems of the sustainability in phosphorus treatment from domestic wastewater. Plants were washed with distilled water, oven dried (60-65°C) and then ground into a fine powder for determination of the percentage of carbon, nitrogen, and phosphorus in the plant. The percentage of carbon and nitrogen were determined by using an elemental analyzer (Leco CHN-2000 model FN24B3). The percentage of phosphorus was determined by digesting plant samples with sulphuric-nitric acid followed by the vanadomolybdophosphoric acid colorimetric method [51]. The percentage of phosphorus in the plant was calculated as follow in section3.3.8.

3.3.13 Biomass of plants before and after phosphorus treatment from domestic wastewater for 20 cycles

E. cordifolius in the control and treatment systems of phosphorus treatment were harvested after treatment for 20 cycles to determine the biomass. Harvested plants were washed with distilled water, the fresh weight was recorded, and the samples were oven dried (60-65 °C) to a constant weight. The plant samples were cooled in desiccators and the dry weight was measured [15].

3.3.14 Microorganism preparation

The study of microorganisms in domestic wastewater found that *Pseudomonas putida* and *Flavobacterium oryzihabitans* were dominant microorganisms [18]. Therefore, enhancement of phosphorus treatment by *E. cordifolius* augmented with microorganisms was studied. *P. putida* and *F. oryzihabitans* were obtained from BIOTEC Culture Collection (BCC) and Department of Medical Sciences (DMST Culture Collection), respectively. Microorganisms were picked up with 2 loops of each plate, inoculated in a flask of 500 mL LB medium, incubated for 24 hrs at room temperature, and then shaken at 200 rpm. The cells were recovered by centrifugation (4,000 rpm for 15 min) and then transferred in sterile distilled water. The cell concentration of each strain and mixed strains were measured by a spectrophotometer to an optical density at 560 nm (OD_{560}) of 0.4 and used as an inoculum.

3.3.15 Bioaugmentation of phosphorus treatment by *Echinodorus cordifolius* with microorganisms (*Pseudomonas putida* and *Flavobacterium oryzihabitans*)

In this experiment, 300 g of plants, 150 g of soil, 1.5 liters of domestic wastewater, and 10% of each strain and mixed strains were inoculated in domestic wastewater. The experiment was performed in triplicate which was comprised of the systems of wastewater including plants + soil + domestic wastewater, plants + soil + domestic wastewater + *P. putida*, plants + soil + domestic wastewater + *F. oryzihabitans* and plants + soil + domestic wastewater + *P. putida* + *F. oryzihabitans*. The water samples were collected at 0, 1, 6, 12, 14, and 17 hr for analysis of the remaining phosphate concentration in the system.

3.3.16 Biomass of plants after bioaugmentation of phosphorus treatment by *Echinodorus cordifolius* with microorganisms (*Pseudomonas putida* and *Flavobacterium oryzihabitans*)

E. cordifolius in the system of phosphorus treatments (plants + soil + domestic wastewater, plants + soil + domestic wastewater + *P.putida*, plants + soil + domestic wastewater + *F. oryzihabitans*, and plants + soil + domestic wastewater + *P. putida* + *F. oryzihabitans*) were harvested after treatment for one month in order to determine the biomass (See section 3.3.13).