

工學碩士 學位論文

**Neural Networks and Molecular  
Analyses of Circulating Piggery Slurry  
Treatment System**

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# ABSTRACT

BOD

1000

가

가

*A. caligenes*

*faecalis* (TSA-3) *Brevundimonas diminuta* (TSA-1),

*A. biotrophia defectiva* (TSA-2)가 ,

MRS-1 ( ) 2 (*Streptococcus* sp. : MRS-3)

*A. caligenes faecalis* (TSA-3)

(polymerase chain reaction; PCR)

가

(glutamine synthetase: GS)

4 )

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(principal component analysis:



PCA)

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PCA

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## **. INTRODUCTION**

The swine wastes may cause a serious degradation of water quality such as eutrophication and spread of pathogens in water bodies (*i.e.*, lakes, rivers and groundwater as water supply sources) (39). The daily volume of livestock wastewater in Korea reached 197,000m<sup>3</sup>, and 50% of the volume was generated from dairy farms that were not target for a legal pollution control. The amount of wastewater is relatively small compared with total wastewater including industrial and domestic wastewater (7% of the total), but contributes significantly to the pollution of the receiving waters because of its high organic nutrient concentration (>BOD 20,000 mg/L) (32). According to the environmental protection law, the large size farms (more than 1,000 heads) are subjected to regulations for treatment facilities whereas small or middle size farms (less than 1,000 heads) are exempt from the regulations. While an activated sludge system has been proven to be effective in the treatment of piggery slurry at large scale farms (more than 1,000 heads), the system may not ensure the effect in small or middle scale farms (less than 1,000 heads) in terms of its operation cost. The number of swine heads under the regulatory control, therefore, takes only 31% of the total number of heads (33).

Recently a circulating reactor system operated under sequential oxic and anoxic conditions for the treatment of swine wastewater has been developed, in which piggery slurry is fermentatively and aerobically treated and then its effluent recycled to the pigsty (9).

This system appears to significantly remove offensive smells (at both pigsty and treatment plant) and BOD, and turns out to be cost effective in the relatively small scale farms.

There are several treatment steps in the system. For its successful operation, it will necessary to monitor microbial population density and treatment parameters. Modeling relationships among these variables will be useful in predicting treatment effects and managing the system. One of the best known models applied for wastewater treatment system so far is the activated sludge model NO. 1(ASM 1) introduced by International Association for Water Quality (IAWQ) in 1987 (15). Application of the model to the field treatment system, however, may have some limitations because the model usually requires many operational parameters and has quite variable kinetic characteristics within the treatment system over time (25).

On the other hand, neural network models that imitate the functions of our human brain have been successfully used to resolve many engineering problems such as complex pattern classification and control of highly nonlinear dynamic systems (4, 26, 31, 42). Those models have the characteristics of massive parallelism, many degrees of freedom, and adaptive learning. It was recently well known that the multi-layer neural networks can approximate a function in  $L^p$  within an arbitrary accuracy (18), and generalize a new data that are not used in learning process (5). Recently a progress has been made in application of neural networks to controlling the biological and chemical engineering processes. There has been, however, no report

dealing with a neural network modeling for biological swine wastewater treatment system, to the best of our knowledge.

This study was carried out to elucidate mechanism of the circulating piggery slurry treatment system using such as variables population dynamics, activity of heterotrophic bacteria, and treatment effects based upon suspended solids (SS), ammonia nitrogen ( $\text{NH}_4^+\text{-N}$ ), total phosphorus (T-P), *ortho*-phosphorus (*o*-P) and chemical oxygen demand (COD) as input or output variables. These variables were used to establish a non-linear model emulator using multi-level neural networks that could eventually allow real time monitoring and prediction of the treatment system. We also tried to elucidate a mechanism for ammonium removal using molecular biological techniques.

## **. LITERATURE REVIEW**

### **2.1 Swine Wastewater Pollution and Treatment Technology**

#### **2.1.1 Pollution Status and Characteristics of Swine Wastewater**

The wastes cause a serious degradation of water quality resulted from spread of pathogenes and a eutrophication in the receiving waters such as lakes and rivers as water supply sources. The daily volume of livestock wastewater in Korea reached 197,000m<sup>3</sup>, while 50% of this volume was generated from dairy farms that are exempt from a legal pollution control (Figure 2.1). The amount of wastewater is relatively small compared with total wastewater including industrial and domestic wastewater (7% of the total), but contributes significantly to the pollution of the receiving waters because of its high organic nutrient concentration (32). The degree of pollution caused by swine wastewater may depend upon the region of water bodies and sometimes reaches almost 20% of the total pollution load (33). In other word, domestic farms of Korea know almost small or middle size farms are exempt from the regulation.

Raw wastewater contains substances to give malodorant smells such as nitrogenous compounds, sulfates, volatile fatty acids, aldehydes and so forth. Besides, the wastewater can allow the growth of health pests so that the living environments would be degraded and use of agricultural water contaminated by swine wastewater could reduce harvest of crops. It has become a target of

public grievances (12).

Physical and chemical characteristics of swine wastewater may be different depending on storage period and treatment process, but BOD concentration may be as high as about 20,000 60,000 (mg/L). Nitrogen concentration as TKN can reach the range of 4,000 6,000mg/L (32).

Table 2.1. Status of the waste, number of heads and percentage rate of the livestock farms in Korea (Environmental Management Research Center, 1998)

Criteria	Permit required	Report required	No regulation	Total
Farms	3,743 (0.7%)	24,118 (4.2%)	539,863 (95.1%)	567,724 (100%)
Heads	3,424,000 (34.5%)	3,726,000 (33.1%)	3,211,000 (32.4%)	9,911,000 (100%)
Wastewater Volume (m <sup>3</sup> /d)	46,700 (23.7%)	51,456 (26.1%)	93,861 (50.2%)	197,017 (100%)

### **2.1.2 Treatment Technologies of Swine Wastewater**

Conventional treatment technologies available so far are activate sludge treatment, trickling filter, oxidation lagoon, oxidation pond, percolation, composting, and landfill disposal. New technologies are HAF (Hyundai Anaerobic Filer), BIMA, and Bio-Ceramic. These treatment technologies, however, are relatively difficult to operate and have comparatively high running costs. One of the reasons for this may be the characteristics of the wastewater itself: its high organic and nutrient content as well as unbalanced carbon, nitrogen, and phosphorus ratios (28).

Treatment plants for swine wastewater using activated sludge and methane fermentation technologies have been disseminated. These methods are effective for removal of BOD and COD but not for nitrogen and phosphorus (29). This is because swine wastewater contains a large amount of nitrogen which corresponding to 20–40% of BOD (28).

## **2.2 Modeling Using Neural Networks**

### **2.2.1 Characteristics of Neural Networks and Principal Component Analysis**

In this study, we used a multi-layer neural network with error back propagation algorithm to model the complex relationship in the

circulating system (10). Artificial Neural Network is a system loosely modeled on the human brain. It attempts to simulate within specialized hardware or sophisticated software, the multiple layers of simple processing elements called neurons. Each neuron is linked to certain of its neighbors with varying coefficients of connectivity that represent the strengths of these connections. Learning is accomplished by adjusting these strengths to cause the overall network to output appropriate results (14).

Designing a neural network consist of:

- Arranging neurons in various layers.
- Deciding the type of connections among neurons for different layers, as well as among the neurons within a layer.
- Deciding the way a neuron receives input and produces output.
- Determining the strength of connection within the network by allowing the network learn the appropriate values of connection weights by using a training data set.

The process of designing a neural network is an iterative process; the figure below describes its basic steps.

As the figure 2.1 shows, the neurons are grouped into layers. The input layer consists of neurons that receive input form the external environment. The output layer consists of neurons that communicate the output of the system to the user or external environment. There are usually a number of hidden layers between these two layers; the figure 2.1 shows a simple structure with only one hidden layer.



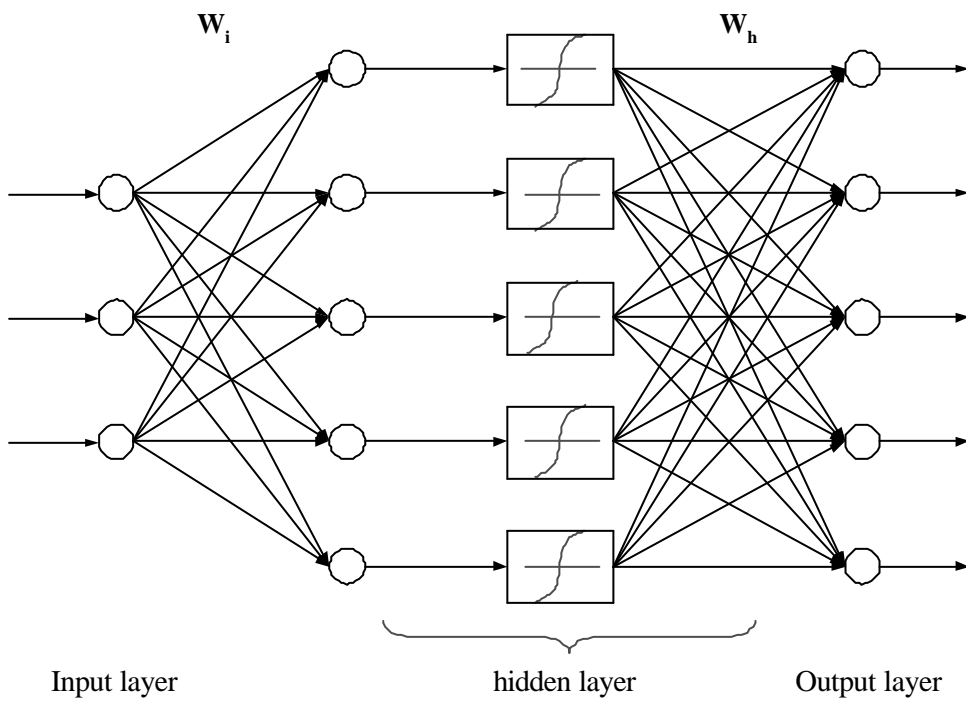


Figure 2.1. Typical structure of a multi-layer neural network

The brain basically learns from experience. Neural networks are sometimes called machine learning algorithms, because changing of its connection weights (training) causes the network to learn the solution to a problem. The strength of connection between the neurons is stored as a weight-value for the specific connection. The system learns new knowledge by adjusting these connection weights.

The learning ability of a neural network is determined by its architecture and by the algorithmic method chosen for training.

The training method usually consists of one of three schemes: Unsupervised learning, Reinforcement learning, Back propagation. Back propagation method is proven highly successful in training of multi-layered neural nets (27). The network is not just given reinforcement for how it is doing on a task. Information about errors is also filtered back through the system and is used to adjust the connections between the layers, thus improving performance. A form of supervised learning (3, 13, 14).

Principal Component Analysis (PCA) is a technique to find the directions in which a cloud of data points is stretched most. These directions represent most of the information in the data and are thus important to know. Knowing these directions allows us to store the data in a compressed form and later reconstruct the data with a minimal amount of distortion. PCA is used in statistics to extract the main relations in data of high dimensionality. A common way to find the Principal Components of a data set is by calculating the eigenvectors of the data correlation matrix. These vectors give the directions in which the data cloud is stretched most. The projections

of the data on the eigenvectors are the Principal Components. The corresponding eigenvalues give an indication of the amount of information the respective Principal Components represent. Principal Components corresponding to large eigenvalues represent much information in the data set and thus tell us much about the relations between the data points (19).

(A)



(B)

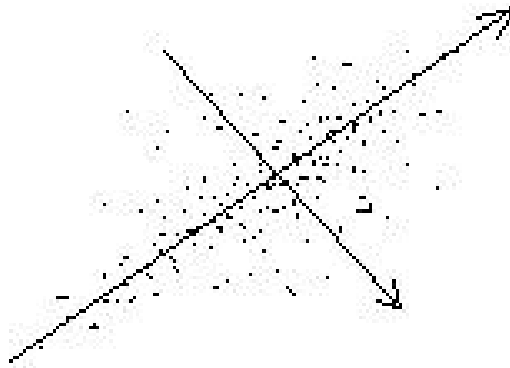


Figure 2.2. Data analysis using principal component analysis (PCA) ((A) measured data and (B) predicted data)

## 2.3 Mechanisms of Biological Nitrogen and Phosphorus Removal

*Alcaligenes faecalis*, commonly found in soil, water, and wastewater treatment systems, was dominant in piggery slurry treatment system. Since denitrifying bacteria were facultative aerobes, a sufficiently high concentration of DO prevents their use of  $\text{NO}_3\text{-N}$  as the terminal electron acceptor. In general, *Alcaligenes faecalis* was far less sensitive than nitrification bacteria *Nitrosomonas* sp. was, reducing or denitrify using nitrate such as electron accepter in anaerobic conditions. *Alcaligenes faecalis* can denitrify both under anaerobic conditions and nitrify under aerobic condition (2, 35). The possibility of aerobic denitrification by *Alcaligenes faecalis* implied that inhibition of denitrification by oxygen did not always occur and hence that nitrification and denitrification may take place simultaneously under aerobic condition. This strain has no nitrate reductases but is able to reduce nitrite to dinitrogen (34).

Figure 2.3 shows a process of amino acid synthesis and pathway of ammonium utilization by bacteria. The amino group of amino acids is often derived from some inorganic nitrogen source in the environment, such as ammonia ( $\text{NH}_3$ ). Ammonia is typically incorporated during the formation of the amino acids glutamate or glutamine by the enzymes glutamate dehydrogenase and glutamine synthetase, respectively (Figure 2.3 a, b). Here the ammonium can be incorporated into  $\alpha$ -ketoglutarate by the transamination reaction of glutamate dehydrogenase, generating glutamate. Glutamate is

subsequently transformed to glutamine by incorporation of ammonium via glutamine synthetase (30).

In addition, this ammonium removal mechanism could be more effective compared with ammonia oxidation bacteria. Therefore, this study focuses on the ammonium uptake and utilizing organisms in the treatment system and tries to elucidate the ammonium removal mechanisms using molecular biological techniques.

Phosphorus removal mechanisms can be affected by oxygen concentration. It is typical in the biological phosphorus removal process that the sludge releases  $P_i$  (with concomitant uptake of wastewater organic carbon) in the anaerobic phase and takes up  $P_i$  rapidly in the aerobic stage (16, 22). The P removed from wastewater is accumulated as a form of polyphosphate (poly P) in the sludge bacteria. Removal of a portion of the growing biomass (waste-activated sludge) results in the net removal of P from the wastewater (7).

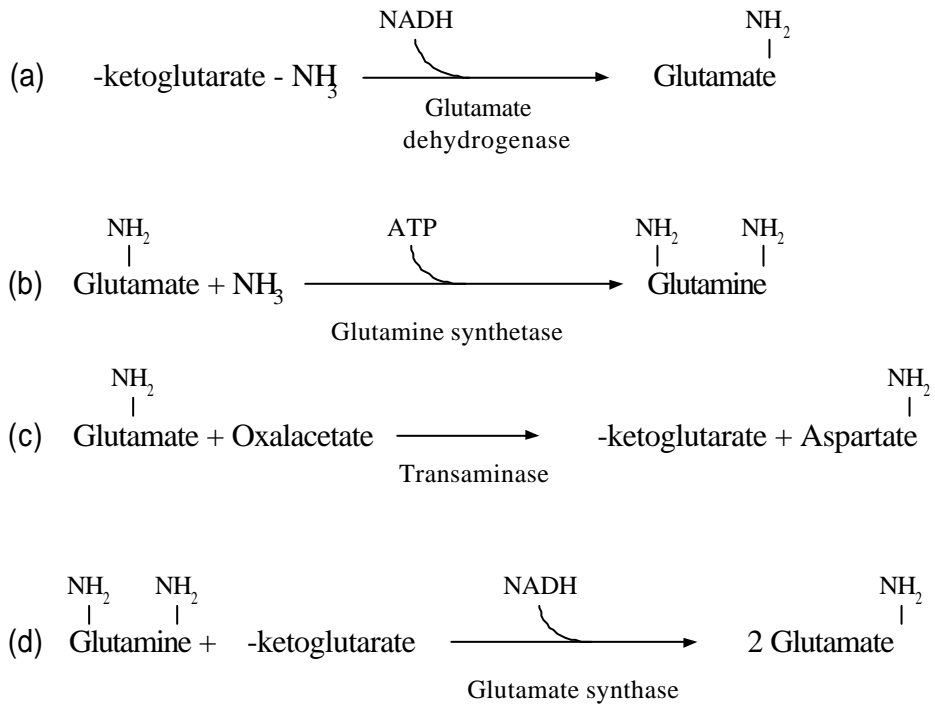


Figure 2.3. Ammonia incorporation in bacteria. Two major pathways for  $\text{NH}_3$  assimilation in bacteria are those catalyzed by the enzymes (a) glutamate dehydrogenase, and (b) glutamine synthetase. (c) Transaminase reactions simply transfer an amino group from an amino acid to an glutamate synthase, two glutamates are formed from one glutamine and one -ketoglutarate (30).

## **. MATERIALS and METHODS**

### **3.1 System Overview**

A scheme for the circulating treatment system at a pilot scale is shown in Figure 3.1. Detail description of the reactor operation has been shown in the previous report (41). Piggery slurry and treated effluent used as a washing water were collected in tank 1, and this influent then flows into the fermentation tank (tank 2; 15L). There is a channel between tank 2 and an aeration tank (tank 3; 15L) so that the fermented wastewater can be transported into tank 3 where oxidative treatment occurs under aeration condition (7.8v/v/m). The treated water then goes through sedimentation process at tanks 6 and 7, and finally is stored at tank 8. A portion of the effluent was used to wash the pigsty.

The wastewater used in this study was collected from a mixing and storage tank at Kimhae Piggery Slurry Treatment Plant (Kimhae, Kyungnam, Korea) and carried COD (*ca.* 4000 mg/L), BOD (*ca.* 7000 mg/L), T-N (*ca.* 2100 mg/L), and T-P (*ca.* 172 mg/L). The influent consisted of piggery slurry (33% v/v), effluent (57%) and tap water (10%) and was supplied every 4 days. Glucose was added to the formulated influent to make a C/N ratio as 100:15 (28) and a microbial agent was also added up to 1 %(w/v). The hydraulic retention time of the system was 4 days and it operated for 47 days.



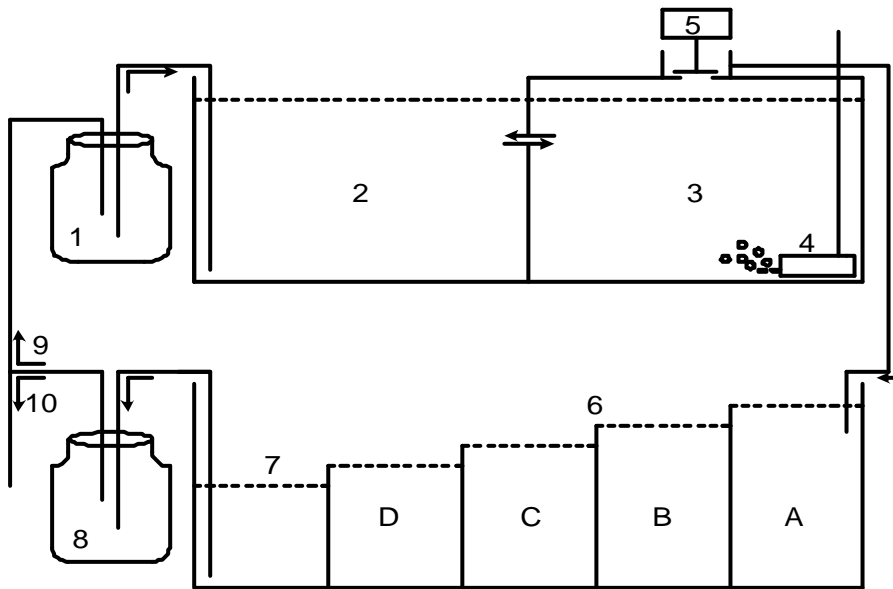


Figure 3.1. Schematic diagram of the circulating treatment system for piggery slurry. 1 influent; 2 fermentation tank; 3 aeration tank; 4 blower; 5 antifoaming device; 6 sediment tanks (A, B, C and D); 7 reservoir; 8 storage tank; 9 recycling flow; 10. for fertilizer).

### **3.2 Isolation, Identification and Quantification of Microorganisms**

Heterotrophic bacteria potentially involved in the piggery slurry treatment within the system were isolated using the appropriate media (23). To isolate and grow lactic acid bacteria (LAB), MRS medium were used. LAB were grown at least 2 weeks before identification and counting were performed. The ingredients of the medium were: Bacto proteose peptone NO. 3 5g/L, yeast extract 2.5g, dextrose 10g, Tween 80g,  $(\text{NH}_4)_2\text{HC}_6\text{H}_5\text{O}_7$  1g,  $\text{CH}_3\text{COONa}_3 \cdot \text{H}_2\text{O}$  4.14g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1g,  $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$  0.04g,  $\text{Na}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$  2.5g, Beef extract 10g; pH 6.5. After autoclaving trace amount of bromophenol blue was added as an indicator. Other heterotrophs were grown on TSA (Trypticase Soy Agar, Difco) at least 1 week, and then identified and counted.

The bacterial communities in the system were quantitatively analyzed based on isolation, identification and measuring their colony forming unit (population density) of dominant populations in each medium. Identification of the bacteria was performed based on cultural, physiological and biochemical characteristic described by Smibert et al. (40) and Holt et al. (17). Utilizations of sugar, amino acids and organic acids by Gram negative bacteria were tested using API Kit (bio Merieux sa, France) and its accompanied protocol.

### **3.3 Analytical Methods for Piggery Slurry samples from the Treatment System**

Monitoring parameters such as SS, T-N,  $\text{NH}_4^+\text{-N}$ , T-P, *o*-P and COD were measured for piggery slurry samples taken daily following Standard Methods for the Examination of Water and Wastewater (1): COD by closed reflux, titrimetric method, T-P and *ortho*-P by ascorbic acid method, suspended solids by total suspended solids gravimetric method, and  $\text{NH}_4^+\text{-N}$  by indole phenol method.

### **3.4 Modeling of the Treatment System Using Neural Networks**

For an optimal treatment of piggery slurry, it is important to understand the physiological characteristics of microorganisms and their relationships, but may be difficult to identify the complicated relationship by a linear analytical method. The relation between population densities of microorganisms and the treatment efficiency may have a nonlinear dynamic characteristic. In this study, we used a multi-layer neural network with error back propagation algorithm to model the complex relationship in the circulating system. Since the multi-layer neural network is able to approximate an arbitrary nonlinear function with sufficient input and output data, the modeling of the circulating piggery slurry treatment system can be realized by the neural network in complex dynamic systems. For modeling the circulating system, we considered cause and effect relation in each

tank that was serially connected. As independent parameters in each tank, population densities of heterotrophic microorganisms MRS-1, TSA-1, TSA-2, and TSA-3 were considered because those could dominantly affect the piggery slurry treatment efficiency. Also, COD, total-P, *ortho*-P, SS and  $\text{NH}_4^+$ -N were considered as treatment parameters in each tank. Thus, we designed multi-layer neural networks in which the input nodes consisted of 4 independent parameters in the current tank and 5 treatment results in previous tank, and the output nodes generated the 5 treatment results in the current tank.

For modeling the circulating system, there were two ways to use the neural network. One was to use a single neural network for modeling the characteristic of whole tanks in the circulating system. The other was to use the neural network for modeling the characteristic of each tank, and then serial connection of each neural network that modeled each tank could allow a monitoring of for the circulating system. In this study, it was difficult to model the overall characteristic of whole tanks by a neural network because each tank of treatment system has different role and characteristic. Thus, we used each neural network that was able to model the characteristic of each tank, and the overall model of the whole treatment system was obtained the connecting each neural network. Figure 3.2 showed a proposed modeling protocol for the circulating system. We used principal component analysis (PCA) as a preprocessor of the neural network. Input of the neural network was reduced to 3 principal values from 9 independent variables. The output values of the neural

network were COD, total-P (T-P), *o*-P, SS and  $\text{NH}_4^+\text{-N}$  in the current tank.

To accomplish a successful modeling, the connectivity within neural networks in the current tank were adjusted to best predict the measured values to be obtained at the next treatment step using SS,  $\text{NH}_4^+\text{-N}$ , T-P, *o*-P and COD as input variables.

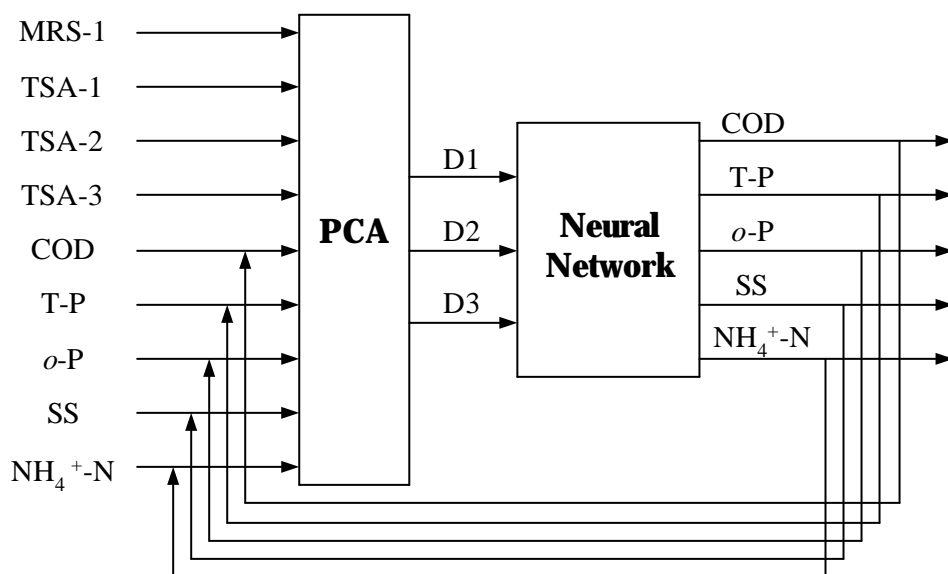


Figure 3.2. A schematic diagram describing training strategy for the neural networks in this study. MRS-1, TSA-1, TSA-2 and TSA-3 denote the population density of the bacterial strains. COD (chemical oxygen demand), T-P (total phosphorus), *o*-P (*ortho*-phosphorus), SS (suspended solid) and  $\text{NH}_4^+\text{-N}$  are parameters for the wastewater treatment. PCA, D1, D2 and D3 denote principal component analysis and dimensions obtained after the analysis, respectively.

### **3.5 Ammonium Uptake and Utilization Test**

Ability of the isolated heterotrophs to uptake ammonium ( $\text{NH}_4^+$ ) was measured to understand an ammonium removal mechanism in the treatment system. The dominant organisms (TSA-1, TSA-3 and TSA-4) were grown in the mineral salts medium (21) containing glucose (0.4% w/v or 3.2% w/v) as a sole carbon source. Unless the organisms were grown in the medium, they were subjected to growth at citrate mineral salts medium (34). Nitrogen source for these media was  $(\text{NH}_4)_2\text{SO}_4$ . The inoculated media were incubated at 26 °C and under rotary shaking (190rpm), and the growth was measured spectrophotometrically (525nm). The ammonium concentrations before inoculation and at stationary phase were measured and the ammonium removal efficiency was calculated.

### **3.6. Extraction of Total DNA**

Cells used for the ammonium removal test were collected by centrifugation and subjected to total DNA extraction that was performed according to Maniatis et al (38). The centrifuged cells were washed once with phosphate buffer and then resuspended in 0.6 ml of lysing buffer (0.15M NaCl, 0.1M EDTA, and 15 mg of lysozyme per ml). After incubation at 37 °C for 3 hr, 0.06 ml of 10% sodium dodecyl sulfate was added and the mixture was incubated at 65 °C for 10 min, and then -70 °C for 5 min. The freeze and thawing procedures were repeated twice. The mixture was then extracted

with phenol-chloroform three times and with chloroform once. The alcohol precipitated DNA was resuspended in TE buffer containing RNase A and incubated at 37 °C for 3 hr to remove residual RNA.

### **3.7. PCR Amplification of Glutamine synthetase Gene and Southern Blot Hybridization**

Degenerate oligonucleotide primers (forward primer GS-L and reverse primer GS-R) targeting glutamine synthetase genes from the isolated organisms were designed from the conserved GS protein sequences of *Bacillus* sp. including *Bacillus subtilis* 168 (KCTC 1326; ATCC 33234 Spizizen strain 168). The protein sequence alignment and analysis were accomplished using the sequence databases of Gene Bank and the Blast sequence analysis protocol available at National Center for Biotechnology Information (National Institute of Health). Their sequences were custom-synthesized by GenoTech (Taejon, Korea):

GS-L: 5' - GTG-AAG-TAT-ATC-CGY-CTT-C-3'

GS-R: 5' - ATA-YTG-WTC-GCG-YTC-CCA-3'

One to 3.3 ng of the extracted total DNA were used as a template. Positive control DNA was from *Bacillus subtilis* 168. The PCR procedures for this study were modified based upon the previous report (36). Each PCR reaction mixture (20  $\mu$ l) contained the following reagents: 10 X *Taq* buffer, MgCl<sub>2</sub> (1.5 mM), dNTPs (250

M, each), forward primer GS-L (10 pM), reverse primer GS-R (10 pM), *Taq* polymerase (1.25 U) (Promega). PCR was performed in a DNA thermocycler (Perkin Elmer model; GeneAmp PCR System 2400). The PCR conditions were denaturation (94 °C, 5min), 30 cycles of the standard PCR (94 °C 1 min; 50 °C 1 min; 72 °C 1 min), and a final chase reaction of (72 °C 5min).

Amplification products were electrophoresed on 1% agarose gel, and stained ethidium bromide. For Southern blots DNA was blotted from the gels to nylon membranes and cross linked to the membranes by dry to 80 °C, 1 hour. The expected PCR product (1269bp) from GS gene of *Bacillus subtilis* 168 was identified and nonradioactively labeled using Nonradioactive Labeling and Hybridization Kit (Boehringer Mannheim, Germany). A GS gene probe was prepared by gel-purified 1296bp GS amplification product from *Bacillus subtilis* 168 with the Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany). Membrane was hybridized overnight at 65 °C in 10% sodium dodecyl sulfate (SDS); 5× SSC; 0.1% N-laurylsarcosine; 1% blocking Agent and then washed twice for 15min each time at 65 °C in buffer contained 0.1× SSC; 0.1% SDS. All the following Southern hybridization procedures were done according to the previous report (21) except using CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-}(5'-chloro)tricyclo[3.3.1.1<sup>3,7</sup>]decan}-4-yl)phenyl phosphate) (Boehringer Mannheim) as a chemiluminescent substrate for the alkaline phosphatase.



## **. RESULTS and DISCUSSION**

### **4.1. Microbial Identification, and Analyses of the Population Dynamics and Piggery Slurry Treatment**

The most dominant heterotrophic bacteria in the treatment system were 4 aerobic bacteria and 3 lactic acid bacteria (LAB). The identified organisms were TSA-1 (*Brevundimonas diminuta*), TSA-2 (*Abiotrophia defectiva*), TSA-3 (*Alcaligenes faecalis*) and MRS-3 (*Streptococcus* sp) (Table 4.1, 4.2). Population dynamics of isolated bacteria were shown Figure 4.1, 4.2, 4.3.

One of the most dominant aerobes was *Alcaligenes faecalis* TSA-3. The most dominant species of LAB was strain MRS-1 that was yet to be characterized. Population dynamics of the representative aerobic bacterium *Alcaligenes faecalis* TSA-3 during the 47-day running period was shown for each tank (Figure 4.3). Interestingly, TSA-3 was a predominant species among aerobes in the aeration tank ( $10^7$ – $10^8$  (c.f.u./ml)) but was also observed in the influent and fermentation tanks (Figure 4.3). Thus the strain appeared to survive and grow under low oxygen tension and anoxic condition. A reported species of *Alcaligenes faecalis* could oxidize ammonia under aerobic condition and denitrify nitrate ions via NO and N<sub>2</sub>O gases under anoxic conditions (2, 35). *Alcaligenes faecalis* was found to accumulate NO<sub>2</sub><sup>-</sup> during exponential growth (34). Population of the strain MRS-1 was more dominant in the influent and fermentation

tanks than aeration and sedimentation tanks, indicating its facultative anaerobic characteristics. The overall population density was in the range of  $10^4$  -  $10^7$  (c.f.u./ml).

Table 4.1. Differential characteristics of the gram-negative bacterial species isolated from circulating treatment system

Characteristic	TSA1	<i>Brevundimonas</i> ** <i>diminuta</i>	TSA3	<i>Alcaligenes</i> ** <i>faecalis</i>
Gram staining	-	-	-	-
Cell shape	rod		rod	rod, coccal
Oxidase	-	+	+	+
Catalase	+	+	+	+
Anaerobic growth	-	-	-	-
*Acid from :				
D-Glucose	-	-	-	-
Mannitol	-		-	-
Inositol	-	-	-	
Salicin	-		-	
D-Melezitose	-		-	
L-Fucose	-		-	
D-Sorbose	-		-	
L-Arabinose	-		-	-
D-Ribose	-	-	-	
D-Sucrose	-	-	-	
Rhamnose	-	-	-	
Maltose	-		-	
*Utilization of :				
Valerate	+		+	+
Citrate	-		+	
2-Ketogluconate	-	-	-	
3-Hydroxybutyrate	+		+	
4-Hydroxybenzoate	-		-	
Itaconate	-		-	-
Suberate	-		-	-
Malonate	-	-	+	+
Acetate	+		+	
DL-Lactate	-		+	
5-Ketogluconate	-		-	
3-Hydroxybenzoate	-		-	
Glycogen	-		-	
*Decomposition of				
Histidine	-		+	
L-Proline	+		+	
L-Alanine	-		+	
L-Serine	-		-	

Symbols: +, 90% or more positive; -, 0-10% positive

\* Tested using API identification program (ID 32 GN: bio Merieux sa, France)

\*\* Data from Bergey's manual of Determinative Bacteriology (Ninth ed.)

Table 4.2. Differential characteristics of the gram-positive bacterial species isolated from circulating treatment system.

Characteristic	TSA2	<i>Abiotrophia defectiva</i> **	MRS3	<i>Enterococcus gallinarum</i> **
Gram staining	+	+	+	+
Cell shape	coccus	coccus	coccus	coccus
Oxidase	+	+	+	+
Catalase	-	-	-	-
Anaerobic growth	+	+	+	+
Motile	-	-	+	+
Hemolysis			-	,
Growth at				
10	+	ND	+	+
45	-	ND	+	+
pH9.6	+	ND	+	+
6.5% NaCl	-	ND	+	+
Voges-Proskauer	-	ND	-	ND
Acid from				
Xylose			+	+
Lactose	-	d	+	+
Sucrose			+	+
Rhamnose			-	-
Melezitose			-	-
Adonitol			-	-
Glycerol			+	+
Raffinose	+	d	-	+
Sorbitol	-	-	-	-
Salicin	+	+		
Trehalose	+	+		
Mannitol	-	-	+	+

Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative; , usually -hemolytic; , ,may be - or - hemolytic; d, 21-79% of strains are positive; ND, not determined.

\*\* Data from Bergey's manual of Determinative Bacteriology (Ninth ed.)

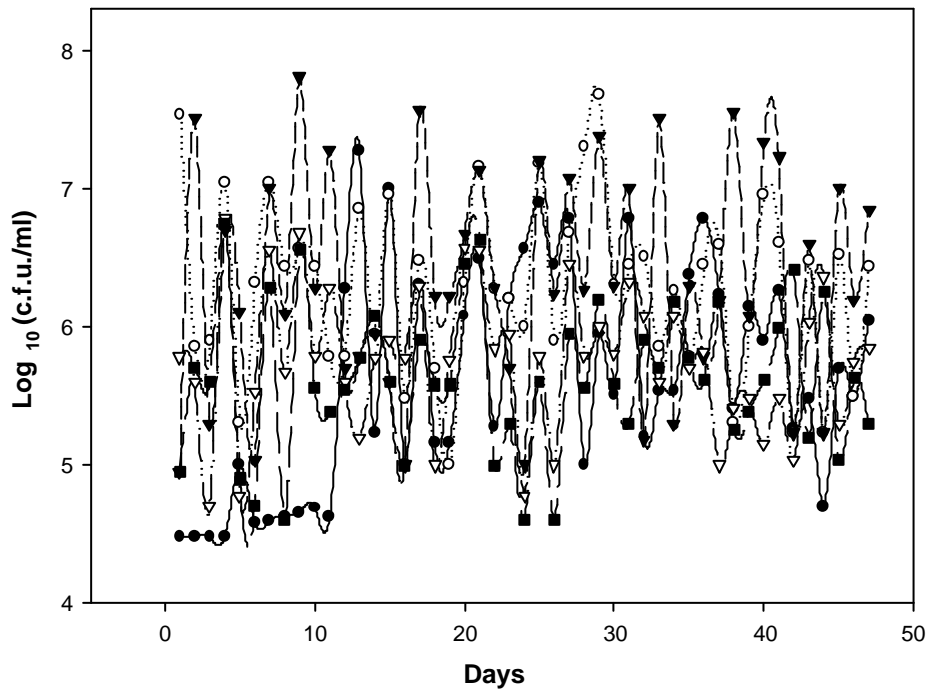


Figure 4.1. Population dynamics of a heterotrophic bacterium (*Brevundimonas diminuta*) in the circulating treatment system (●- Influent tank; ○- Fermentation tank; - Aeration tank; - Sedimentation tank A; - Sedimentation tank D)

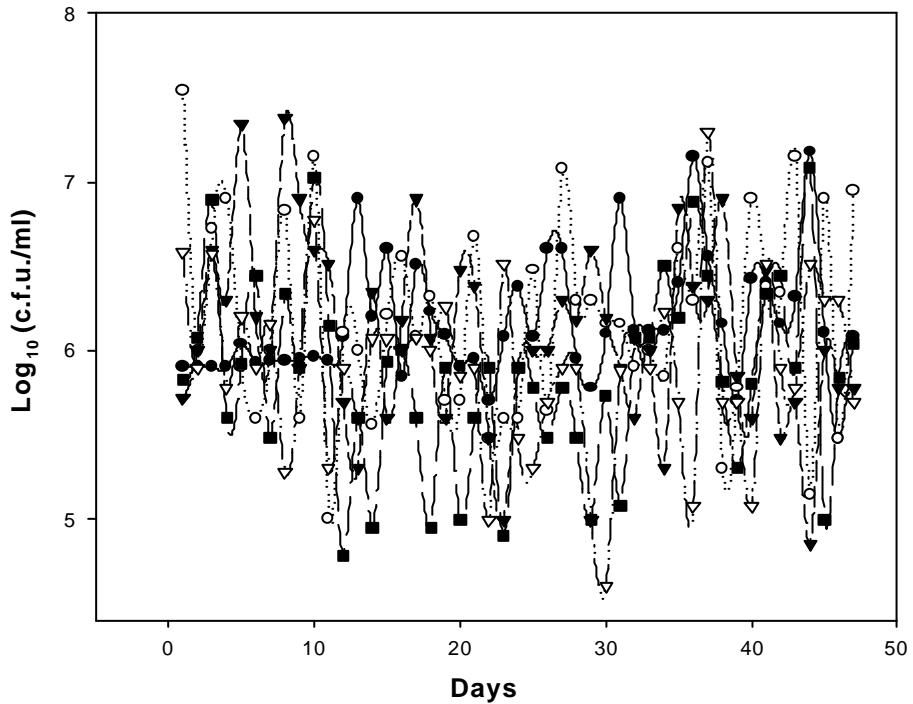


Figure 4.2. Population dynamics of a heterotrophic bacterium (*Abiotrophia defectiva*) in the circulating treatment system (●- Influent tank; ○- Fermentation tank; ■ - Aeration tank; △ - Sedimentation tank A; ▽ - Sedimentation tank D)

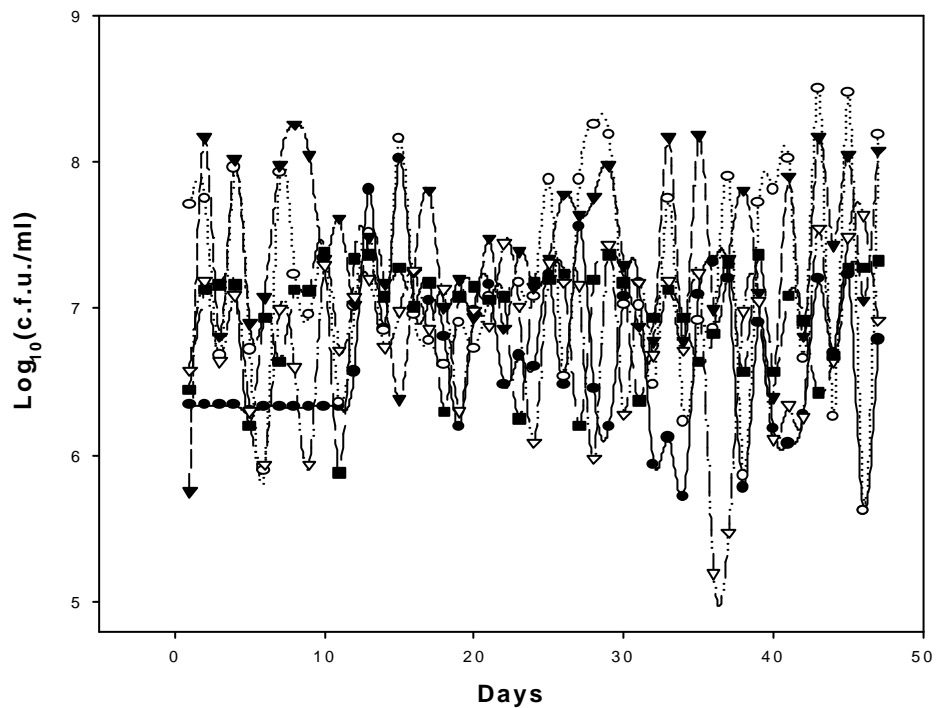


Figure 4.3. Population dynamics of a heterotrophic bacterium (*Alcaligenes faecalis*) in the circulating treatment system (●- Influent tank; ○- Fermentation tank; □ - Aeration tank; △ - Sedimentation tank A; ▽ - Sedimentation tank D)

## 4.2 Wastewater Treatment Efficiency of the Treatment System

The ammonium removal during the extent of the experiment is shown, for tank, in Figure 4.4. The ammonium removal efficiency reached 41% as a maximum (Table 4.3). The reason for this rather low efficiency was not clear but unbalanced (presumably, lower) C/N ratio would be one of the causes. Here, however, offensive smells appeared to be significantly reduced for the effluent.

The COD removal during the extent of the experiment is shown, for tank, in Figure 4.5. The overall COD treatment efficiency was about 54% (Table 4.3). The COD removal may be mostly accomplished by biological oxidation or absorption (or uptake) of organic compounds derived from livestock feeds that carried abundant carbonaceous, nitrogenous and phosphorus materials, since livestock wastewater contains generally little recalcitrant compounds.

The *ortho* or total phosphorus removal during the extent of the experiment is shown, for tank, in Figure 4.6, 4.7. The *ortho* or total phosphorus removal effect was also obvious in the aeration and sedimentation tanks (at least 40%). The possible mechanism for the phosphorus removal would be an uptake of phosphorus by cells under aerobic condition and a subsequent sedimentation of the cells. Surplus phosphorus to be uptaken may be transformed to poly-phosphorus as a storage material within the cells (21). A discharge of phosphorus is known to occur under anaerobic conditions (7, 37).



In this study the best removal effect of suspended solids (SS) (63%) was first observed in the aeration tank. This seemed to be due to a transport hole between fermentation and aeration tanks, which screened out most of the sedimented solids. The suspended solids (SS) removal during the extent of the experiment is shown, for tank, in Figure 4.8.

Table 4.3. Wastewater treatment efficiency of the piggery slurry treatment system

Parameter	Efficiency (%)	Efficiency for 40 days				
		Influent (Mg/ )	Fermentation tank % (Mg/ )	Aeration tank % (Mg/ )	Sedim. tank-1 % (Mg/ )	Sedim. tank-4 % (Mg/ )
COD	54	3341	18.5 (2723.76)	45.5 (1819.5)	51.1 (1634.2)	56 (1461)
SS	65.5	0.54	25.9 (0.4)	63 (0.2)	63 (0.2)	63 (0.2)
T-P	42	45.24	17.5 (37.3)	45.2 (24.8)	46.3 (24.3)	46 (24.5)
<i>Ortho</i> -P	33	43	18.8 (34.9)	39.5 (26)	39.1 (26.2)	38 (26.8)
NH <sub>4</sub> <sup>+</sup> -N	39	1431	26.4 (0.4)	33.5 (952)	37.8 (890)	41 (848)

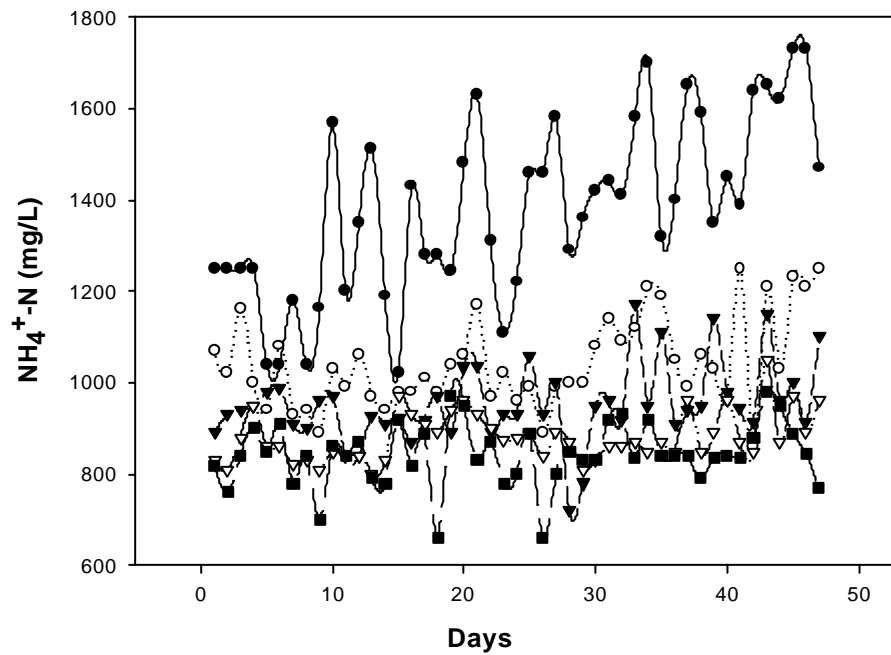


Figure 4.4. Dynamics of ammonium removal in the circulating treatment system (●- Influent tank; ○- Fermentation tank; △ - Aeration tank; ■ - Sedimentation tank A; ▽ - Sedimentation tank D)

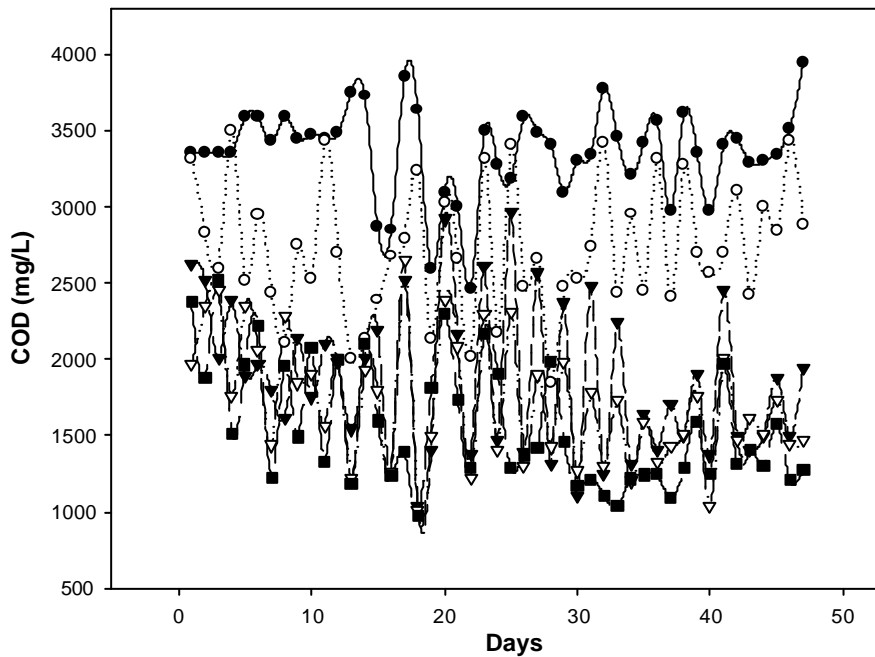


Figure 4.5. Dynamics of chemical oxygen demand (COD) removal in the circulating treatment system (●- Influent tank; ○- Fermentation tank; - Aeration tank; - Sedimentation tank A; - Sedimentation tank D)

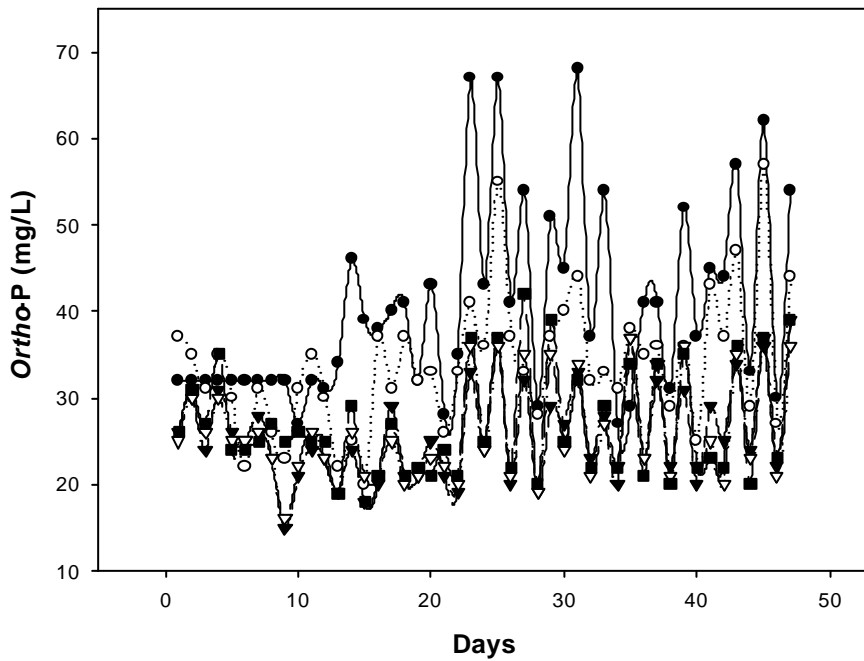


Figure 4.6. Dynamics of *ortho*-phosphorus removal in the circulating treatment system (●- Influent tank; ○- Fermentation tank; ■ - Aeration tank; △ - Sedimentation tank A; ▽ - Sedimentation tank D)

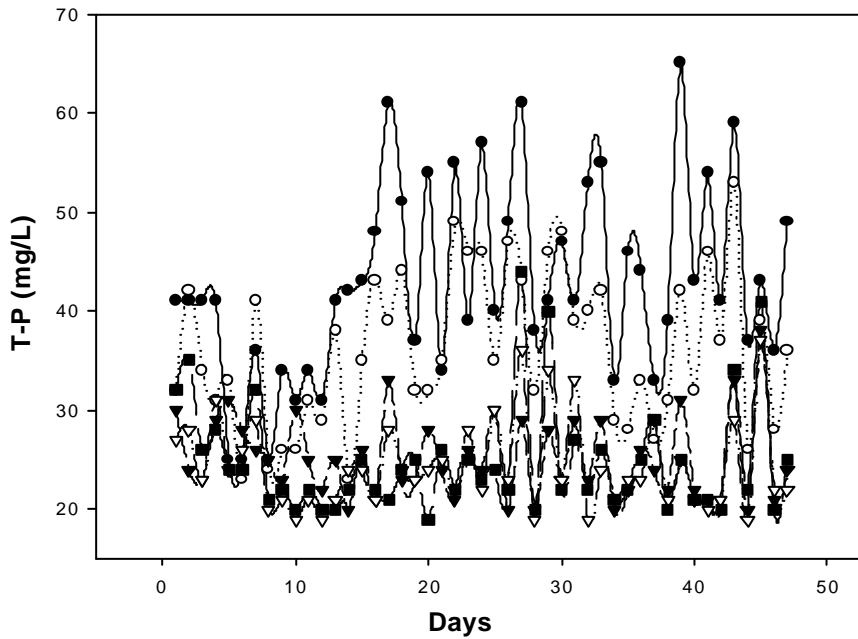


Figure 4.7. Dynamics of total phosphorus removal in the circulating treatment system (●- Influent tank; ○- Fermentation tank; ■ - Aeration tank; ▲ - Sedimentation tank A; △ - Sedimentation tank D)

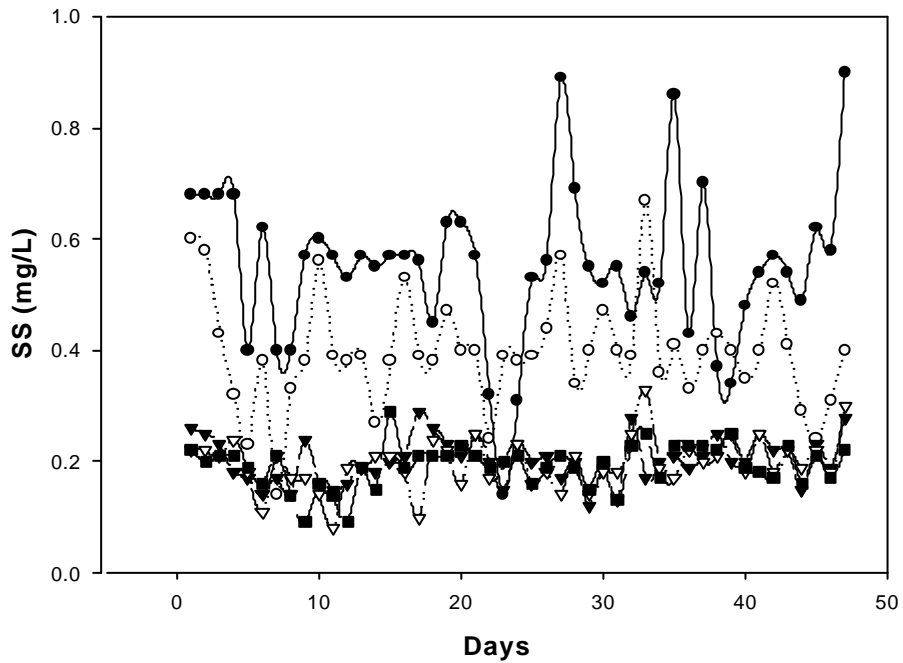


Figure 4.8 Dynamics of suspended solid removal in the circulating treatment system (●- Influent tank; ○- Fermentation tank; ■ - Aeration tank; △ - Sedimentation tank A; ▽ - Sedimentation tank D)

### **4.3 Principal Component Analysis of Input Data**

The neural networks can estimate output about the ignorant input data that were not used to learning neural network. This is called the generalization capability and important characteristic of neural networks. For the effective generalization, the number of training data must be more than 100 times the number of input dimension, at least. But it was rather difficult to obtain enough training data because the biological process by microorganisms usually takes long time to reach the stable treatment efficiency. Moreover, the input and output dimensions of the neural networks was 9 and 5, respectively. Training data measured for 47 days, were not enough to figure out the complex correlation between input and output in each tank, and also it was a little hard to expect a generalization. Moreover, there were some noises in data due to a measuring error or unstable bioprocess. In order to reduce the input and output dimensions and also remove the noisy data, we first used the principal component analysis (PCA) method to analyze the training data. PCA projects high dimensional data onto low dimensional coordinates that consist of principal component axes. In other words, PCA finds the first component that can best express variation for high dimensional data and other orthogonal component of first component and represents high dimensional data on the new orthogonal coordinates with low dimensional ones by projection. PCA method is applied to analysis statistical data because the projected data on 2 or 3 dimensional coordinates are visual and can be classified easily.

To find new orthogonal coordinates for the measured data, we should get eigenvalues from correlation matrix of the measured data, and then selects a few eigenvalues after ordering in magnitude. Finally the new orthogonal coordinates become eigenvectors for the selected eigenvalues. Appearing the values to be represented by eigenvectors on planes makes easy to understand the correlation of each high dimensional data. If  $N$  dimension of the training data can be expressed by vector  $X$  and also  $C$ ,  $N \times N$  correlation matrix is given as following.

$$C = \langle X^T X \rangle \quad (1)$$

Where  $X$  is  $N$  dimension input vector and  $\langle \rangle$  means expectation. The eigenvalue and eigenvector of this correlation matrix  $C$  can be obtained by following equations.

$$CX = \lambda X \quad (2)$$

$$\det(\lambda I - C) = 0 \quad (3)$$

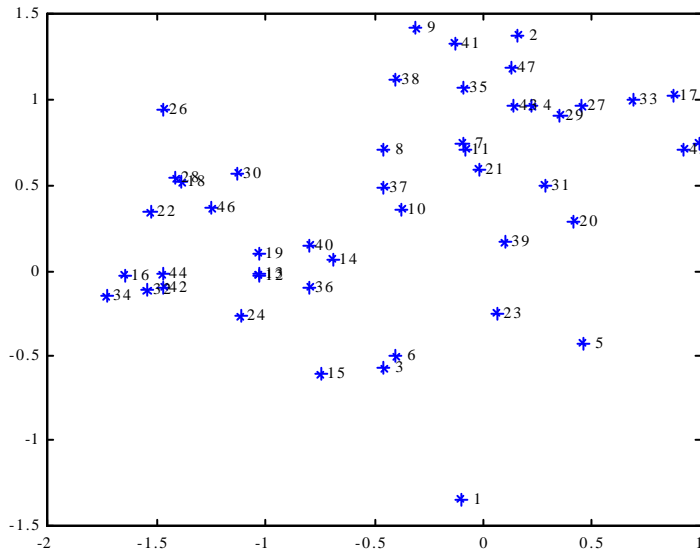
Where  $\lambda$  is eigenvalue and  $X$  means eigenvector of  $C$  corresponding to  $\lambda$ . Principal components are obtained by taking eigenvectors of a few eigenvalues whose magnitudes are more than any other eigenvalues and the projected data onto the low dimension coordinates



are given by inner production with these eigenvectors.

In this study, we used three axes as orthogonal coordinates. These axes were obtained by analysis of the PCA analysis to remove the data with one-to-many mapping that gives different output of the same inputs. Figure 4.9 showed the PCA results for the measured data in aeration tank. The X-axis denoted the mapping result by the first eigenvector, and Y-axis denoted the result by the second eigenvector. The number of each point in Figure 4.9 (A) indicates the passage from starting day. Figure 4.9 (B) showed the PCA results for target data measured in sedimentation tank 1, and each axis was same with that of Figure 4.9 (A). As shown in Figure 4.9, there were several data with one-to-many mapping property but we removed these values in the training process.

(A)



(B)

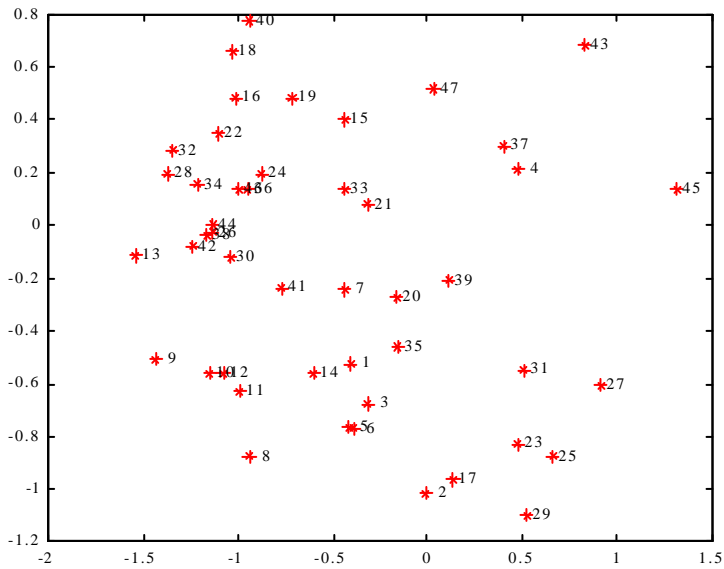


Figure 4.9. Principal component analysis of input (A) output (B) of aeration tank data during the 47 days' running period

## 4.4 Modeling of Treatment System by Neural Networks

Among 47 training data, we reversed the 6th, 11th, 16th, 21st, 26th, 31st, 36th, 41st, 46th, 47th data for training phase, which were randomly selected and used as test data to evaluate the generalization performance of the neural network. The neural network has one hidden layer with 30 nodes that were determined by an *ad hoc* method and nonlinear function of each layer except that input layer has a sigmoid. The weight values were adjusted by error back-propagation algorithm.

Through computational experiment we could assure that the learned neural network successfully imitated each tank of treatment system and approximated the target values of the input pattern well. Figure 4.10 showed the graphic estimations of COD,  $\text{NH}_4^+\text{-N}$ , *o*-P and SS values based upon the neural network analysis. The X-axis represented the tanks from 46th days influent tank to 47th days sedimentation tank 2. As shown in Figure 4.10, the proposed neural network could successfully monitor the treatment results according to the population densities of microorganisms. A dramatic increase of the measured SS in the influent at 47th day (Figure 4.10 D) was an outlier due to a sampling error.

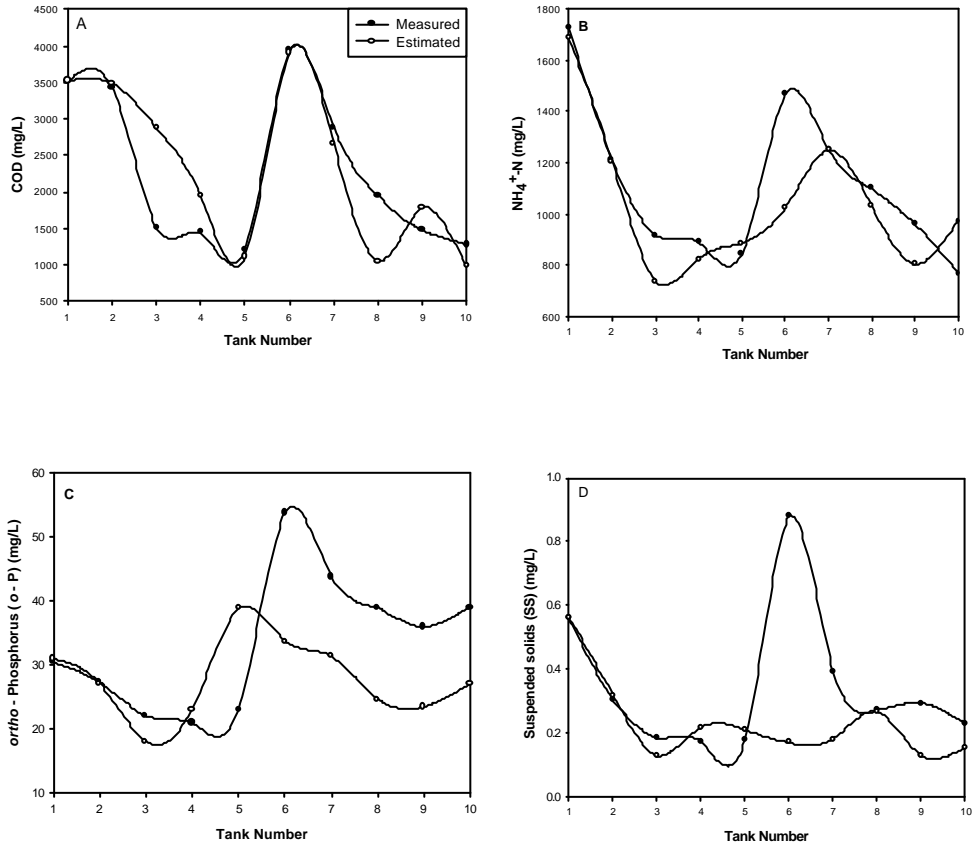


Figure 4.10. Prediction of various treatment parameters COD (A),  $\text{NH}_4^+\text{-N}$  (B), *ortho*-P (C) and SS (D) by the neural network modeling. COD and  $\text{NH}_4^+\text{-N}$  data were cited from reference 25. The serial numbers in X-axis indicate samples taken from tanks of influent, fermentation, aeration, sedimentation-1 and sedimentation-4 at 46 and 47 day's running in order, respectively.

## 4.5 Molecular Analysis of Ammonium Removal

The isolated heterotrophs TSA-1 (*Brevundimonas diminuta*), TSA-3 (*Alcaligenes faecalis*), TSA-4 (not identified) were tested for their ammonium uptake. These three strains could utilize  $(\text{NH}_4)_2\text{SO}_4$  as a sole nitrogen source for their growth. Ammonium appeared to be almost utilized since little ammonium was detected at the stationary phase. Phosphorus removal efficiency was observed up to 60%.  $\text{NH}_4^+$ -N and *ortho*-phosphorus utilization rates appeared to be species or strain specific. This indicates a direct utilization of  $\text{NH}_4^+$  by a heterotroph and hence removal of nitrogen from the system by circumventing nitrification process that is energy and oxygen consuming pathway. It, therefore, appeared that the ammonium uptake and utilization could contribute to the nitrogen removal in the treatment system (particularly aeration tank).

Amplifications of GS gene with the GS-L and GS-R primers from the isolated heterotrophic bacteria were performed. The GS gene product amplified from *Bacillus subtilis* 168 could hybridize with one of the PCR products from the isolated bacteria (Figure 4.11). Therefore, the presence of ability of ammonium utilization and GS gene in the heterotrophs isolated from the treatment system indicates the possibility that ammonium removal in the system occurs via GS system of these organisms being involved in amino acid synthesis.

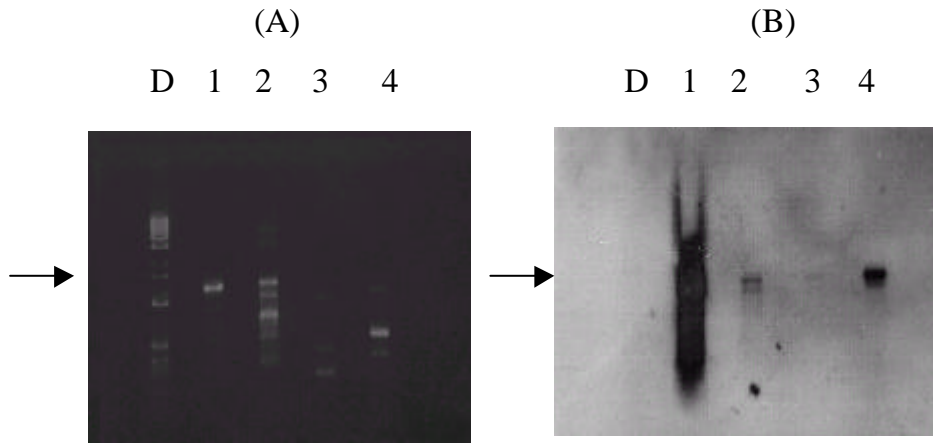


Figure 4.11. Amplification of glutamine synthetase (GS) gene using GS-L and GS-R primers from the total DNA of heterotrophic bacteria (A) and southern hybridization with the PCR products using the putative GS gene product (1269 bp indicated by arrow heads) of *Bacillus subtilis* 168 (B) as a DNA probe. Hybridization and washing were done under a stringent condition (65 °C). D: DNA 1kb ladder; 1 *Bacillus subtilis* 168; 2 *Alcaligenes faecalis* TSA-3; 3 *Brevundimonas diminuta* TSA-1; 4 TSA-4

## . Conclusion

In this study a novel monitoring system of piggery slurry circulating treatment system has been proposed. Multi-layer neural networks combined with PCA successfully modeled the tank characteristics. It was possible to train the neural network with the given data by reducing the input dimension with minimal loss of information and removing the noisy data with one-to-many mapping property. The proposed model may be useful to develop a reverse neural network model that could be used to determine optimal microbial densities critical for a desired quality level of the treated wastewater.

The following conclusions can be drawn based on the results from this study:

1. *Alcaligenes faecalis* appeared to survive and grow under low oxygen tension and anoxic condition and may oxidize ammonia under aerobic condition resulting in the ammonium removal.
2. LAB were dominantly observed in anoxic condition, indicating the BOD removal under anaerobic condition.
3. Multi-layer neural network combined with PCA successfully modeled the treatment system using relatively small amount of data.
4. The proposed model may be useful to develop a reverse neural network model that could be used to determine optimal microbial

densities critical for a desired quality level of the treated wastewater.

5. A significant removal of ammonium may be attributed to an uptake by glutamine synthetase.



## **. FUTURE RESEARCH**

The following needs to be considered for a further research:

1. The C/N ratio could be adjusted to increase the treatment efficiency.
2. The application of reverse neural network modeling to the system will allow an elucidation of optimal microbial species and population densities to optimize the treatment system.
3. Biosensors and chemical sensors will be highly useful for the collection and analysis of data.
4. Molecular sensors such as gene chips for heterotrophic and autotrophic ammonium oxidizers and other degraders will be quite useful for a rapid and sensitive monitoring of the treatment system.
5. Transformation of nitrate to ammonium and its removal under anoxic condition.

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# CURRICULUM VITAE

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## Publications

### 1. Thesis:

Neural Networks and Molecular Analyses of Circulating Piggery Slurry Treatment System, Korea Maritime University, 2001.

### 2. Journal Papers:

1. J. I. Sohn, M. Lee, J. H. Choi and S. C. Koh (2000) Monitoring of recycling treatment system for piggery slurry using neural networks. Journal of the Korean Sensors Society. Vol. 9 No. 2:127-133.

2. Jung-Hye Choi, Jun-Il Shon, Hyun-Sook Yang, Young-Ryun Chung, Minhoo Lee, Sung-Cheol Koh. (2000) Modeling of recycling oxic and anoxic treatment system for swine wastewater using neural networks. *Biotechnol. Bioprocess Eng.* Vol. 5 No. 5:355-361.
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### **3. Conference papers:**

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