Doctoral Dissertation

Construction of a new water treatment system based on material circulation for static water environment

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Construction of a new water treatment system based on material circulation for static water environment
(止水域における物質循環を基とした新規水処理システムの構築)

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General introduction

The study of water treatment systems which are able to improve water quality of aquatic environments earns the attention of many scientists in industrial biotechnology. The establishment of water treatment system approaches are limited, involving several concepts to purify water with various aspects. The water treatment system is applied for many purposes, such as: supply for agricultural activity [1] [2], drinking water industry [3] [4] and the necessity for ecological protection [5]. The high demand on the effective and efficient water treatment system attracts many scientists to develop new effective water treatment system. Several approaches have conducted to provide water treatment systems with effective results, but most of them are running in complicated systems and/or require high energy. Moreover, some of them are not suitable to be applied in a large scale and natural water environments.

During the past decades, research on water treatment system is developing including construction of the new concepts, applications, and developments to create the most effective system. High effectivity to purify water from various contaminants such as organic materials [6], inorganic materials [7], suspended solid materials [8], chemicals, microorganisms and other substances is the main consideration. In case of water treatment system for drinking water, suspended solid materials and microorganisms gives negative impact on human health, but these pollutants can be eliminated for other purposes. In early 1970, several technologies such as aeration and flocculation are established to purify waste

water from human activities including industry and agriculture. Then, the concept moved to the use of membrane technology in 1980s to improve the quality of treated water.

However, waste water treatment systems using techniques such as aeration and flocculation or activated sludge require high energy. The utilization of this facilities in US consumes 2% of the total amount of electricity produced in US. Moreover, approximately 1,100 to 2,400 MJ of electricity are required to process 1000 L of water during operation of activated sludge technology. Aeration is the main treatment that need high energy in a water treatment system. Similar to activated sludge, membrane filtration technology shows inefficient results because of contaminants backwashing process. Both activated sludge and membrane filtration technology are less efficient to be applied in a large scale and natural environments. A simple and less-energy required water treatment system is necessary to be constructed for small scale, large scale, and for natural aquatic environments.

In this study, a new water treatment system was constructed based on material circulation to purify water of a natural static water environment and evaluate the efficiency of the system in a small-scale experiment. After being constructed, the new water treatment system was applied in a real static water environment (pond). The efficiency of the system was analyzed by observing the ability of system to purify the pond water for 2 years of experiment. An investigation on the sediment properties of southern and northern Lake Biwa was carried out to know the current environmental conditions of those area, and compared with the environmental condition of paddy field and Kitanoshin pond. This study is divided into 3 chapters.

In Chapter 1, a new water treatment system was constructed based on material circulation in a small-scale experiment (200 L). This system used a water circulation to allows continuous contact between organic materials and environmental bacteria in a set of microbial columns, called as 3-columns unit and 6-columns unit. The microbial columns were created based on different water flow rates in the column: faster water flow rate in the 3-columns unit and slower water flow rate in the 6-columns unit. To evaluate the efficiency of the system, COD, TC, and TN removal rates were analyzed during one year of treatment.

In Chapter 2, the new water treatment system was applied in a real static water environment (pond). This study was aimed to evaluate the efficiency of the new water treatment system in the real natural pond. Similar to the first study, 3-columns unit and 6-columns unit were used to treat water. Then, a water pump with the flow rate of 1.15×10^6 L/day was used to circulate the water in the pond and activate the environmental bacteria in the pond. To evaluate the efficiency of the system, DO, COD, TC, TN, turbidity, colority, pH and EC of the water were analyzed every week, while TC, TN, TP, TK, total bacterial number, pH, and EC of the sediment were analyzed every 3 months for 2 years of treatment.

In Chapter 3, an investigation on the sediment properties of several aquatic environments was carried out. This study was aimed to compare the environmental conditions of the sediment in the southern and northern parts of Lake Biwa, and to compare with the condition of Kitanoshin pond which was equipped with the new water treatment system. The environmental condition in Lake Biwa was also compared with the sediment of paddy field based on Soil Fertility Index (SOFIX) to observe their current conditions.

Chapter 1

Construction of a new water treatment system based on material circulation

1.1 Introduction

Oceans and rivers have a self-purification process that occurs through water circulation to maintain the organic matter load at a low level [9]. Through this process, organic materials in the water that are derived from anthropogenic activities [10] [11] and natural sources [12] [13] are decomposed to CO_2 by aerobic bacteria (genus *Bacillus*, *Pseudomonas*, *etc.*) [14] [15], while nitrogen is removed through denitrification via microbial conversion of nitrate to N_2 by α, β, and γ proteobacteria [16] [17].

However, limited water flow in lakes and ponds often causes inefficient self-purification [18]. Several experiments have attempted to solve this problem through various methods, such as the use of activated sludge, which supplies air for aerobic microorganisms to form a bacterial consortium to purify the water [19] [20], but such systems require high energy [21]. Slow-water circulation can be used to reduce carbon and nitrogen in water by allowing continuous contact between organic materials and environmental bacteria [22]. In addition, efficient water purification requires activation of both aerobic and anaerobic bacteria to decompose organic materials in the aquatic environment [23].

This chapter describes the development and construction of a new water treatment system based on material circulation by environmental microorganisms to purify water in a static water environment.

1.2 Materials and methods

1.2.1 Sampling site and water sample

The water sample used in this study was taken from a small pond (Kitanoshin pond) located in Kusatsu, Shiga, Japan (34°98' N, 135°96' E). A 200 L water sample was taken from the pond every 14 days from 16 April 2014 to 16 February 2015.

1.2.2 Construction of a new water treatment system based on material circulation

Before construction of the new water treatment system, the efficiencies of various treatments were evaluated in a preliminary experiment (20 L water tank). The treatments used were: water circulation with faster water flow rate columns (3-columns unit) and slower water flow rate columns (6-columns unit), circulation with 3-columns unit only, circulation with 6-columns unit only, circulation only, and control (without any treatment). The columns were filled with polyvinylalcohol (PVA) sponge and the chemical oxygen demand (COD) removal rate was used to evaluate the efficiency of each treatment. The preliminary experiment showed that a combination of water circulation with 3-columns unit and 6-columns unit was most efficient (COD removal rate > 70%). A new water treatment system was then constructed using a combination of water circulation with 3-columns unit and 6-columns unit in a 200 L water tank.

1.2.3 Experimental period of the water treatment system

The efficiency of the new water treatment system was evaluated by measuring COD, total carbon (TC), and total nitrogen (TN) in a 200 L experimental tank. The measurement

was performed from April 2014 to February 2015 throughout the spring (March to May), summer (June to August), autumn (September to November), and winter (December to February). The water was treated in the experimental tank for 14 days, after which it was exchanged with fresh water from the pond.

1.2.4 Analysis of water quality

Water quality parameters (COD, TC, and TN) were analyzed during treatment. The COD was analyzed using the permanganate based titrimetric method [24]. Briefly, a 100 mL aliquot, 10 mL of diluted H_2SO_4 (48%), 10 mL of 5 mM $KMnO_4$, and 0.2 g of Ag_2SO_4 were mixed in an Erlenmeyer flask before digestion under heating at 90°C. Next, 10 mL of 12.5 mM $Na_2C_2O_4$ were added to the flask and mixed well. Finally, the remaining $Na_2C_2O_4$ from the solution was determined by titration with 5 mM $KMnO_4$ until pink color is observed. The COD was then calculated by the formula:

COD (mg/L) = (mL sample titration – mL blank titration) × 1 × dilution × 0.2

where 1 is the $KMnO_4$ factor in 5 M and 0.2 is the amount of oxygen in 5 M $KMnO_4$. The COD removal rates were obtained through the formula:

$$\text{COD removal rate (\%)} = \frac{\text{COD}_0 - \text{COD}_{14}}{\text{COD}_0} \times 100$$

where COD_0 is the value of COD before treatment and COD_{14} is the value of COD after treatment.

The TC and TN were measured using a TOC and TN analyzer (Shimadzu Corporation, Kyoto, Japan) and solid sample combustion unit (Shimadzu Corporation,

Kyoto, Japan). Sodium hydrogen phthalate was used to make the standard of TC. A 2.125 g of sodium hydrogen phthalate was diluted to 1,000 mL of distilled water to obtain the stock of carbon standard solution with concentration 1,000 mg/L. Serial dilution of 0, 1, 10, and 100 mg/L was made from the standard solution stock to provide the linear regression. To make the TN standard with concentration 1,000 mg/L, a 7.219 g of potassium nitrate was diluted in 1,000 mL of distilled water. After that, the stock was diluted to 0, 1, 10, and 100 mg/L to create the linear regression.

1.2.5 Estimation of total bacteria

The number of total bacteria in the microbial columns was measured from a sample collected from the PVA sponge filled in the 3-columns unit and 6-columns unit. Briefly, the slow-stirring method was used to extract the environmental DNA (eDNA) of bacteria [25]. Next, 1.0 g of wet PVA was mixed with 8 mL of DNA extraction buffer (Table 1) and 1.0 mL 20% (w/v) sodium dodecyl sulfate (SDS) solution. The suspension was then mixed using a propeller (1,500 rpm) under constant agitation for 20 min at room temperature before centrifugation at 6,000 × g for 10 min. Next, 700 µL of supernatant was transferred into a 1.5 mL microtube, mixed with chloroform-isoamylalcohol mixture (24:1 (v/v)) in equal volume, and centrifuged at 18,000 × g for 10 min. The aqueous phase (500 µL) was subsequently mixed with 300 µL of isopropanol to precipitate the pellet of crude nucleic acids by centrifuging the mixture at 18,000 × g for 20 min. After drying, the pellet was rinsed with 1 mL of 70% ethanol and dissolved in 50 µL of 1× TE buffer (Tris/EDTA = 10:1 mM). The extracted eDNA was quantified based on the intensity of eDNA bands

generated during 1% agarose gel electrophoresis using the KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company, CT, USA).

Table 1. Composition of DNA extraction buffer (pH 8.0)

Reagent	Concentration (g/L)
Ethylenediaminetetraacetic acid disodium salt dihydrate	37.22
Hexadecyltrimethylammonium bromide	10.00
Sodium chloride	87.66
Sodium dihydrogen phosphate	12.00
Tris(hydroxymethyl)aminomethane	12.11

1.2.6 Amplification of 16*S* rRNA bacterial gene

The 16*S* rRNA bacterial gene was amplified using primers DGGE-F (5'-CGCCC GCCGC GCCCC GCGCC CGTCC CGCCG CCCCC GCCCG CCTAC GGGAG GCAGC AG-3') and DGGE-R (5'-CCGTC AATTC CTTTG AGTTT-3') [26]. The amplification reaction was carried out in a 50 µL PCR mixture containing 0.01 ng/µL of DNA template, 1.5 U rTaq DNA polymerase, 5.0 µL of 10× buffer, 5.0 µL of 2 mM dNTPs, and 1.0 µL of 10 mM of each primer as listed in Table 2. DNA polymerase, dNTPs and PCR buffer were purchased from Toyobo (Toyobo Co. LTD, Osaka, Japan), while all primers were synthesized by Sigma-Aldrich (Sigma-Aldrich Co. LLC, Tokyo, Japan). The thermal PCR profile was as follows: initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 30 sec, and extension at 72°C for 1 min and then final extension at 72°C for 5 min (Table 3). Finally, the amplified 16*S*

rRNA bacterial genes were used for denaturing gradient gel electrophoresis (DGGE) analysis.

Table 2. PCR mixture

Reagent	Quantity (µL)
rTAQ	0.2
rTAQ Buffer	5.0
dNTPs	5.0
Forward primer	1.0
Reverse primer	1.0
Milli Q water	32.8
DNA template	5.0
Total volume	50.0

Table 3. Thermal profile for amplification of 16*S* rRNA gene

Step	PCR condition	Cycle
Initial denaturation	95ºC, 1 min	1
Denaturation	95°C, 1 min	35
Annealing	55°C, 30 sec	35
Extension	72°C, 1 min	35
Final extension	72°C, 5 min	1

1.2.7 DGGE analysis

DGGE was performed using a D Code System (BioRad Laboratories Inc., California, USA). A total of 20 µL of PCR product was loaded into 40% (w/v)

polyacrylamide gel with a denaturant gradient of 27.5% - 67.5% (Table 4 and 5, respectively). The gel was the run in 1× TAE buffer (40 mM Tris (pH 7.6), 20 mM acetic acid, and 1 mM EDTA) (Table 6) at a constant voltage of 70 V at 60°C for 15 h. Next, the gel was stained using SYBR Gold for 20 min, then rinsed with distilled water. Cluster analysis of the DGGE band pattern was subsequently conducted using the FPquest Bioinformatics Software (BioRad Laboratories Inc., California, USA).

Table 4. Composition of the low denaturant (27.5%)

Reagent	Quantity
Urea	11.55 g
Formamide	11.00 mL
50× TAE	2.00 mL
40% Acrylamide	20.00 mL
Total volume	100.00 mL

Table 5. Composition of the high denaturant (67.5%)

Reagent	Quantity
Urea	28.35 g
Formamide	27.00 mL
50× TAE	2.00 mL
40% Acrylamide	20.00 mL
Total volume	100.00 mL

Table 6. Composition of 1× TAE Buffer for DGGE

Reagent	Concentration
Tris(hydroxymethyl)aminomethane (pH 7.6)	40 mM
Acetic acid	20 mM
EDTA	1 mM

1.2.8 Analysis of carbon and nitrogen in a fish-cultivated environment using the new water treatment system

The new water treatment system was evaluated in water containing high levels of organic materials in a fish tank. A tank containing 200 L of tap water, 20 kg of sand sediment, and 12 goldfish maintained at room temperature (25°C) was used in the experiment. The experiment was divided into two stages: before operation of the microbial columns (stage I) and after the operation of the microbial columns (stage II). Stage I was conducted for 21 weeks, and stage II for 18 weeks. Fish were fed with 0.5 g fish food every day. Composition of TC and TN in the fish food is listed in Table 7. Thus, the total carbon input during the experiment was 40,900 mg (22,050 mg in stage I and 18,900 mg in stage II), and the nitrogen input was 6,140 mg, with 3,310 mg in stage I and 2,830 in stage II. TC and TN input from the fish food is shown in Table 8. The amounts of TC and TN (in 200 L of water and 20 kg of sediment) at the beginning and at the end of stage I and stage II were measured to analyze their mass balance (input and accumulation). The differences in TC and TN accumulation in CO_2/N_2 gas and goldfish between stage I and stage II were used to analyze the removal efficiency of TC and TN. Finally, the removal rate of TC and TN in the environment during treatment was analyzed based on the removal efficiency value.

Table 7. Composition of TC and TN in the fish food

Material	Value
TC (mg/kg)	300,000
TN (mg/kg)	45,000

Table 8. Input of TC and TN from fish food during the experiment (39 weeks)

Parameter	Composition	Input/day (mg)	Input in 39 weeks (mg)
TC (mg/kg)	300,000	150	40,950
TN (mg/kg)	45,000	23	6,140

1.3 Results

1.3.1 Construction of a new water treatment system based on material circulation and activated environmental microorganisms

A new water treatment system was constructed using water circulation and activated aerobic and anaerobic bacteria. The schematic diagram of the new water treatment system is shown in Figure 1. This system consists of two units of microbial columns with a 200 L water tank, and each unit has a different water flow rate. A stainless steel microbial column (347 mm in length with a 52 mm inner diameter) was prepared and filled with 770 mL of PVA sponge. The first unit had six parallel columns, while the second had three parallel columns. The first unit provided a lower water flow rate (1.8 L/min/column), and the water circulation was 13 cycles/day. The flow rate of the second unit was 2.9 L/min/column, and the water circulation was 21 cycles/day. The first (column with slower water flow rate) and the second units (faster flow rate) were defined as 6-columns unit and 3-columns unit, respectively. The faster water flow rate seems to provide relatively higher aeration in the 3-

columns unit. Similarly, slower water flow rate might have provided relatively low aeration in the 6-columns unit. The water passed through both columns units and went back to the tank, where the water was mixed and recirculated to the both columns units, separately.

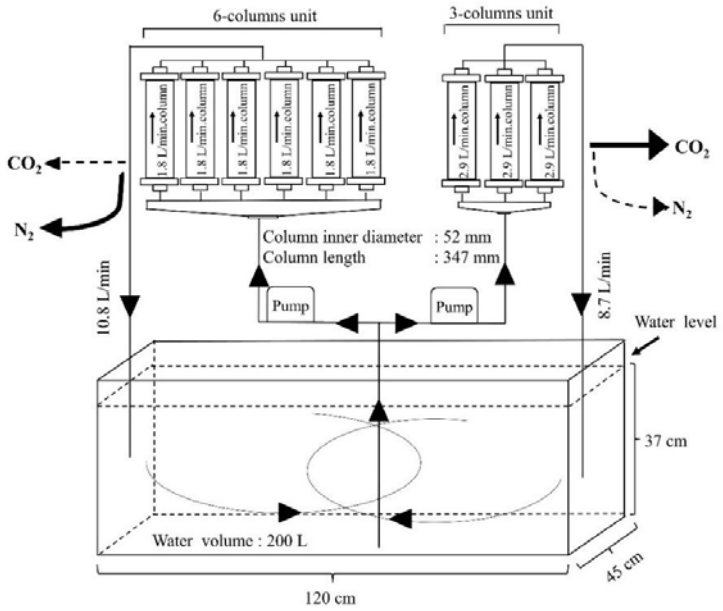

Figure 1. Schematic diagram of the new water treatment system based on material circulation. Black arrow represents the direction of water flow in the system.

1.3.2 Efficiency of the new water treatment system for treating water from a natural pond

Water used in this study was taken from Kitanoshin pond, Shiga, Japan (34°98' N, 135°96' E). The COD, TC, and TN values in all seasons are shown in Table 9. Analysis of the water showed that the average TC (4.7 mg/L) was 10 times higher than that of the TN (0.41 mg/L), indicating high carbon biomass in the water. The average COD during all seasons was 5.6 mg/L, which was still higher than the standard for agricultural use in Japan (< 5 mg/L).

Table 9. Values of COD, TC, and TN in the water and their removal rates during the 14 days treatment period (experiments were carried out from 16 April 2014 to 16 February 2015).

Season		COD				TC				TN			
	Date	Day 0 (mg/L)	Day 14 (mg/L)	Removal rate (%)	Average removal rate (%)	Day 0 (mg/L)	Day 14 (mg/L)	Removal rate (%)	Average removal rate (%)	Day 0 (mg/L)	Day 14 (mg/L)	Removal rate (%)	Average removal rate (%)
Spring	April 16	3.4	3.4	0.0	7.5	3.5	3.5	0.0	0.7	0.1	0.1	0.0	18.0
	May 1	3.2	3.2	0.0		3.5	3.5	0.0		0.3	0.2	34.4	
	May 16	4.0	3.1	22.5		4.9	4.8	2.0		0.4	0.3	37.5	
Summer	June 1	3.8	3.2	15.8	24.6	5.9	4.3	27.1	20.5	0.3	0.3	0.0	28.0
	June 16	5.0	3.0	40.0		5.7	4.1	28.1		0.8	0.1	81.8	
	July 1	8.1	7.1	12.4		5.6	5.2	7.1		0.4	0.4	0.0	
	July 16	8.0	5.8	27.5		6.2	5.7	8.1		0.6	0.3	49.2	
	August 1	6.1	5.2	14.8		5.4	4.7	13.0		0.6	0.6	0.0	
	August 16	8.1	5.1	37.0		6.8	4.1	39.7		0.5	0.3	37.0	
Autumn	September 1	6.6	5.1	22.7	23.0	5.0	4.7	6.0	18.6	0.3	0.3	0.0	12.8
	September 16	13.4	10.4	22.4		7.1	5.6	21.1		0.7	0.6	6.2	
	October 1	8.5	6.0	29.4		5.5	4.5	18.2		0.8	0.4	54.4	
	October 16	6.5	4.8	26.2		5.2	3.6	30.8		0.3	0.3	16.1	
	November 1	6.7	4.9	26.9		4.7	3.4	27.7		0.3	0.3	0.0	
	November 16	4.3	4.3	0.0		3.8	3.5	7.9		0.5	0.5	0.0	
Winter	December 1	2.8	1.9	33.2	21.7	3.3	1.9	42.4	17.8	0.4	0.2	47.5	21.5
	December 16	2.0	2.0	0.0		2.1	2.1	0.0		0.3	0.3	0.0	
	January 16	3.7	2.0	46.0		2.8	2.2	21.4		0.3	0.2	40.7	
	February 1	3.3	3.3	0.0		2.6	2.3	11.5		0.3	0.2	19.2	
	February 16	4.4	3.1	29.6		2.9	2.5	13.8		0.3	0.3	0.0	
Average					19.2				14.4				20.1

The water was treated using the new water treatment system to evaluate the efficiency of this system to reduce COD, TC, and TN in the water. The COD removal rate increased from spring (7.5%) to summer (24.6%), after which the removal rates became stable from autumn (23.0%) to winter (21.7%) (Table 9). A similar tendency was observed in the TC and TN removal rates. The average removal rates of COD, TC, and TN were 19.2%, 14.4%, and 20.1%, respectively, during all seasons. These results indicate that environmental bacteria together with water flow led to constant and efficient purification of the pond water.

1.3.3 Analysis of bacterial number and diversity in the columns

The total bacterial number in both 3-columns unit and 6-columns unit were analyzed for environmental DNA (Table 10 and Figure 2). During the experiment, the average total number of bacteria in the 3-columns unit was almost six times higher (1.4×10^9 cells/g-wet PVA) than that in the 6-columns unit (2.5×10^8 cells/g-wet PVA). The similarity between 3-columns unit and 6-columns unit were below 86%, but the similarity among 3-columns unit and 6-columns unit were above 90%. Figure 3 shows the PCR DGGE analysis of 16*S* rRNA bacterial genes in microbial columns, while Figure 4 shows the cluster analysis of 16*S* rRNA bacterial genes in microbial columns. These results indicate that the difference in water flow rate in the columns lead to supply of different aerobic conditions. The faster water flow rate in the 3-columns unit appears to supply oxygen to enhance the total bacterial number. Different bacterial diversities were also shown in 3-columns unit and 6-columns unit owing to differences in oxygen level.

Table 10. Total bacterial number in the microbial columns

Month	in the 3-columns unit ($\times 10^8$ cells/g-wet PVA)	in the 6-columns unit ($\times 10^8$ cells/g-wet PVA)
April	5.2	3.7
June	14.4	3.0
August	19.4	1.8
October	13.4	1.3
December	18.2	1.5
February	13.8	4.2
Average	14.1	2.5

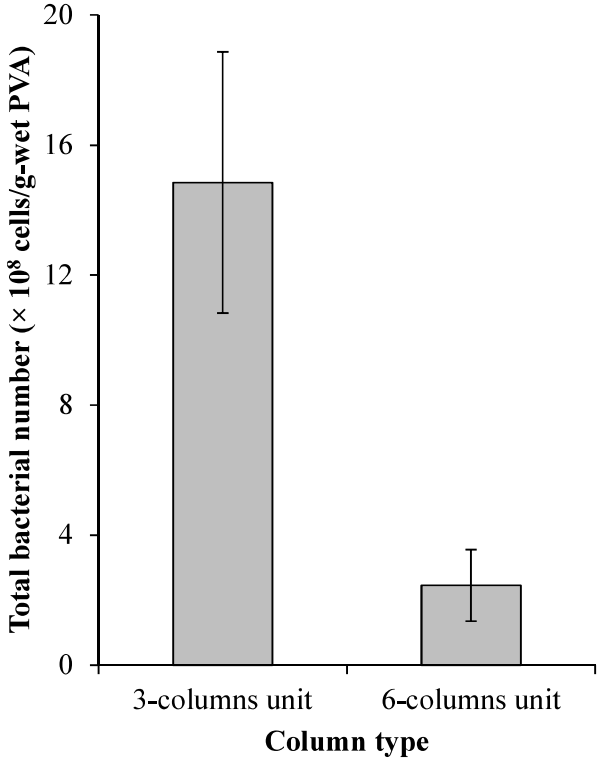

Figure 2. Total bacterial number in the 3-columns unit and 6-columns unit.

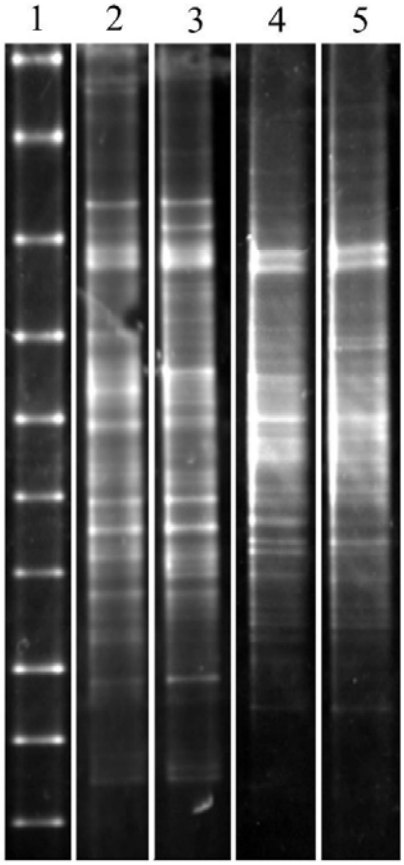

Figure 3. PCR DGGE analysis of 16*S* rRNA bacterial genes in microbial columns (lane 1: Marker; lanes 2–3: 3-columns unit; and lanes 4–5: 6-columns unit)

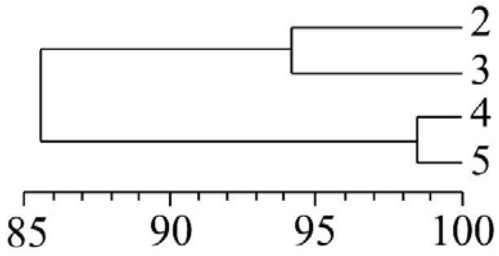

Figure 4. Cluster analysis of 16*S* rRNA bacterial genes in microbial columns (lanes 2–3: 3-columns unit and lanes 4–5: 6-columns unit)

1.3.4 Effect of the water treatment system in a fish-cultivated aquatic environment

The new water treatment system was used for evaluation of water treatment in a fish-cultivated aquatic environment. Before starting the operation (stage I), 22,050 mg of carbon and 3,310 mg of nitrogen were added into the environment by fish food (Table 11). The concentration of TC in the water was gradually increased from 2.24 to 11.49 mg/L in the stage I, while concentration of TC in the sediment was increased from 300 to 500 mg/kg-sand sediments (Table 12). During this stage, the carbon accumulation in 200 L of water and 20 kg of sand sediment were 1,850 mg and 4,000 mg, respectively. In the case of nitrogen, there was gradual increase of TN in the water from 0.00 to 6.33 mg/L and also in the sediment from 30 to 120 mg/kg-sand sediments. A total 1,270 mg of nitrogen was accumulated in 200 L of water and 1,800 mg was accumulated in the sediment. Figure 5 and 6 show the time course of TC and TN concentration in the water and sediment with the addition of fish food.

Table 11. Mass balance of TC and TN during treatment of a fish-cultivated aquatic environment.

Parameter	Stage I				Stage II			
	Input (mg)	Water	Sediment	CO_2/N_2 and accumulation in fish	Input (mg)	Water	Sediment	CO_2/N_2 and accumulation in fish
TC	22,050	1,850	4,000	16,200	18,900	-700	0	19,600
TN	3,310	1,270	1,800	240	2,830	-880	-1,200	4,910

Table 12. Concentrations of TC and TN in the water and sediment during treatment

Time (Week)	TC of Water (mg/L)	TC of Sediment (mg/kg-sand sediment)	TN of Water (mg/L)	TN of Sediment (mg/kg-sand sediment)
1	2.24	300	0.00	30
2	2.69	305	0.00	35
3	3.14	310	0.00	35
4	3.99	315	0.00	40
5	4.21	320	0.13	40
6	4.55	325	0.66	45
7	4.92	330	0.76	50
8	5.25	335	0.83	65
9	5.99	340	0.91	60
10	6.11	345	1.03	65
11	6.39	350	1.72	70
12	6.57	356	1.84	80
13	6.84	362	2.70	85
14	7.21	371	3.07	90
15	8.91	378	3.55	90
16	9.35	393	3.81	95
17	9.88	422	4.22	95
18	10.21	449	4.66	100
19	10.46	456	4.96	100
20	10.89	470	2.87	110
21	11.49	500	6.33	120
22	10.89	501	7.76	125
23	9.67	490	7.83	130
24	8.99	490	6.83	124
25	8.79	495	6.84	110
26	8.76	505	6.66	100
27	7.50	500	6.26	90

28	7.50	498	5.86	80
29	7.70	503	5.71	75
30	7.90	489	5.46	70
31	7.70	504	5.46	70
32	8.00	501	5.16	80
33	8.20	501	5.06	70
34	7.80	490	4.66	70
35	8.10	488	5.26	80
36	8.30	489	5.26	70
37	7.90	495	5.06	70
38	8.10	499	4.80	70
39	7.73	500	3.41	70

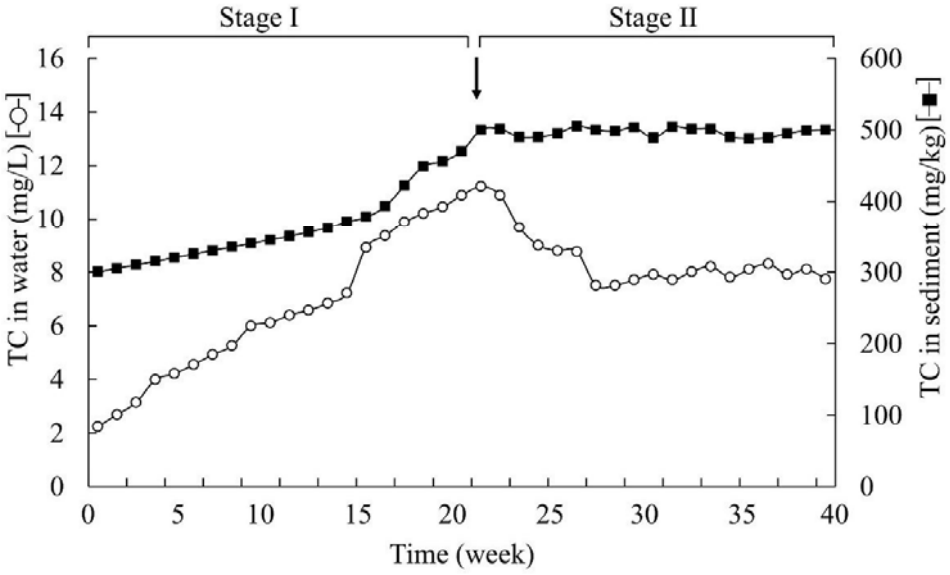

Figure 5. Time course of TC concentration in the water and sediment in the fish-cultivated aquatic environment. Black arrow represents the starting time of the new water treatment system operation.

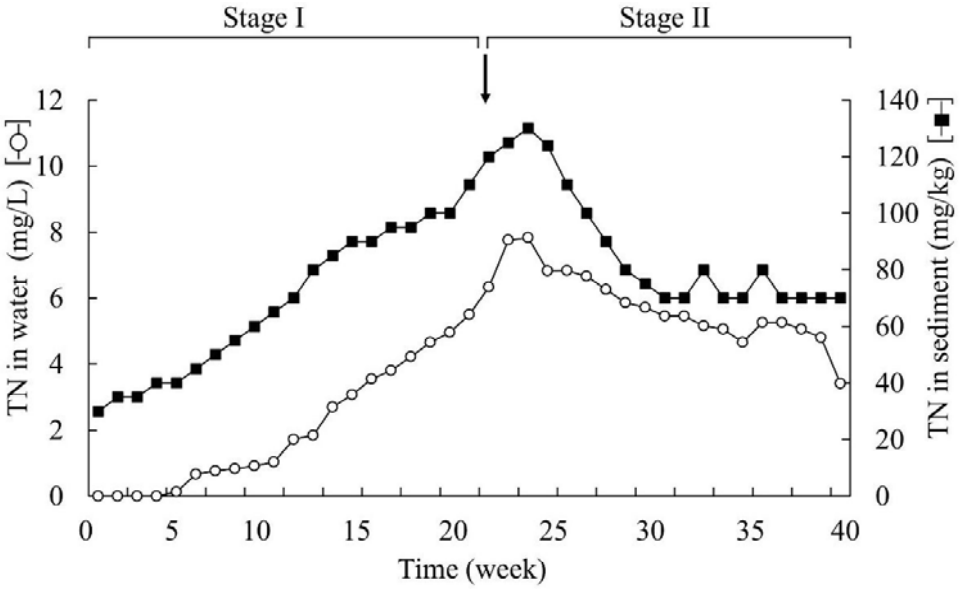

Figure 6. Time course of TN concentration in the water and sediment in the fish-cultivated aquatic environment. Black arrow represents the starting time of the new water treatment system operation.

The new water treatment system was operated to purify the water after 21 weeks (stage II). During stage II, there was no accumulation of carbon and nitrogen in either the water or sediment (Table 11). The removal rates of TC and TN in the water and sediment during stage II are shown in Table 13. The removal values of TC and TN during stage II were 3,400 mg and 4,670 mg, respectively, which was about 190 mg/week and 260 mg/week, respectively. These results suggest that the amounts of carbon and nitrogen in the water and sediment were efficiently reduced after using the new water treatment system.

Table 13. TC and TN removal during treatment of a fish-cultivated aquatic environment.

Parameter	CO_2/N_2 gas and accumulation in fish (mg)		Total removal value by the system (mg)	Removal value in a week by the system (mg)
	Stage I	Stage II		
TC	16,200	19,600	3,400	190
TN	240	4,910	4,670	260

The accumulation of TC and TN during the experiment (stages I and II) were analyzed (Figure 7). From a total 40,950 mg of carbon input (22,050 mg in stage I and 18,900 mg in stage II), the accumulation in the water and the sediment were 2.8% and 9.8%, respectively (87.4% was removed as CO_2 gas and/or accumulated in the fish). In the case of TN, 6.4% was accumulated in the water and 9.8% was accumulated in the sediment (83.9% was removed as N_2 gas and/or accumulated in the fish). These results indicate that the new water treatment system efficiently removed TC and TN, even in the fish-cultivated aquatic environment.

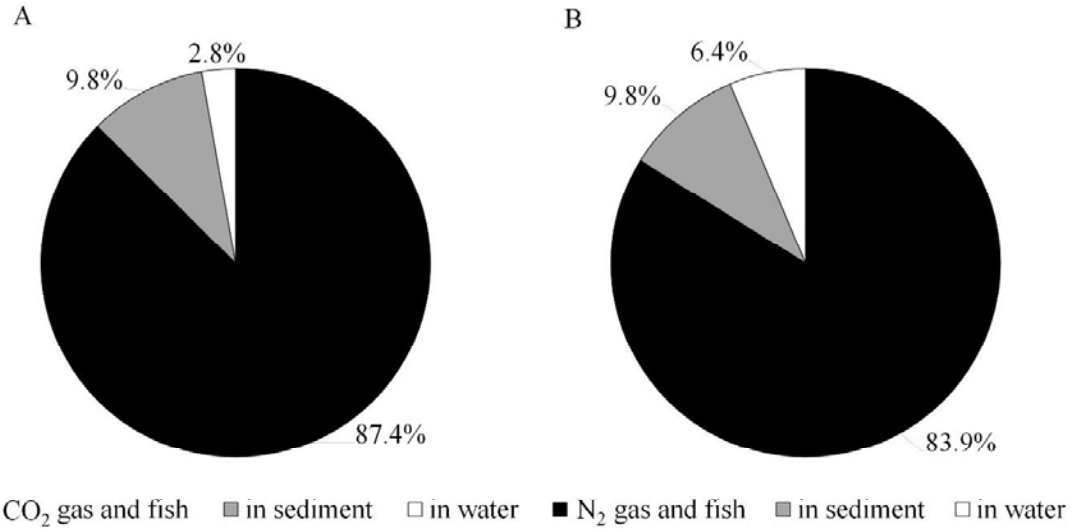

Figure 7. Accumulation of TC and TN in the water and sediment during treatment in the fish-cultivated aquatic environment: TC (A) and TN (B).

1.4 Discussion

A new water treatment system based on material circulation was constructed for purification of water in a natural static environment. The system was designed based on a water flow environmental self-purification mechanism [9]. The system consisted of two pumps that contributed to environmental bacterial immobilization and water circulation without filtration [27]. Water in the aquarium was circulated (34 cycles/day) by the system at a rate faster than that of Lake Biwa (ancient lake) in Japan [28]. The microbial immobilization and water circulation by the system appeared to efficiently improve the values of COD and TC and lead to stabilization of the TN level in the water [29].

Enhanced aerobic and anaerobic bacteria accelerated the carbon and nitrogen removal in the water through several processes [14] [17]. Removal of TC to CO_2 by the system was attributed to the decomposition of organic materials by aerobic bacteria in the columns. Similarly, TN removal was associated to the anaerobic bacteria in the 6-columns unit. The difference in bacterial community structure as shown in Figure 3 might be due to the different aeration in the columns. The bacterial number in the columns was higher than that in lake environments [30], indicating that the system had a high capacity to decompose organic materials in the aquatic environment. The new water treatment system also worked efficiently in a fish-cultivated aquatic environment, and the nitrogen removal rate became higher. The high nitrogen concentration in the aquatic environment may stimulate activation of denitrifying bacteria in the 6-columns unit [31]. Although the denitrification and nitrification activities were not analyzed in this study, but successful reduction of nitrogen indicates that the denitrification was taken place in the system.

Use of activated sludge system is one of the extensively used methods for treating polluted water. Activated sludge system carries both aerobic and anaerobic tanks, but it requires huge energy [19]. The new water treatment system also carries both aerobic and anaerobic bacterial columns, but the system does not require high energy and large space. Therefore, the system seems to be suitable for water treatments of natural pond and lake with lower energy. In a study of treating fish-pond water using water circulation system, TN level was about 10 mg/L [24]. But our system using water circulation and microbial columns with different water flow rates provided TN level less than 5 mg/L. The system successfully removed carbon and nitrogen from the water, but accumulations of phosphorus and potassium were remained in the environment. Addition of mechanisms for removal of phosphorous and potassium will be needed in the system; therefore, a new machine to accomplish this is currently being developed.

1.5 Summary

A new water treatment system based on material circulation was constructed for purification of naturally polluted pond water in an aquarium. The water treatment system consisted of microbial columns with different flow rates (1.8 L/min/column in 6-columns unit and 2.9 L/min/column in 3-columns unit). Two hundred liters of water from a naturally polluted pond were treated for 14 days using the water treatment system. After treatment, the COD, TC, and TN had been reduced by up to 19.2%, 14.4%, and 20.1%, respectively. High bacterial number in the microbial columns indicates the high decomposition of organic materials. The difference in bacterial community structure might be due to the

different aeration in the columns. The new water treatment system also worked efficiently in a fish-cultivated aquatic environment, with TC and TN removal rates of 190 mg/week and 260 mg/week, respectively.

Chapter 2

Application of the new water treatment system for purification of static water environment

2.1 Introduction

Lotic environment, such as river and stream, has the ability to maintain the balance of organic materials in the environment through self-purification mechanism [9]. In this mechanism, microorganisms play an important role to purify the water through decomposition of organic materials in the sediment. In contrast, lake and pond are lack of water flow and less water circulation occurs in the ecosystem [33]. The limited water circulation causes slow material circulation, so organic materials in the water are not transferred to the sediments. As a result, microbial number and microbial activities in the sediment become low [34].

In a rich-oxygen sediment within top view centimeters, several aerobic heterotroph bacteria perform extracellular enzymatic hydrolysis of organic materials to smaller and simple forms [35] [36]. About 19% of total cellulose in the sediment of natural lake is decomposed by aerobic heterotroph bacteria, especially genus *Cytophaga* and *Cellulomonas* [37] [38]. Enzyme β-glucosidase is one of many cellulose enzymes activated during this process [39]. Another study showed that bacteria genus *Bacillus* and *Streptomyces* are responsible for the decomposition of hemicellulose by activating xylannase, mannase, and galactanase [40]. These aerobic bacteria have respired about 8% of total glucose taken up from the sediment of water environment [41]. In the case of lignin decomposition, peroxidase and phenol oxidase are activated in aerobic condition by several

genera of bacteria, such as *Alcaligenes*, *Arthrobacter*, *Nocardia*, *Pseudomonas*, and *Strepomyces*, have ability to degrade lignin [42] [43].

On the other hand, anaerobic bacteria performed denitrification process in the sediment by activating nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR) [44] [45]. Denitrification is a part of nitrogen cycle converting nitrate to nitrogen gas form [46]. Nitrate is converted to nitric oxide by NaR, NiR, and NOR [47] [48], and followed by conversion to nitrous oxide by the assistance of N_2OR [49] [50]. It is finally released to the atmosphere in molecular nitrogen gas form. Several anaerobic bacteria, such as α-, β-, and γ-proteobacteria, are known to perform this denitrification process [15] [16].

In the previous study, a new water treatment system was established based on material circulation. The system consists of microbial columns with different water flow rates: 3-columns unit with 2.9 L/min/column of water flow rate and 6-column unit with 1.8 L/min/column of water flow rate. The system provided water circulation value of 34 cycles/day and reduced the COD, TC, and TN of the water up to 19.2%, 14.4%, and 20.1%, respectively. However, it is necessary to evaluate the efficiency of the new water treatment system to purify water in the real aquatic environment. In this study, the relationship between sediment properties and the water quality in a static water environment (pond) was investigated. The aim of this study was to evaluate the efficiency of the new water treatment system to improve water quality and sediment properties of a static water environment.

2.2 Materials and methods

2.2.1 Experimental site and sampling

The evaluation of the new water treatment system was carried out in Kitanoshin pond located in Kusatsu city, Shiga, Japan (34° 98' N, 135° 96' E) (Figure 8), from July 2014 to July 2017. The total area of the pond is approximately 1,716 m^2 with 121 m in length and 2.0 to 43.5 m in width. The depth of the pond is 0.5 to 4.0 m, suggesting a 2.34 × 10^6 L of water volume in the pond. The average rainfall in the location was 1,465 mm/year resulting water circulation of 1.1 cycle/year. The water circulation before the treatment in Kitanoshin pond was estimated by using following formula:

$$\text{Water circulation} = \frac{\text{Volume input}}{\text{Total volume}}$$

where, water circulation is the water circulation in the pond before the treatment, volume input is the water volume obtained from the average of rainfall during the experiment and total volume is the total volume of water in the pond.

During the experiment, the pond was divided into two zones: zone A and zone B (Figure 12). Zone A was in the north side of the pond which was highly influenced by the new water treatment system, while zone B was in the south side of the pond which was less influenced by the new water treatment system. Water samples were collected from different sites in zone A and zone B every week from July 2014 to July 2017. Sediment samples were taken from zone A (11 sites) and zone B (5 sites) every 3 months: April (spring), July (summer), October (autumn), and January (winter) from April 2015 to July 2017. The date of sediment sampling is shown in Table 14.

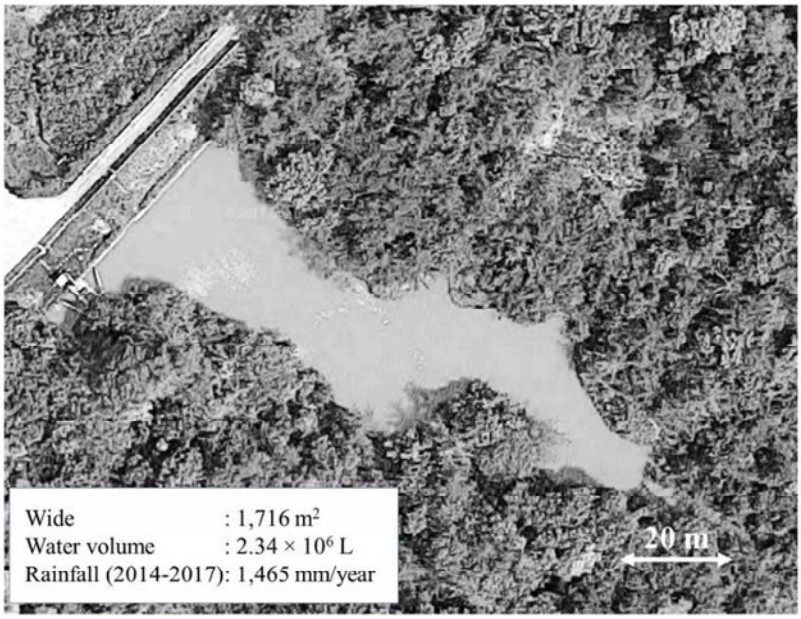

Figure 8. Kitanoshin pond (a natural pond surrounded by natural forests)

Table 14. Date of sediment sampling

Date	Season
26 April, 2015	Spring
15 July, 2015	Summer
21 October, 2015	Autumn
12 January, 2016	Winter
17 April, 2016	Spring
13 July, 2016	Summer
18 October, 2016	Autumn
12 January, 2017	Winter
16 April, 2017	Spring
25 July, 2017	Summer

2.2.2 Application of the new water treatment system in Kitanoshin pond

The new water treatment system was applied in Kitanoshin pond to treat the water and sediment of the pond. The experiment was carried out from July 2014 to July 2017 throughout the summer (June to August), autumn (September to November), winter (December to February), and spring (March to May). Water circulation after the treatment was estimated to observe the increase of circulation in the pond. The water circulation after the treatment in the pond was estimated by using a following formula:

$$\text{Water circulation} = \frac{\text{Volume input} + \text{Machine capacity}}{\text{Total volume in the pond}}$$

where water circulation is the water circulation after treatment (cycles/year), volume input is the water volume obtained during the treatment period (L), machine capacity is the volume of water circulated by the new water treatment system, and total volume in the pond is the total volume of water in the pond.

2.2.3 Analysis of water properties

Water properties of the pond before treatment was measured on 7 July 2014 to know the water condition at the beginning of experiment. Dissolved oxygen (DO), COD, TC, and TN of the water at the beginning of experiment were 1.3 mg/L, 5.4 mg/L, 7.5 mg/L, and 0.5 mg/L, respectively. Next, measurement of water properties was continued after applying the new water treatment system in the pond. The measurement of DO, COD, TC, and TN in the water of the zone A was started from July 2014 to July 2017. In order to compare the water properties in the zone A and zone B, water properties in the zone B was

started to be analyzed in April 2015. Several parameters, such as turbidity, colority, pH and electrical conductivity (EC), were also analyzed in the both zone A and zone B.

DO of water was measured using DO meter OM 12 (Horiba, Ltd., Kyoto, Japan). In the zone A, measurement of DO was performed at the surface and bottom (±3 m), while measurement of DO in the zone B was carried out in the surface only because of the shallow depth (<1 m).

Analysis of the COD was carried out by using permanganate based titrimetric method [24]. A 100 mL of water sample was mixed with 10 mL of diluted H_2SO_4 (48%), 10 mL of 5 mM $KMnO_4$, and 0.2 g of Ag_2SO_4 in Erlenmeyer flask. The mixture was digested at 90°C for 30 min, and allow cooling for next 30 min. Next, 10 mL of 12.5 mM $Na_2C_2O_4$ were added to the flask and mixed well. The remaining $Na_2C_2O_4$ from the solution was determined by titrating 5 mM $KMnO_4$ until pink color appeared. The COD was then calculated by the formula:

$$\text{COD (mg/L)} = (\text{mL sample titration} - \text{mL blank titration}) \times 1 \times \text{dilution} \times 0.2$$

where, 1 is the $KMnO_4$ factor in 5 M and 0.2 is the amount of oxygen in 5 M $KMnO_4$.

Total organic carbon (TOC), total nitrogen (TN) analyzer, and solid sample combustion unit (Shimadzu Corporation, Kyoto, Japan) were used for analyzing the TC and TN of water. A 1,000 mg/L of TC standard solution was made from 2.125 g of sodium hydrogen phthalate in a total volume of 1,000 mL distilled water. The TN standard solution (1,000 mg/L) was made by mixing 7.219 g of potassium nitrate and distilled water in total volume of 1,000 mL. Serial dilutions of TC and TN standard solution (0, 1, 10, and 100

mg/L) were made to create the linear regression and to obtain the TC and TN value of the water samples.

The pH of water was measured using LAQUA pH/ion meter F-72 (Horiba, Ltd., Kyoto, Japan) after 2-point calibrations at pH 6.84 and pH 7.01, while EC of water was measured using HI 98331 EC and temperature sensor (Hanna Instruments, Inc., Chiba, Japan). Turbidity and colority of water were analyzed using TCR 30 turbidity and colority sensor (Kasahara Chemical Instruments Corp., Saitama, Japan).

2.2.4 Analysis of sediment properties

Sediment of the pond was analyzed to investigate the sediment properties in Kitanoshin pond. The sediment samples from both of zone A and zone B were analyzed in every 3 months: April (spring), July (summer), October (autumn), and January (winter), for 2 years from April 2015 to July 2017. To analyze TN, total phosphorus (TP), and total potassium (TK), soil sample was digested in a Kjeldahl digestion unit (Gerhardt, Königswinter, Germany). A 0.5 mg of sediment sample was mixed with 0.5 mg of $CuSO_4$ in a Kjeldahl tube. Subsequently, 5 mL of H_2SO_4 and 5 mL of H_2O_2 were added to the mixture in a fume hood. The mixture was digested at 420^{o}C for 1.5 h. After that, allow cooling for 30 min and filtered the extract using ADVANTEC filter paper no 6 (Toyo Roshi Kaisha, Ltd., Tokyo, Japan).

The TN of sediment sample was determined using the indophenol blue method [51]. Indophenol blue solution (Table 15) and sodium hypochlorite solution (Table 16) were prepared to carry out the TN analysis. Subsequently, 1 mL of Kjeldahl extract was mixed

with 400 µL of indophenol blue solution and 600 µL of sodium hypochlorite solution. The mixture was incubated at room temperature for 45 min for the color development. The absorbance of TN was observed using U 1900 UV visible spectrophotometer (Hitachi High-technologies Corporation, Tokyo, Japan) at 635 nm. Ammonium sulfate was used for preparing the standard solution [$(NH_4)_2SO_4$, (0.56 g/50 mL)] (Table 17). The standard curve for TN analysis is shown in Figure 9.

Table 15. Composition of Indophenol blue solution

Reagent	Quantity (g/L)
Trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$)	30.0
Trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	30.0
EDTA	3.0
Sodium pentacyano-nitrosulferrate(III) dihydrate	0.2
Phenol granules	60.0

Table 16. Composition of sodium hypochlorite solution

Reagent	Quantity (mL)
Sodium hypochlorite	20.0
1 M NaOH solution	400.0

Table 17. Absorbance of standard ammonium solutions

Concentration (mg–NH_4^+–N/L)	Absorbance (at 635 nm)
0.0	0.000
0.1	0.080
0.2	0.175
0.5	0.439
1.0	0.894
1.5	1.406

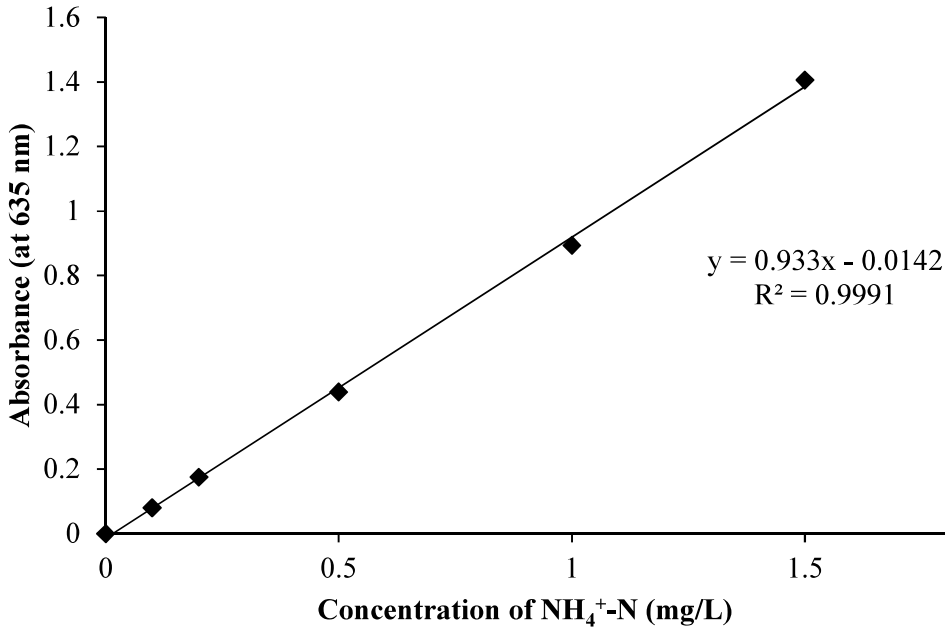

Figure 9. Standard curve of TN

Determination of TP in the sediment was performed using molybdenum blue method [52]. Ammonium molybdate solution (Table 18) and ascorbic acid solution (Table 19) were prepared for the TP analysis. A 1 mL of Kjeldahl extract was mixed with 100 µL of 1:5 ammonium molybdate and ascorbic acid solution. The mixture was incubated at 30^{o}C for 30 min to obtain color development. The absorbance was observed using UV visible spectrophotometer at 710 nm. Standard curve was obtained from serial dilution of 1000 mg/L Phosphorus standard solution (Table 20 and Figure 10).

Table 18. Composition of ammonium molybdate solution

Reagent	Quantity
Ammonium molybdate tetrahydrate	6.00 g
Antimony potassium tartrate	0.24 g
1:2 H_2O:H_2SO_4	120 mL
Distilled water	up to 500 mL

Table 19. Composition of ascorbic acid solution

Reagent	Quantity
L ascorbic acid	7.2 g
Distilled water	up to 100 mL

Table 20. Absorbance of standard P solutions

Concentration (mg–P/L)	Absorbance (at 710 nm)
0.0	0.000
0.1	0.054
0.2	0.109
0.5	0.259
1.0	0.523
1.5	0.762

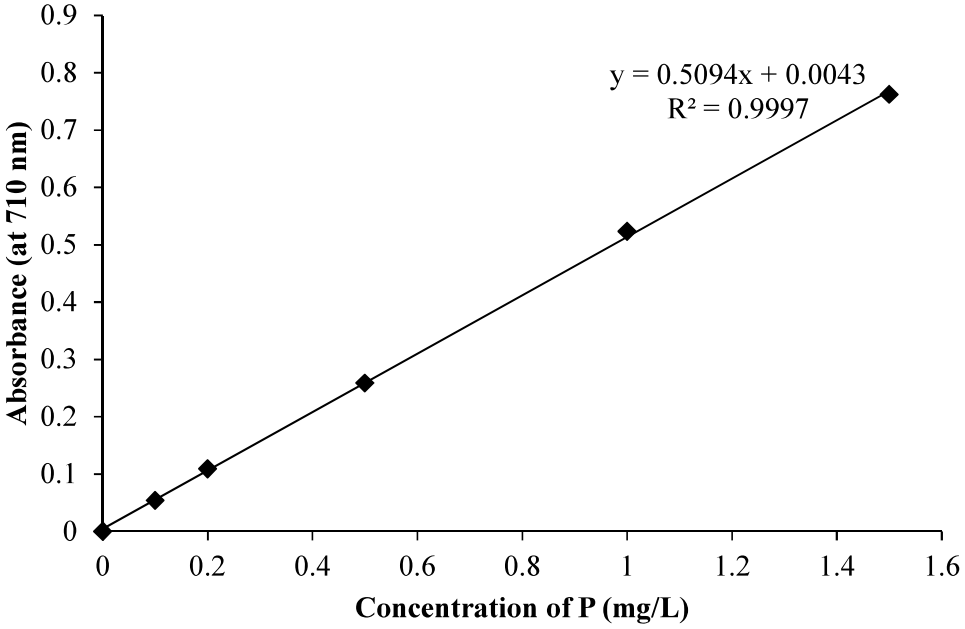

Figure 10. Standard curve of TP (mg/L)

Analysis of TK in the sediment was performed using Z-2300 atomic absorption spectrophotometer (Hitachi High-technologies Corporation, Tokyo, Japan). Standard solution was obtained from Potassium standard solution 1000 mg/L (Table 21). The standard curve for TP analysis is shown in Figure 11.

Table 21. Absorbance of standard K solutions

Concentration (mg-K/L)	Absorbance (at 248.3 nm)
0.00	0.0000
1.00	0.0315
2.00	0.0937
5.00	0.3108
10.00	0.6567

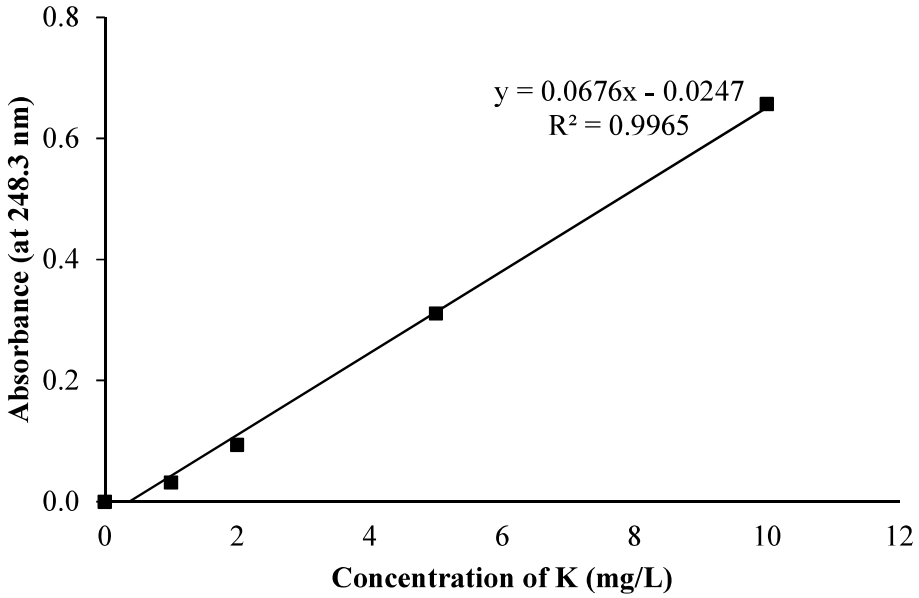

Figure 11. Standard curve of TK (mg/L)

The pH and EC of sediment was analyzed using LAQUA pH/ion meter F-72 (Horiba, Ltd., Kyoto, Japan) and HI 98331 EC and temperature sensor (Hanna Instruments, Inc., Chiba, Japan) after diluting the sediment with distilled water in a ratio of 2:5.

2.2.5 Estimation of total bacterial number in sediment

Total bacterial number in the sediment was estimated by quantification of environmental DNA (eDNA) using the low stirring method [17]. One g of soil sample was mixed with 8.0 mL of eDNA buffer and 1 mL of 20% sodium dodecyl sulfate (SDS) solution. The suspension was stirred at 1,500 rpm for 20 min by using a mechanical stirrer, followed by centrifugation of the suspension at 6,000 × g for 10 min. The supernatant was mixed with a mixture of chloroform-isoamyl alcohol (24:1 (v/v)) in equal volume, and centrifuged at 18,000 × g for 10 min. Precipitation of the crude nucleic acid was performed by mixing 500 μL of aqueos phase and 300 μL of isopropanol, followed by further centrifugation at 18,000 × g for 20 min. The remained pellet was dissolved in 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Quantification of the eDNA was performed based on the intensity of the band after electrophoresis of 1% agarose gel using KODAK 1 D 3.6 Image Analysis Software (Eastman Kodak Company, CT, USA).

2.3 Results

2.3.1 Construction of a new water treatment system with water circulation and enhanced microorganisms

The new water treatment system was set up to improve the water quality of the pond. The system was composed of 3 water pumps, 6 microbial columns with faster water flow rate (3.2 L/min/column), and 3 microbial columns with slower water flow rate (2.0 L/min/column) (Figure 12). The columns with faster flow rate were constructed to create aerobic condition and activate aerobic bacteria in the column, namely 3-columns unit. The other columns with slower water flow rate were constructed for creating anaerobic condition and activate anaerobic bacteria, called as 6-columns unit. During the operation of the system, two water pumps with different flow capacity were used to take the water from the pond, and transferred to the microbial columns. Water containing activated aerobic and anaerobic bacteria from the columns were transferred back to the pond. Then, another water pump (with capacity 1.15×10^6 L/day) was used to circulate the water in the pond. The system was able to circulate 4.20×10^8 L of water in the pond for 180 cycles/year. These results suggest that the new water treatment system increases the water circulation in Kitanoshin pond up to 164 times higher than before the application of the new water treatment system (1.1 cycle/year).

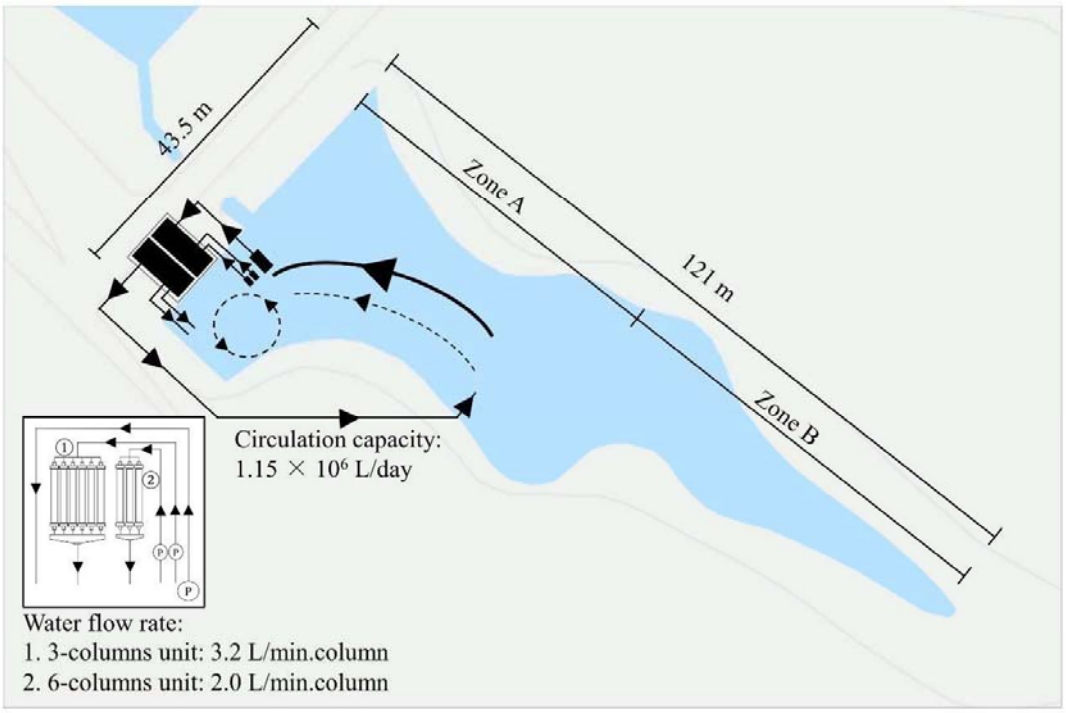

Figure 12. Construction of a new water treatment system in Kitanoshin pond. P is the water pump used in this experiment, and black arrow indicate the direction of water flow

2.3.2 Effect of the new water treatment system on the water quality of Kitanoshin pond

Table 22 shows the summary of water properties from July 2014 to July 2017. Measurement of water properties in the zone A was started from July 2014, while it in the zone B was started from May 2015. The result showed that there were no clear differences on the values of water properties between zone A and zone B.

Table 22. Characteristic of water properties in the zone A and zone B of Kitanoshin pond during the treatment

Year	Month	Zone	DO (mg/L)	COD (mg/L)	TC (mg/L)	TN (mg/L)	Turbidity (Degree)	Colority (Degree)	pH	EC (mS/cm)
2014	July	A	3.17	5.94	7.48	0.5	-	-	-	-
		B	-	-	-	-	-	-	-	-
	August	A	3.69	12.04	7.18	0.5	-	-	-	-
		B	-	-	-	-	-	-	-	-
	September	A	4.47	9.11	5.20	0.4	-	-	-	-
		B	-	-	-	-	-	-	-	-
	October	A	5.67	6.39	3.85	0.3	-	-	-	-
		B	-	-	-	-	-	-	-	-
	November	A	5.98	4.33	3.44	0.4	-	-	-	-
		B	-	-	-	-	-	-	-	-
	December	A	7.41	3.6	3.38	0.5	-	-	-	-
		B	-	-	-	-	-	-	-	-
2015	January	A	8.29	3.38	3.40	0.4	-	-	-	-
		B	-	-	-	-	-	-	-	-
	February	A	8.03	3.60	2.80	0.4	-	-	-	-
		B	-	-	-	-	-	-	-	-
	May	A	6.5	6.0	3.4	0.2	9.4	29	6.6	0.08
		B	8.6	4.5	3.4	0.2	10.1	13.2	6.6	0.08
	June	A	6.7	5.3	4.6	0.3	18.0	51.0	6.5	0.07
		B	7.8	6.5	4.3	0.3	13.6	53.0	6.5	0.06
	July	A	5.7	7.6	7.0	0.5	27.4	95.7	6.5	0.06
		B	5.5	7.1	7.0	0.5	23.7	86.2	6.5	0.07
	August	A	5.7	7.8	7.1	0.7	21.6	86.7	6.6	0.07
		B	5.9	7.8	7.2	0.7	22.5	88.7	6.6	0.07
	September	A	4.4	6.3	6.4	0.7	15.7	63.1	6.6	0.07
		B	4.3	6.5	6.4	0.7	16.7	67.4	6.6	0.07
	October	A	7.3	3.8	4.5	0.7	17.1	53.9	6.7	0.09
		B	7.0	4.0	4.5	0.6	19.7	58.3	6.8	0.08
	November	A	8.2	4.4	5.4	0.6	16.5	41.7	6.9	0.08
		B	7.3	4.5	5.2	0.6	15.2	37.0	7.0	0.08

	December	A	9.6	3.7	5.2	0.6	10.2	31.6	6.8	0.09
		B	8.4	3.8	5.0	0.5	11.9	34.5	6.8	0.08
2016	January	A	11.9	3.2	4.2	0.6	7.2	23.2	6.7	0.09
		B	9.9	3.5	4.4	0.5	6.4	22.3	6.7	0.09
	February	A	10.2	4.9	3.4	1.0	5.5	13.8	6.0	0.07
		B	8.5	4.9	3.2	0.8	2.2	12.0	6.0	0.08
	March	A	9.8	2.9	2.6	0.8	9.1	21.9	6.2	0.09
		B	8.7	2.6	2.4	0.8	10.0	6.8	6.2	0.09
	April	A	8.3	5.5	5.1	0.7	13.9	43.0	6.1	0.08
		B	7.4	4.1	4.7	0.6	12.9	38.8	6.1	0.08
	May	A	6.9	5.0	5.5	0.6	21.7	53.7	6.8	0.09
		B	8.1	5.5	5.5	0.8	19.0	54.5	6.7	0.08
	June	A	6.6	5.4	4.7	0.7	21.4	53.7	6.4	0.07
		B	6.5	5.4	5.0	0.6	21.7	54.5	6.4	0.07
	July	A	5.1	6.9	7.1	1.0	16.3	56.9	6.7	0.07
		B	5.5	6.7	6.7	0.8	17.4	59.8	6.8	0.07
	August	A	4.7	6.5	6.9	1.2	34.7	108.1	6.5	0.09
		B	4.7	6.6	7.1	1.0	36.9	111.3	6.4	0.08
	September	A	5.4	6.4	6.5	1.0	25.6	94.2	6.3	0.06
		B	5.5	6.2	6.1	1.0	24.4	85.1	6.4	0.06
	October	A	6.1	6.9	5.9	0.8	19.5	53.0	6.1	0.06
		B	5.4	6.7	5.9	0.7	17.5	49.2	6.2	0.06
	November	A	7.6	3.0	3.2	0.6	14.1	36.6	6.2	0.09
		B	6.5	2.9	3.2	0.5	10.7	28.6	6.4	0.09
	December	A	8.9	2.6	3.1	0.4	6.5	23.9	6.8	0.09
		B	8.4	2.6	3.0	0.5	6.0	21.9	6.9	0.09
2017	January	A	10.7	2.0	1.6	0.4	4.2	13.9	6.9	0.09
		B	8.5	1.7	1.6	0.4	3.9	12.1	6.8	0.09
	February	A	10.4	2.0	1.5	0.5	5.1	20.3	6.9	0.09
		B	8.5	2.3	1.7	0.4	3.5	15.9	6.9	0.09
	March	A	9.5	2.1	2.4	0.9	10.1	24.7	6.8	0.09
		B	9.15	2.1	2.3	0.6	8.7	25.5	6.9	0.09
	April	A	7.3	3.5	3.8	1.1	14.1	37.2	6.9	0.09
		B	6.6	3.4	3.6	1.2	14.2	38.2	6.8	0.09
	May	A	6.1	4.2	4.2	0.7	18.9	55.6	6.9	0.09

	B	5.8	4.0	4.0	0.6	16.7	38.5	6.9	0.09
June	A	5.7	4.3	4.5	1.0	12.0	33.1	7.4	0.09
	B	6.8	4.6	4.5	1.0	16.1	40.9	7.5	0.09
July	A	5.5	7.0	7.7	1.3	29.3	82.2	7.6	0.09
	B	5.5	7.3	7.8	1.3	30.0	91.0	7.4	0.09

Time course of DO during the treatment in the pond is shown in Figure 13. The result showed that the average value of DO was increased from summer 2014 to summer 2015 (from 3.4 mg/L to 6.1mg/L), and kept in a stable value in summer 2016 and 2017 (5.5 mg/L and 5.9 mg/L, respectively). The similar tendency was also shown in the other seasons. This result suggest that the system has ability to increase the DO of water.

In the case of COD, the result showed that there was a gradual decrease from summer 2014 to summer 2015 (from 9.0 mg/L to 6.9 mg/L), and kept in stable level in the summer 2016 and 2017 (6.3 mg/L and 5.7 mg/L, respectively) (Figure 14). This result indicates that the system has ability to improve the water quality by decreasing the COD of water during the treatment period. The COD of water was corresponded to the DO of water indicating high supply of oxygen obtained from the system which might inhibit the COD of water in the pond.

Analysis on the TC of water during the treatment period is shown in Figure 15. The result showed that there were no clear decreased of TC from summer 2014 to summer 2017. In the case of TN, the concentration of TN in the water was gradually increased from summer 2014 to summer 2017. The time course of TN value during the treatment period is shown in Figure 16. These result reveal that several amounts of nitrogen are remained in the water during the treatment period.

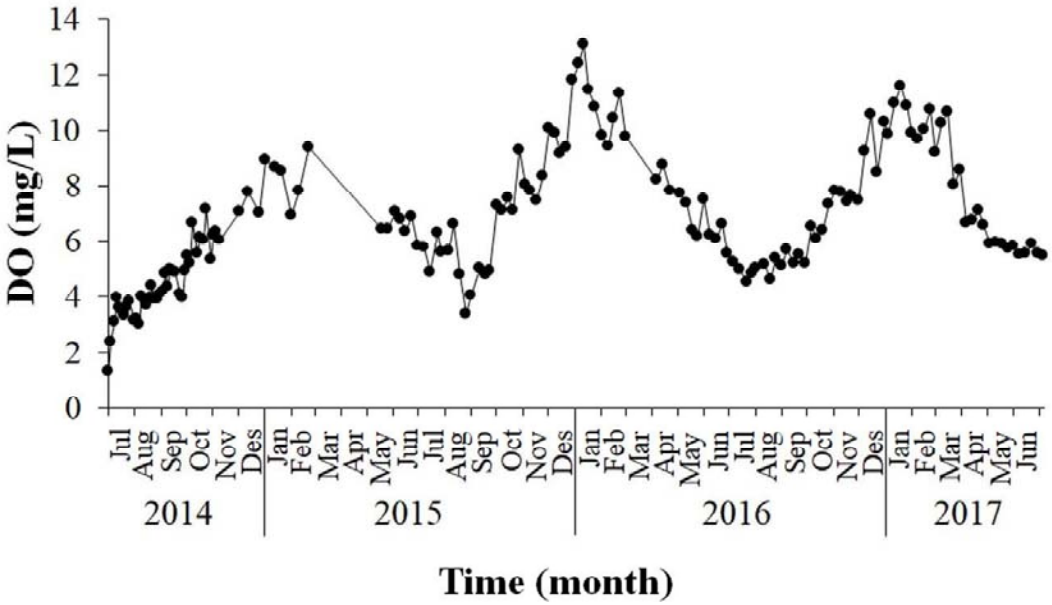

Figure 13. Time course of DO in zone A during treatment period

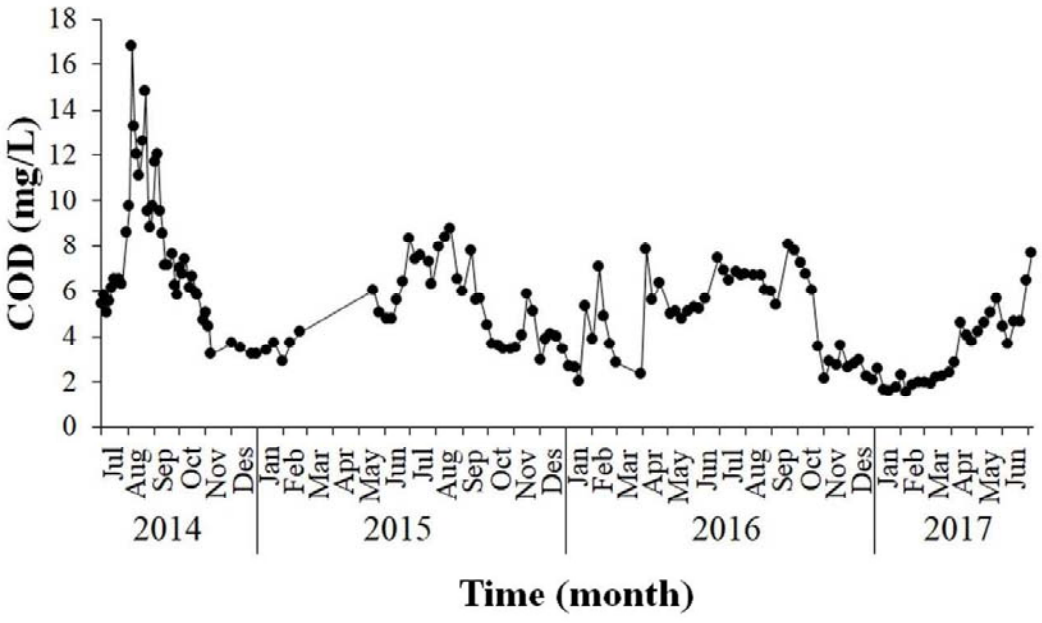

Figure 14. Time course of COD in zone A during treatment period

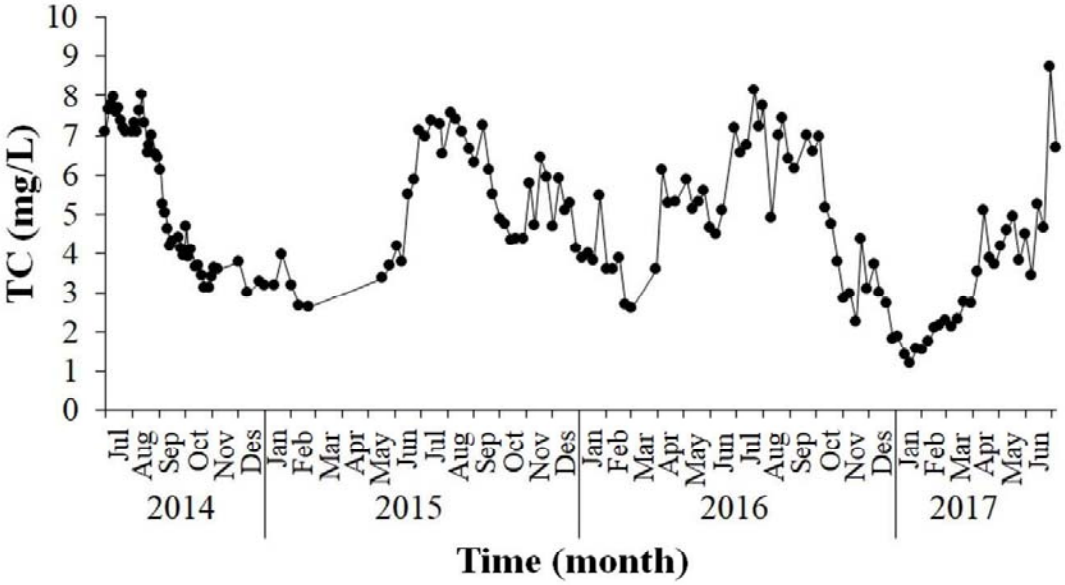

Figure 15. Time course of TC in zone A during treatment period

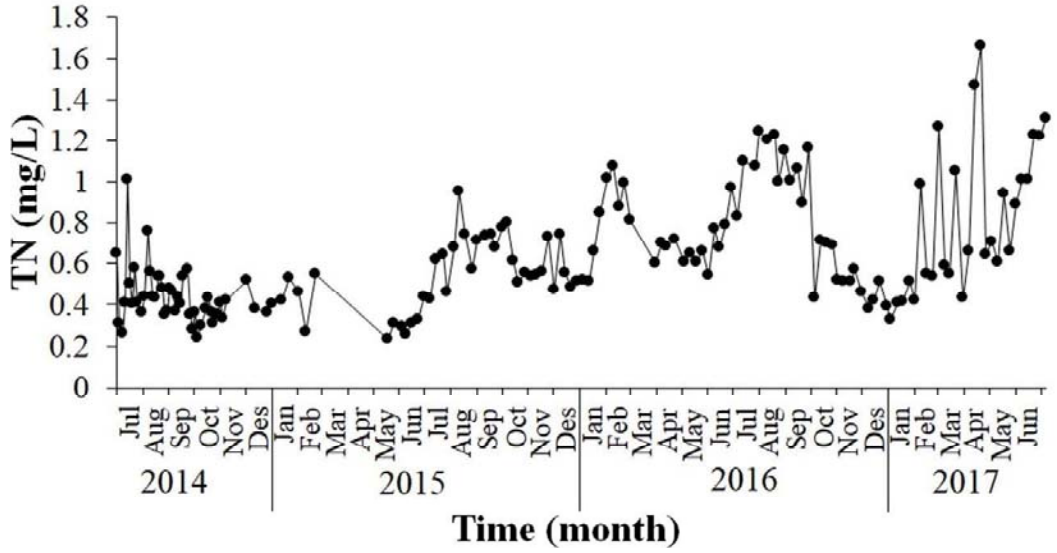

Figure 16. Time course of TN in zone A during treatment period

Analysis on the turbidity of water in the zone A of Kitanoshin pond is shown in Figure 17. The result showed that there was no clear difference between summer 2015 (22.35 degree) and summer 2016 (24.13 degree). Similarly, turbidity in winter 2015 (7.64 degree) and 2016 (5.25 degree) was not clearly different. The analysis of water colority in the pond also showed that there were no clear differences between summer 2015 and 2016, and also between winter 2015 and 2016. The time course of colority in the zone A of the pond is shown in Figure 18. These results suggest that there is no accumulation of suspended solid in the water during the treatment, and the new water treatment system does not affect the appearance of the water.

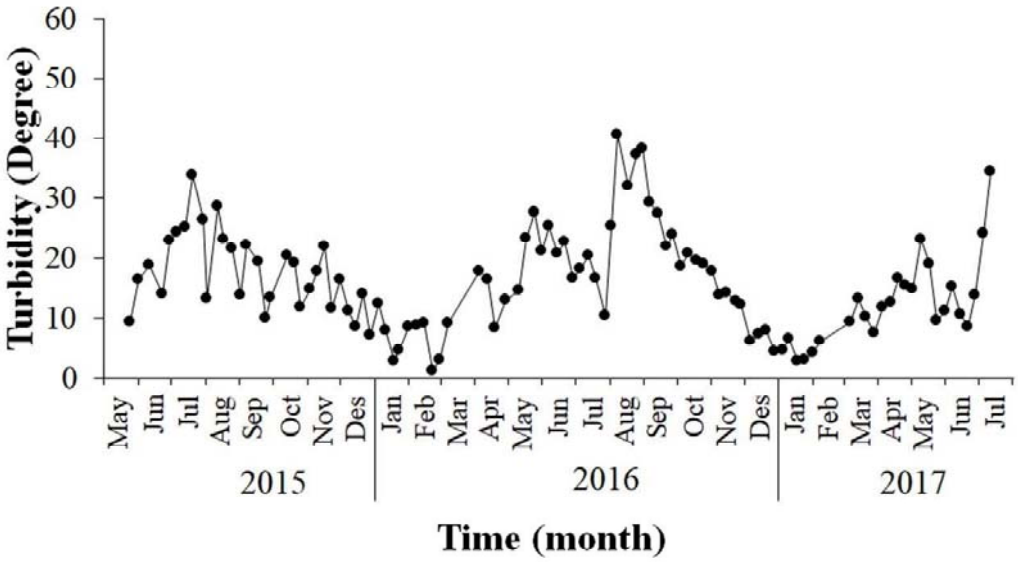

Figure 17. Time course of turbidity in zone A during the treatment period

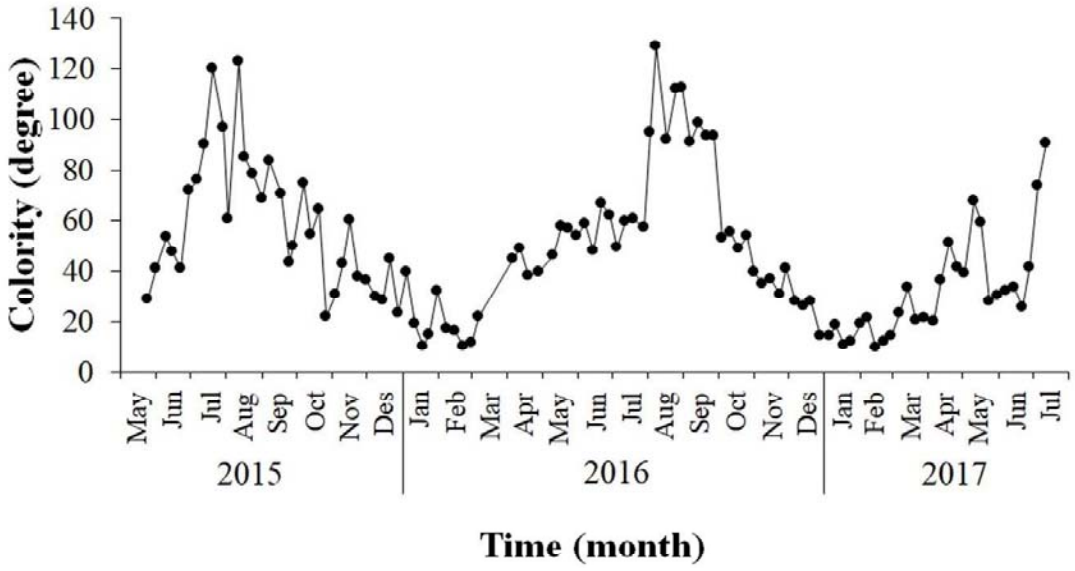

Figure 18. Time course of colority in zone A during the treatment period

2.3.3 Effect of the new water treatment system on the sediment properties of Kitanoshin pond

Sediment samples were analyzed from April 2015 to July 2017 in the zone A and zone B. Characteristic of sediment properties in zone A and zone B during the treatment period is shown in Table 23. Total bacterial number in sediment was increased from April to October 2014 in both of zone A and zone B (7.1 to 12.7 × 10^8 cells/g-sediment and 5.9 to 12.0 × 10^8 cells/g-sediment, respectively), and kept in a relatively stable condition until July 2017 (Figure 19). The total bacterial number in the sediment of zone A was slightly higher (10.1 × 10^8 cells/g-sediment) than that in the sediment of zone B (8.4 × 10^8 cells/g-sediment) (Table 23). This result suggests that the treatment has capacity to improve environmental bacterial number in sediment.

Table 23. Properties of sediment in Kitanoshin pond during the treatment

Year	Month	Zone	TC (mg/kg-sediment)	TN (mg/kg-sediment)	TP (mg/kg-sediment)	TK (mg/kg-sediment)	Total bacterial number ($\times 10^8$ cells/g-sediment)	pH	EC (mS/cm)
2015	May	A	11,000	530	690	3,430	7.1	4.8	0.06
		B	12,800	570	810	3,440	5.9	4.4	0.12
	July	A	7,700	440	440	2,980	8.9	4.8	0.09
		B	10,300	550	630	3,330	9.9	4.3	0.13
	October	A	7,300	320	480	2,580	12.7	4.5	0.14
		B	10,400	480	640	2,560	12.0	4.0	0.29
2016	January	A	6,400	260	550	3,120	9.3	5.1	0.10
		B	9,300	380	720	3,200	6.7	4.7	0.13
	May	A	6,300	240	610	3,410	13.0	4.9	0.07
		B	9,100	360	760	3,450	8.9	4.5	0.13
	July	A	5,300	310	350	2,800	9.3	5.3	0.06
		B	8,900	340	450	3,200	6.8	4.7	0.11
	October	A	6,300	260	330	2,600	13.0	5.4	0.06
		B	10,200	360	470	2,550	10.9	4.9	0.09
2017	January	A	6,300	220	290	2,920	7.7	6.1	0.02
		B	8,700	350	310	3,350	6.0	5.9	0.03
	May	A	6,200	230	290	3,010	10.1	5.3	0.12
		B	8,800	360	320	3,120	9.1	5.0	0.10
	July	A	6,400	210	300	3,000	9.8	5.4	0.11
		B	9,000	300	310	3,200	8.3	5.0	0.12

TC of sediment in both of zone A and zone B were gradually decreased from the beginning of treatment (April 2015) to the end of treatment (July 2017) (Table 23). Similarly, TN of sediment in both of zone A and zone B were gradually decreased. Spatial analysis of TC and TN in zone A and zone B showed that average value of TC and TN in the zone A were 1.5 times lower than that in the zone B. The time courses of TC and TN in the sediment of Kitanoshin pond are shown in Figure 20 and 21. These results indicate that the treatment affect the sediment in the pond by decreasing the TC and TN content.

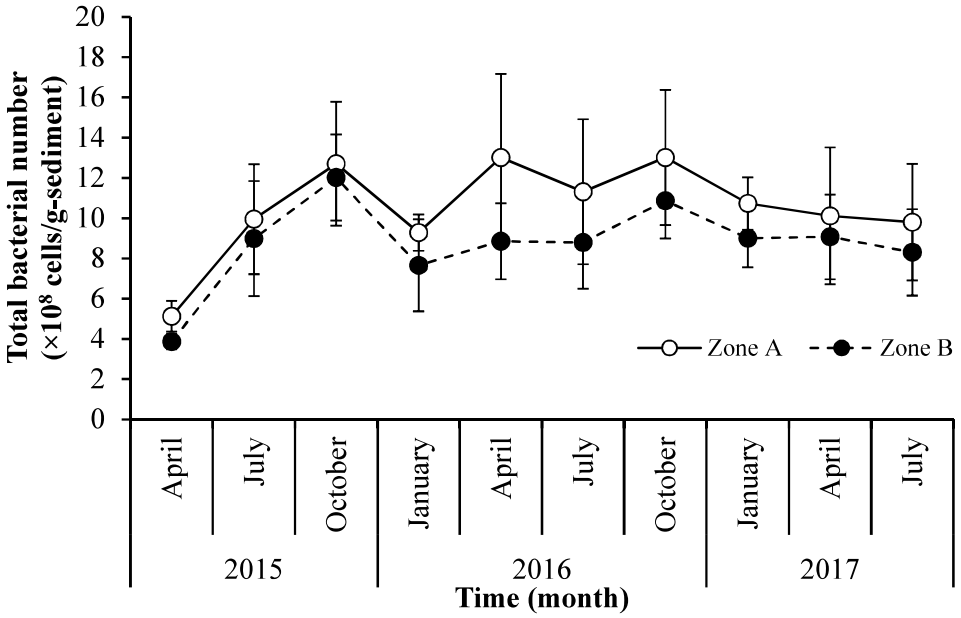

Figure 19. Time course of total bacterial number in sediment during treatment period

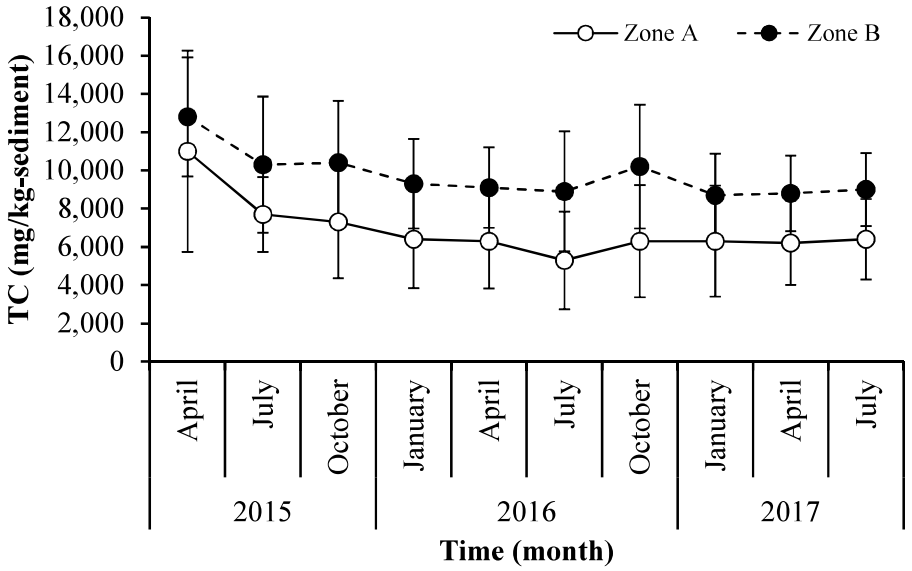

Figure 20. Time course of TC in sediment during treatment period

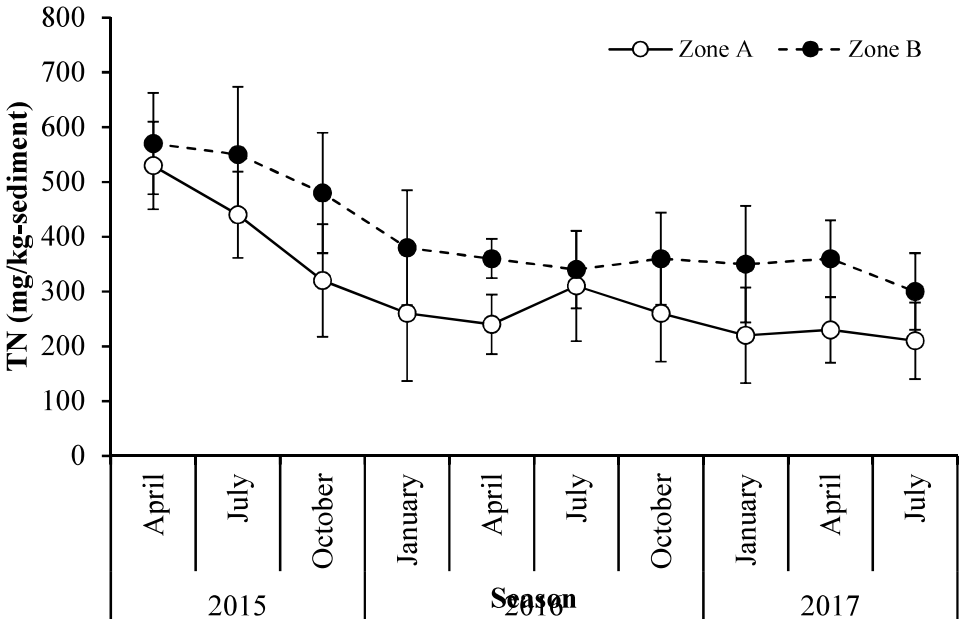

Figure 21. Time course of TN in sediment during treatment period

2.4 Discussion

A new water treatment system is applied in Kitanoshin pond which is polluted by natural organic materials. The water treatment system might improve the water quality in the pond through several mechanisms. Based on the calculation, the water circulation of the pond increased up to 180 cycles/year after the treatment, which might be higher than that of water circulation in Lake Biwa with water velocity less than 15 cm/s [28]. The increase of water circulation might increase the contact between organic materials in the water and environmental bacteria in the sediment. High water circulation causes higher sediment turnover and transports organic materials to the sediment. The high turnover of organic materials from the water to the sediment increases the number of environmental bacteria in sediment [53]. After that, environmental bacteria in the sediment decompose those organic materials [34], indicated by the gradual decrease of TC and TN in the sediment.

The increase of water circulation also enhance the dissolved oxygen of the water. The high availability of oxygen in the pond optimizes the decomposition of organic materials in the water by the environmental bacteria [54], and decreases the COD of water. A similar study showed that the decrease of COD was associated with the reduction of TC [55]. In the case of TN in the water, the increase of TN during the treatment might be related with the conversion of organic materials in the sediment to inorganic materials such as ammonium, nitrate and nitrite in the water [56]. This finding is supported by other studies that in a flowing water ecosystem, organic materials are rapidly decomposed through ammonification and nitrification [57] [58]. Furthermore, nitrogen released from the water by denitrification process is low because of the high oxygen level in the water. The

increase of oxygen supply inhibits the nitrate and nitrite reductase activities in the sediment [59] [60] [61]. In addition, the emission of N_2O and N_2 in the water is decreased with the increase of oxygen supply [62] [63].

Based on this fact, the application of the new water treatment system in the pond might improve the sediment properties by increasing of total bacterial number and decreasing the TC and TN. The water quality in the pond, therefore, are improved by increasing the DO and decreasing the COD and TC, even there is slight increase on the TN of water.

2.5 Summary

A new water treatment system based on material circulation was evaluated in a real natural pond. The experiment was carried out in Kitanoshin pond (Kusatsu city, Shiga, Japan) from August 2014 to July 2017. Measurement of water quality before treatment showed that DO, COD, TC, and TN of water were 1.3 mg/L, 5.4 mg/L, 6.1 mg/L, and 0.65 mg/L, respectively. The new water treatment system was applied in the pond by utilizing fast water flow and slow water flow microbial columns. A high capacity water pump (1.15×10^6 L/day) was used to circulate the pond, and providing 180 cycles per year. There were no clear differences on the water properties between zone A and zone B, but time course of water properties analysis showed that there was clear effect of the water treatment system on the DO, COD, TC, and TN. The system affects the sediment of the pond by increasing the total bacterial number. As a result, TC and TN of sediment was gradually decreased during the treatment. The system has higher effect on the area that was highly influenced by

the water circulation, indicated by the lower TC and TN in the sediment of zone A than that of zone B.

Chapter 3

Investigation of sediment properties in aquatic environments

3.1 Introduction

Lake Biwa is the biggest lake or aquatic environment in Japan with total area of 650 km^2, and becomes one of view ancient lake in the world (2 - 5 million years). The lake is functioned as habitat of various endemic species [64] and gives ecological services for people such as residential, agriculture, and industrial purposes [65]. However, several human activities are known to decrease the environmental condition of Lake Biwa during the past few centuries [66] [67] [68] [69]. Basically, Lake Biwa consists of two main basins: the northern and southern basin. The average depth of the northern basin is 44 m and it has a maximum depth of 104 m. The southern basin is shallower than the northern basin with 3 m of the average depth.

The ecosystem in Lake Biwa has changed since 1960s indicating by the significant deterioration of water quality and overgrowth of submerged water plants. The coverage of submerged water plants has increased from 43 km^2 in 2002 to 47 km^2 in 2007, which is almost seven times larger than in 1969 (7.1 km^2) [70]. The high loading of organic materials caused eutrophication and accelerated the population of the submerged water plants [71] [72]. Several studies were carried out to analyze the water quality in Lake Biwa [73] [74], but it lacked information about its sediment properties. Sediment of an aquatic environment usually related to the water quality involving several processes by environmental bacteria [75] [76] [77]. Decomposition of organic materials is mainly

performed in the sediment through contact between organic materials and environmental bacteria in the sediment. Several mechanisms, such as respiration, nitrification [78] [79] and denitrification [80], are occurred in the sediment and convert organic materials into simple inorganic forms.

In recent years, a new method has been developed to evaluate the environmental condition of agricultural soil [81]. A suitable or poor soil could be determined by observing the relation between total bacterial number and TC of the soil. Hence, an investigation of sediment properties in the southern and northern Lake Biwa by utilizing this method become an advantageous effort to determine the environmental status of the Lake Biwa. A comparison of sediment properties between Lake Biwa, Kitanoshin pond (equipped with the new water treatment system), and paddy field (agricultural aquatic environment) is meaningful to be studied. This chapter describe the analysis of sediment properties in the southern and northern Lake Biwa compared with sediments of Kitanoshin pond and paddy field.

3.2 Materials and methods

3.2.1 Sediment sampling and sampling site

Sediment samples used in this study were taken from southern Lake Biwa, northern Lake Biwa, Kitanoshin pond (more detail in Chapter 2, section 2.2.1), and 93 paddy field in Japan. Sediment samples from the southern Lake Biwa (near Otsu, Shiga) were taken every month from May to October 2016 and April to June 2017 at 5 different sites (S1 to S5), while sediment samples from the northern Lake Biwa (near Nagahama, Shiga) were taken in January and February 2015 from 9 different sites (N1 to N9) (Figure 22). Sediment of

paddy field was taken from several areas in Japan during 2016. A total 93 sediment samples were collected from paddy fields and analyzed during this study.

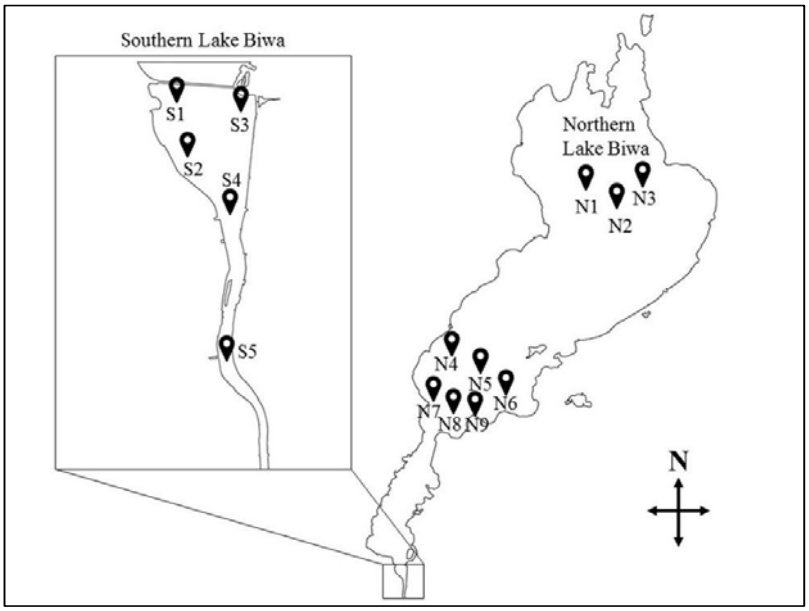

Figure 22. Sampling location in Lake Biwa

3.2.2 Analysis of sediment properties

Sediment samples from the southern Lake Biwa, northern Lake Biwa, Kitanoshin pond, and paddy field were analyzed to know the condition of sediment properties. Several parameters measured in this study were total bacterial number, TC, TN, TP, TK, pH, and EC.

3.2.2.1 Digestion of samples for analyzing TN, TP, and TK

Analysis of TN, TP, and TK was carried out by digesting the sediment sample using a Kjeldahl digestion unit (Gerhardt, Königswinter, Germany). The Kjeldahl extracts were

obtained by digesting 0.5 mg of sediment sample with concentrated H_2SO_4 and 30% H_2O_2 at 420^oC for 1.5 h. A 0.5 mg of $CuSO_4$ was used as the digestion catalyst during the heating process.

3.2.2.2 Analysis of TN in sediment

TN in sediment was analyzed following the procedures explained in Chapter 2, section 2.2.4. One mL of Kjeldahl extract was mixed with 400 µL of indophenol blue solution and 400 µL sodium hypochlorite solution. The mixture was incubated at room temperature for 45 min for the color development, and the absorbance was observed at 635 nm.

The TN of sediment sample was determined using indophenol blue method, as explained in Chapter 2. A 1 mL of Kjeldahl extract was mixed with 400 µL of indophenol blue solution and 400 µL sodium hypochlorite solution. The mixture was incubated at room temperature for 45 min. A UV Vis spectrophotometer was used to observe the absorbance at 635 nm. A standard solution was obtained from ammonium sulfate [$(NH_4)_2SO_4$, (0.56 g/50 mL)].

3.2.2.3 Analysis of TP in sediment

TP of sediment was determined using molybdenum-blue method following the procedures explained in Chapter 2, section 2.2.4. A 1 mL of Kjeldahl extract was mixed with 100 µL of 1:5 of ammonium molybdate (6 g of ammonium molybdate tetrahydrate, 0.24 g of antimony potassium tartrate, and 120 mL of H_2O:H_2SO_4 solution (H_2O:H_2SO_4 : H_2O = 2 : 1) was mixed and meshed up to 500 mL using distilled water) and ascorbic acid

solution (7.2 g of L ascorbic acid mixed with distilled water to reach total volume of 100 mL). After 30 min of incubation at 30°C, the absorbance was observed at 710 nm. The serial concentration of standard used in this experiment were 0, 0.1, 0.2, 0.5, 1, and 1.5 mg/L.

3.2.2.4 Analysis of TK in sediment

TK of sediment was analyzed using an atomic absorption spectrophotometer (Hitachi High-technologies Corporation, Tokyo, Japan), as explained in the Chapter 2. Serial concentrations of potassium (0, 1, 2, 5, and 10 mg/L) was used to provide a standard curve. A 2 mL of Kjeldahl extract was used during the measurement, and TK value was shown in mg/L.

3.2.2.5 Analysis of pH and EC in sediment

The pH of sediment was measured by using LAQUA pH/ion meter F-72 (Horiba, Ltd., Kyoto, Japan), while EC of sediment was measured by using HI 98331 EC and temperature sensor (Hanna Instruments, Inc., Chiba, Japan). Sediment samples were mixed with distilled water at ratio 2:5 (w/v) and shaking at 100 rpm for 1 h. After shaking, pH meter and EC meter was dipped in to the soil mixture to analyze the pH and EC of sediment.

3.2.2.6 Estimation of total bacterial number in sediment

A low stirring method was used to extract eDNA from the sediment samples. The eDNA extract was used for estimating the total bacterial number. The same protocol as explained in Chapter 2 (section 2.2.5) was used for the quantification of total bacterial

number. 1.0 g of sediment sample was mixed with 8.0 mL of eDNA buffer and 1.0 mL of SDS solution. A mechanical stirrer was used to stir the mixture at 1,500 rpm for 20 min. A 1.4 mL of sediment suspension was centrifuged at 6,000 × g for 10 min. An equal volume of supernatant and chloroform-isoamyl alcohol (1:24) was centrifuged at 18,000 × g for 10 min. Precipitation of the crude nucleic acid was carried out by centrifuging the aqueous phase (500 µL) and isopropanol (300 µL) at 18,000 × g for 20 min. To dissolve the pellet after drying, a 50 µL of 1× TE buffer was added to the microtube. The extracted eDNA was quantified based on the band intensity in 1% agarose gel electrophoresis.

3.3 Results

3.3.1 Analysis of sediment in the southern Lake Biwa

Sediment samples from the southern Lake Biwa were analyzed from May to October 2016, and May to June 2017. The value of sediment properties in the southern Lake Biwa is shown in Table 24. TC of sediment in the southern Lake Biwa was ranging from 2,070 to 12,040 mg/kg-sediment, with average 5,800 mg/kg-sediment. Sites S4 and S5 showed the highest value of TC (6,370 and 6,230 mg/kg-sediment, respectively) among all sites in the southern Lake Biwa (Figure 23).

On the other hand, TN in the sediment ranged from 100 to 630 mg/kg-sediment, with an average of 310 mg/kg-sediment. The highest concentration of TN was observed in the sediment of site S5 (390 mg/kg-sediment) (Figure 24). The average value of C/N ratio was 18.

Table 24. Analysis of sediment properties in the southern Lake Biwa.

Month	Site	Total bacterial number ($\times 10^8$ cells/g-sediment)	TC (mg/kg-sediment)	TN (mg/kg-sediment)	TP (mg/kg-sediment)	TK (mg/kg-sediment)	pH	EC (mS/cm)
May 2016	S1	3.8	2,810	340	490	1,740	6.8	0.09
	S2	8.2	7,120	340	210	4,050	6.9	0.09
	S3	9.7	7,350	340	240	2,220	6.9	0.09
	S4	11.3	8,490	370	370	3,250	6.9	0.11
	S5	4.3	3,040	260	260	1,640	7.1	0.10
June 2016	S1	5.2	4,240	190	400	2,940	7.1	0.09
	S2	7.6	6,110	440	760	2,350	7.3	0.12
	S3	6.7	6,210	400	200	1,570	6.9	0.12
	S4	9.5	6,400	190	280	1,280	7.2	0.26
	S5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
July 2016	S1	5.9	4,700	510	480	2,260	7.4	0.07
	S2	7.0	6,490	290	390	1,960	7.8	0.04
	S3	8.5	6,180	410	250	2,440	7.7	0.06
	S4	6.9	6,280	290	260	1,940	7.3	0.06
	S5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
August 2016	S1	6.2	5,790	400	360	2,040	7.4	0.09
	S2	9.2	6,680	290	640	2,470	7.8	0.09
	S3	9.7	8,210	500	480	2,590	7.5	0.09
	S4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	S5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
September	S1	4.5	4,860	210	480	1,710	6.7	0.09

2016	S2	6.3	6,210	130	90	1,700	6.9	0.09
	S3	6.9	6,400	370	190	1,740	6.9	0.09
	S4	8.4	6,280	630	240	1,700	6.8	0.10
	S5	6.1	5,870	470	140	1,700	6.8	0.11
October 2016	S1	5.6	7,590	410	480	3,210	7.4	0.26
	S2	5.3	8,100	360	820	3,650	7.5	0.10
	S3	6.3	8,210	240	350	4,130	7.5	0.12
	S4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	S5	7.0	7,050	540	260	2,180	7.3	0.16
April 2017	S1	15.1	4,710	110	330	2,540	7.5	0.36
	S2	21.0	7,790	210	610	2,660	7.5	0.11
	S3	9.4	2,570	510	230	2,860	7.3	0.14
	S4	8.4	5,500	130	230	2,510	7.9	0.12
	S5	2.3	2,070	100	60	1,460	7.5	0.19
May 2017	S1	10.9	3,500	360	410	2,310	4.8	0.46
	S2	8.4	2,860	160	760	2,370	6.1	0.19
	S3	12.8	3,500	160	160	1,910	6.4	0.07
	S4	6.9	3,500	110	170	2,830	7.4	0.13
	S5	14.6	7,290	380	190	3,030	7.9	0.07
June 2017	S1	8.5	3,930	130	1,070	760	7.5	0.17
	S2	8.7	3,140	340	160	790	6.9	0.06
	S3	7.3	4,930	140	360	1,500	7.4	0.10
	S4	9.9	8,140	210	410	2,160	7.6	0.14
	S5	10.9	12,040	560	520	2,590	6.9	0.05

Analysis of the total bacterial number in the southern Lake Biwa ranged from 2.3 to 21.1 × 10^8 cells/g-sediment, with an average of 8.1 × 10^8 cells/g-sediment. Site S2 showed the highest value of the total bacterial number (8.6 × 10^8 cells/g-sediment) compared to other sites in the southern Lake Biwa (Figure 25).

The values of TP in the sediment was quite different among sites ranging from 60 to 1,070 mg/kg-sediment with average value of 370 mg/kg-sediment. Site S1 and S2 showed the highest TP (500 and 490 mg/kg-sediment, respectively) among all sites (Figure 26). TK also showed high difference among sites ranged from 760 to 4,130 mg/kg-sediment, with an average of 2,270 mg/kg-sediment (Figure 27).

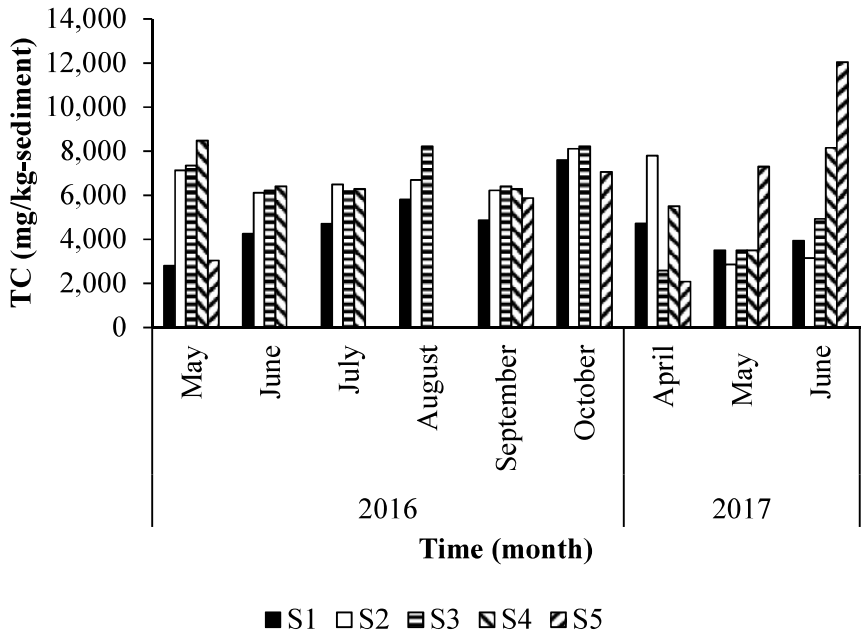

Figure 23. TC of sediment in the southern Lake Biwa

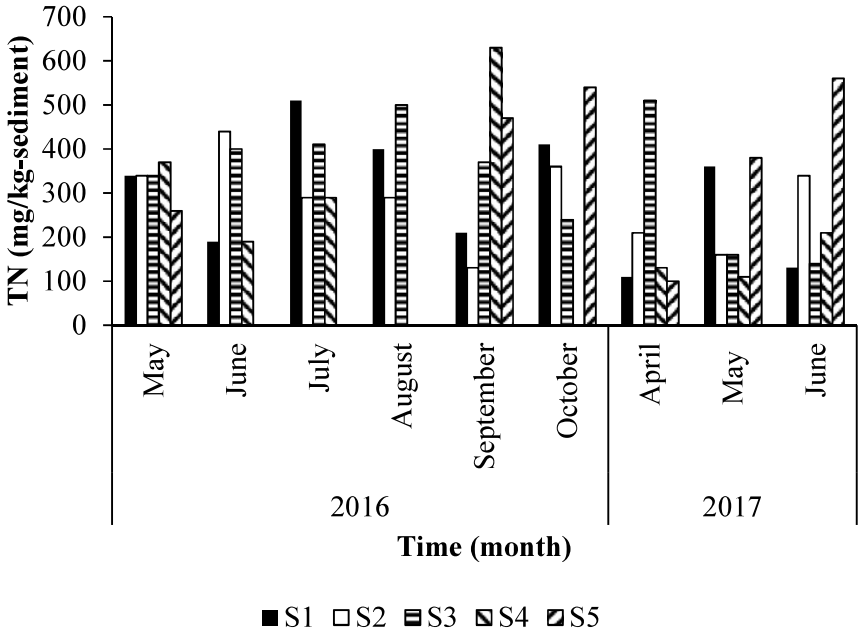

Figure 24. TN of sediment in the southern Lake Biwa

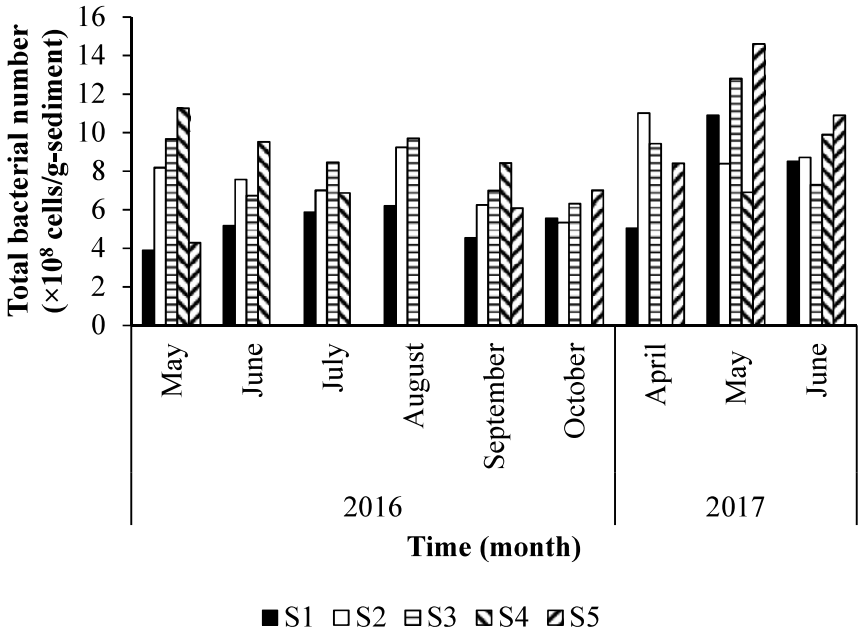

Figure 25. Total bacterial number of sediment in the southern Lake Biwa

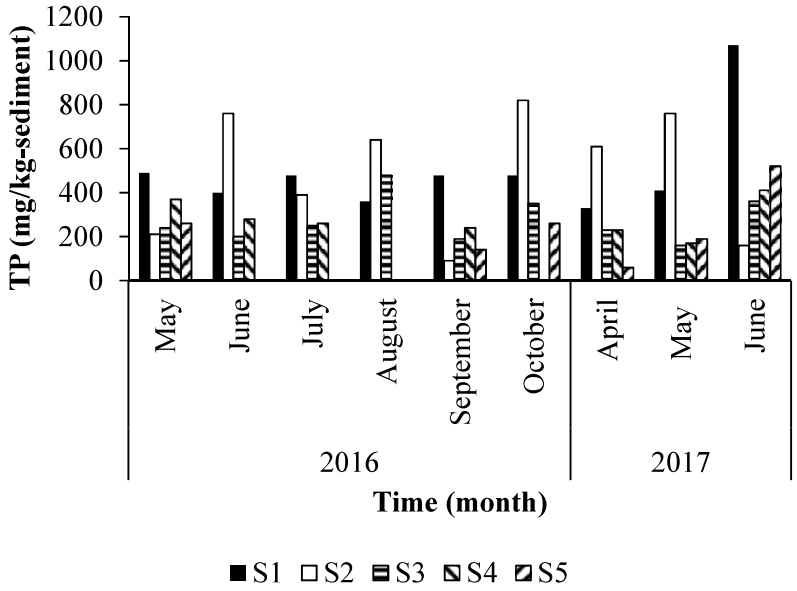

Figure 26. TP of sediment in the southern Lake Biwa

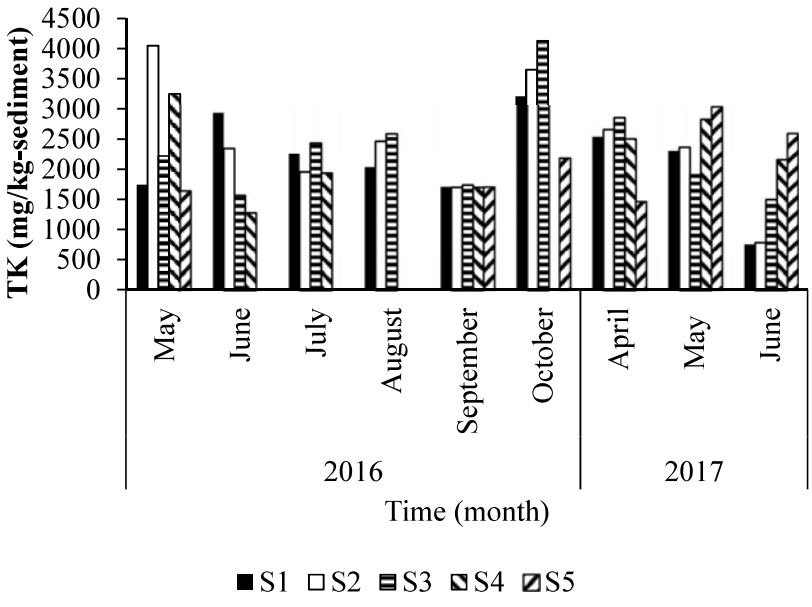

Figure 27. TK of sediment in the southern Lake Biwa

3.3.2 Analysis of sediment properties in the northern Lake Biwa

Sediment samples from the northern Lake Biwa was analyzed on January and February 2015. The analysis of sediment properties in the northern Lake Biwa is shown in Table 25. The result showed that the average value of total bacterial number was 10.1 $\times 10^8$ cells/g-sediment. The average value of TC and TN in sediment were 10,300 and 500 mg/kg-sediment. Based on these value, the C/N ratio of sediment was 20. TP of sediment was ranging from 430 to 520 mg/kg-sediment, and the average value was 490 mg/kg-sediment. The average value of TK in the northern Lake Biwa was 2,930 mg/kg-sediment. The sediment sample was also analyzed for pH and EC. The average value of pH and EC of the sediment were 7.1 and 0.08 mS/cm, respectively.

Table 25. Analysis of sediment properties in the northern Lake Biwa.

Site	Total bacterial number ($\times 10^8$ cells/g-sediment)	TC (mg/kg-sediment)	TN (mg/kg-sediment)	TP (mg/kg-sediment)	TK (mg/kg-sediment)	pH	EC mS/cm
N1	15.1	9,880	550	430	3,620	7.2	0.08
N2	16.8	9,620	490	520	2,740	7.1	0.09
N3	17.8	11,410	470	510	2,440	7.0	0.08
N4	12.4	11,430	510	570	4,560	7.1	0.09
N5	12.6	12,180	540	590	4,380	7.2	0.09
N6	13.2	11,600	580	670	3,140	6.9	0.09
N7	11.0	11,780	670	690	3,620	7.0	0.09
N8	11.9	11,200	580	460	2,740	7.1	0.09
N9	7.4	12,620	580	530	2,440	7.0	0.09
Average	10.1	11,300	550	550	3,300	7.1	0.09

3.3.3 Analysis of paddy field in Japan

Paddy field analysis was carried out in this study to compare with the environmental condition in Lake Biwa and Kitanoshin pond. A total of 93 samples of paddy soil were analyzed in this study (Table 26). TC and TN of the paddy soil varied greatly, ranging from 7,990 to 45,960 mg/kg-soil for TC, and 380 to 3,200 mg/kg-soil for TN. The average value of TC was 15,320 mg/kg-soil, while TN was 1,000 mg/kg-soil. Based on this result, the C/N ratio of the paddy soil was 15. Analysis on the total bacterial number also varied greatly, ranging from N.D. to 9.7×10^9 cells/g-soil. The average value of the total bacterial number in the paddy soil was 12.1×10^8 cells/g-soil. Analysis on the TP of paddy soil showed value ranging from N.D. to 1,920 mg/kg-soil, while TK was varied from 410 to 9,100 mg/kg-soil. The average value of pH in the paddy soil was 5.9, while the average value of EC was 0.9 mS/cm.

In this study, environmental condition in the paddy field was categorized into 4 groups (Figure 28). Proportion of each group is shown in Figure 29. The groups were categorized based on the value of TC and total bacterial number. The first group characterized with TC value more than 15,000 mg/kg-soil and bacterial biomass more than 12×10^8 cells/g-soil. The second group has TC value less than 15,000 mg/kg-soil, but the bacterial number was more than 12.0×10^8 cells/g-soil. The third group characterized with TC value more than 15,000 mg/kg-soil, but their total bacterial numbers were less than 12.0×10^8 cells/g-soil. The last group was the lowest condition with TC value less than 15,000 mg/kg-soil and the total bacterial number less than 12.0×10^8 cells/g-soil. The group category is shown in Table 27, and a proportion of each group is shown in Table 28.

Table 26. Soil properties of paddy field in Japan

Sample	Total bacterial number ($\times 10^8$ cells/g-soil)	TC (mg/kg-soil)	TN (mg/kg-soil)	TP (mg/kg-soil)	TK (mg/kg-soil)	pH	EC (mS/cm)
1	N.D.	11,830	530	730	2,400	5.3	0.10
2	N.D.	12,800	1,060	941	3,750	6.1	0.05
3	N.D.	24,940	2,740	N.D.	3,920	6.2	0.12
4	N.D.	25,700	400	N.D.	1,030	6.0	0.04
5	N.D.	45,960	3,200	N.D.	4,160	6.3	0.11
6	0.5	19,990	680	820	2,660	5.5	0.00
7	0.5	13,560	590	860	2,190	5.1	0.10
8	0.8	11,770	630	840	1,920	5.8	0.00
9	0.9	13,070	600	1,010	2,930	5.4	0.00
10	0.9	18,510	680	520	3,250	5.7	0.00
11	1.2	14,820	610	580	2,960	5.7	0.00
12	1.3	13,090	980	1,080	2,350	5.2	1.40
13	1.3	15,220	640	640	1,500	6	0.10
14	1.4	20,690	770	480	2,340	5.1	0.00
15	1.5	10,830	630	790	2,240	5.0	1.30
16	1.7	19,090	790	720	2,240	5.5	0.10
17	1.8	17,780	620	870	3,520	5.6	0.00
18	2.0	17,110	640	550	2,320	5.5	0.10
19	2.4	12,490	560	980	1,900	5.8	0.78
20	2.5	17,150	900	690	3,010	5.7	0.10
21	2.6	16,000	1,000	340	1,400	0.0	5.80
22	2.7	13,700	600	990	1,690	4.8	0.30

23	3.0	13,357	1,200	1,190	3,670	6.2	0.11
24	3.2	17,640	640	750	1,710	5.9	0.10
25	3.4	12,560	1,190	1,210	4,300	6.6	0.10
26	3.6	17,170	1,690	1,350	3,210	6.6	0.11
27	3.9	9,300	750	770	2,140	5.6	0.00
28	4.0	14,600	1,290	1,470	4,450	6.7	0.10
29	4.1	15,640	590	660	1,700	5.7	0.10
30	4.2	8,400	380	1,450	5,040	5.5	0.10
31	4.4	19,000	1,500	440	2,400	7.4	5.60
32	4.5	8,240	760	1,000	3,210	6.9	0.07
33	5.2	7,990	660	800	2,200	6.8	0.04
34	5.3	16,270	950	880	2,660	5.4	0.10
35	5.4	14,760	720	900	1,750	5.0	1.10
36	5.6	16,190	750	770	1,790	6.2	0.10
37	5.6	9,570	590	590	9,100	5.6	0.03
38	6.4	8,770	720	640	2,240	5.7	0.00
39	6.6	23,250	910	1,640	4,030	7.2	0.40
40	6.9	11,850	930	1,090	2,090	5.8	0.00
41	7.0	17,920	840	850	2,900	6.1	0.10
42	7.2	13,510	1,050	1,070	3,020	5.7	0.00
43	7.3	13,640	710	620	1,520	5.8	0.10
44	7.5	14,540	880	750	1,490	5.6	0.10
45	7.8	10,010	930	850	2,370	6.1	0.00
46	8.1	11,950	1,000	770	2,330	5.8	0.00
47	8.2	12,070	770	1,660	4,520	5.7	0.10
48	8.2	15,380	1,350	1,330	2,150	5.7	0.00

49	9.0	10,980	1,080	900	2,500	6.2	0.00
50	9.6	13,470	1,500	1,080	2,090	6.8	0.10
51	9.7	13,010	1,170	700	2,030	6.2	0.10
52	9.7	26,640	1,370	1,920	7,190	6.0	0.04
53	9.9	10,900	610	820	3,880	5.3	0.00
54	9.9	17,960	890	970	2,410	5.2	0.20
55	10.7	12,570	810	1,130	6,290	6.6	0.00
56	10.9	13,850	1,150	970	3,480	5.4	0.00
57	11.1	9,890	940	1,040	3,300	6.1	0.00
58	11.1	10,750	890	1,780	3,940	5.7	0.10
59	12.3	14,490	740	870	2,510	6.3	0.10
60	12.5	9,980	570	1,580	3,500	5.7	0.10
61	12.8	10,320	830	830	2,430	5.2	0.10
62	12.8	12,560	1,240	1,380	3,200	6.1	0.10
63	13.0	14,000	860	190	1,400	9.2	6.10
64	13.1	18,840	1,000	1,230	2,460	4.9	0.10
65	14.0	13,460	740	860	1,990	6.3	0.00
66	14.0	20,000	1,600	120	410	4.4	5.70
67	14.4	20,140	980	1,030	2,590	7.1	0.10
68	14.5	12,020	810	710	2,170	5.5	0.10
69	16.0	17,830	1,220	750	3,220	6.9	0.04
70	16.8	16,140	1,100	1,480	2,250	5.5	0.10
71	17.0	12,000	780	100	1,600	6.8	5.60
72	17.0	14,140	870	870	2,590	5.7	0.10
73	17.3	9,980	800	770	2,350	5.2	0.10
74	17.8	11,900	750	1040	2,470	5.3	0.00

75	18.0	9,590	970	680	3,000	6.8	0.02
76	18.1	13,850	1,020	850	2,180	5.6	0.10
77	19.0	16,000	1,400	740	4,700	18.0	6.10
78	20.0	18,790	1,670	940	3,560	7.0	0.05
79	23.0	18,000	1,600	1600	4,800	9.2	5.80
80	24.0	19,000	1,400	190	3,100	5.6	5.60
81	24.0	20,700	1,690	960	3,080	6.1	0.10
82	25.0	11,560	1,140	750	2,700	6.9	0.02
83	26.8	11,150	720	1190	2,220	7.1	0.00
84	27.0	18,000	1,200	1100	3,300	5.6	6.50
85	28.0	14,000	990	510	2,600	0.0	5.70
86	31.0	19,000	1,900	900	4,500	0.0	6.20
87	33.0	21,000	1,900	620	4,700	13.2	5.70
88	33.8	15,200	820	900	7,870	7.1	0.10
89	34.2	23,010	1,660	1660	3,970	6.2	0.10
90	38.0	15,000	1,000	90	520	6.1	0.00
91	44.0	24,000	1,500	240	3,600	6.0	0.10
92	50.0	17,000	1,400	160	1,000	5.4	0.00
93	97.0	12,500	750	430	2,320	5.9	0.03

Table 27. Categories of paddy field

Group	Total bacterial number ($\times 10^8$ cells/g-soil)	TC (mg/kg-soil)	Sediment property
1	≥ 12.0	≥ 15,000	High bacterial number and high TC
2	≥ 12.0	< 15,000	High bacterial number and low TC
3	< 12.0	≥ 15,000	Low bacterial number and high TC
4	< 12.0	< 15,000	Low bacterial number and low TC

Table 28. The average value of paddy soil properties in four groups

Group	Sample number	Total bacterial number ($\times 10^8$ cells/g-soil)	TC (mg/kg-soil)	TN (mg/kg-soil)	TP (mg/kg-soil)	TK (mg/kg-soil)
1	18	26.4	18,800	1,390	820	3,300
2	17	22.0	12,200	860	800	2,430
3	23	3.6	20,100	1,070	750	2,760
4	35	5.5	12,000	840	970	3,070

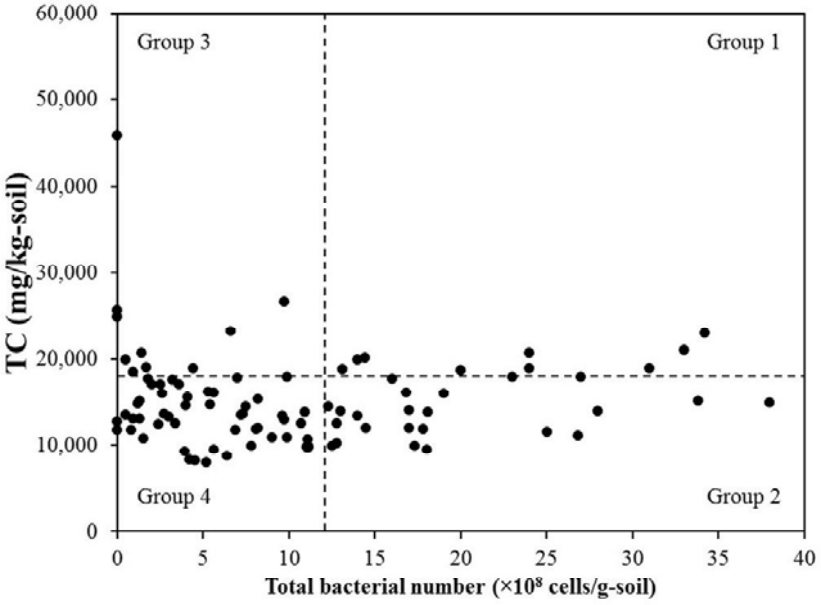

Figure 28. Relationship and categories between TC and total bacterial number. Dashed line shows the average values of TC and bacterial biomass in paddy field.

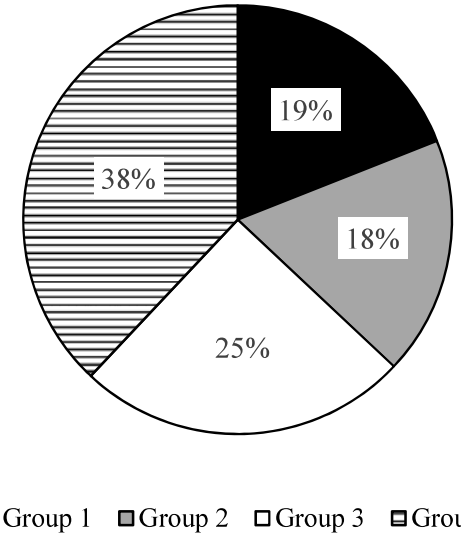

Figure 29. Proportion of soil sample in each group of paddy field

3.3.4 Comparison of sediment properties in southern Lake Biwa, northern Lake Biwa, Kitanoshin pond, and paddy fields

The sediment properties of southern and northern Lake Biwa were compared with the sediment properties of Kitanoshin pond (equipped with the new water treatment system) and paddy soil (agricultural aquatic environment) to know the current condition of Lake Biwa environment. The average value of TC in of southern Lake Biwa was relatively similar to that average value in Kitanoshin pond (Figure 30), but the average value of TC in the northern Lake Biwa was two times higher than those in the southern Lake Biwa and Kitanoshin pond. Even high TC was observed in the northern Lake Biwa, but it was lower than that in the paddy field. This result suggests that organic material content in the sediment of static water environments is lower than that in the terrestrial aquatic sediment.

Similarly, the same pattern was shown in the TN analysis. TN of sediment in the northern Lake Biwa was relatively higher than that in the southern one, but it was 2 times lower than the TN of paddy field (Figure 31). This result suggests that there is high accumulation of nitrogen-containing organic materials in the paddy field. This result related with the lower C/N ratio in the sediment of paddy field (C/N ratio 15) than that in the Lake Biwa and Kitanoshin pond (C/N ratio 20). The difference on the C/N ratio also affected the total bacterial number in sediment. Even high TC and TN observed in the paddy field, but their total bacterial number was not clearly different with the total bacterial number in Lake Biwa and Kitanoshin pond (Figure 32).

Analysis on the TP of sediment showed that average value of TP in the sediment of southern Lake Biwa was similar to Kitanoshin pond, but slightly lower than that in the

northern Lake Biwa (Figure 33). In contrast, a high amount of TP was observed in the sediment of paddy field indicating high input of phosphorus to the paddy field environment. There were no big differences of TK in all sediment types (Figure 34).

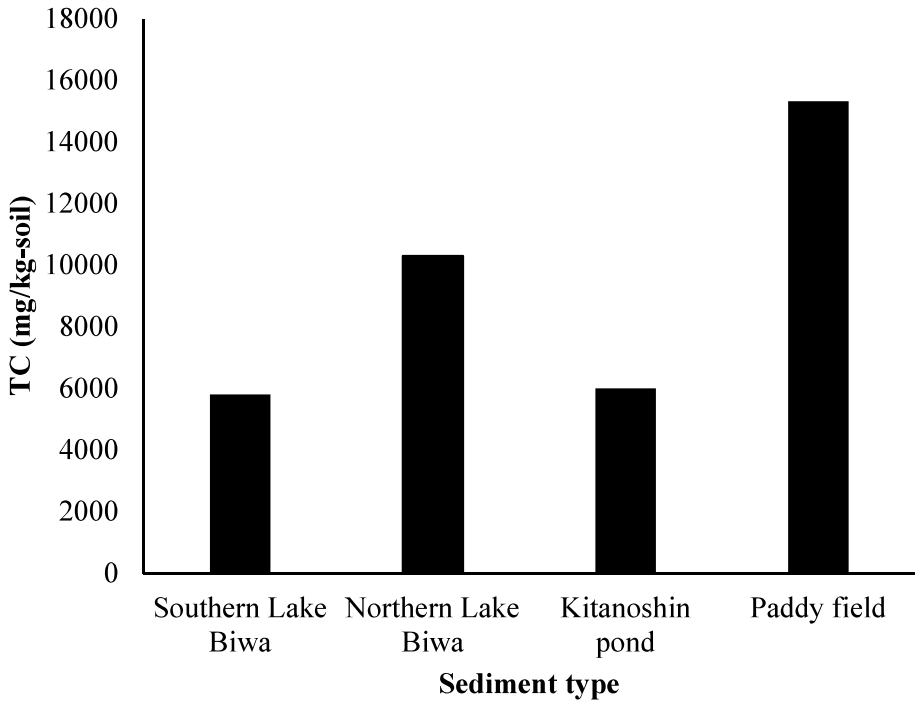

Figure 30. Comparison of TC concentration among sediment or soil types

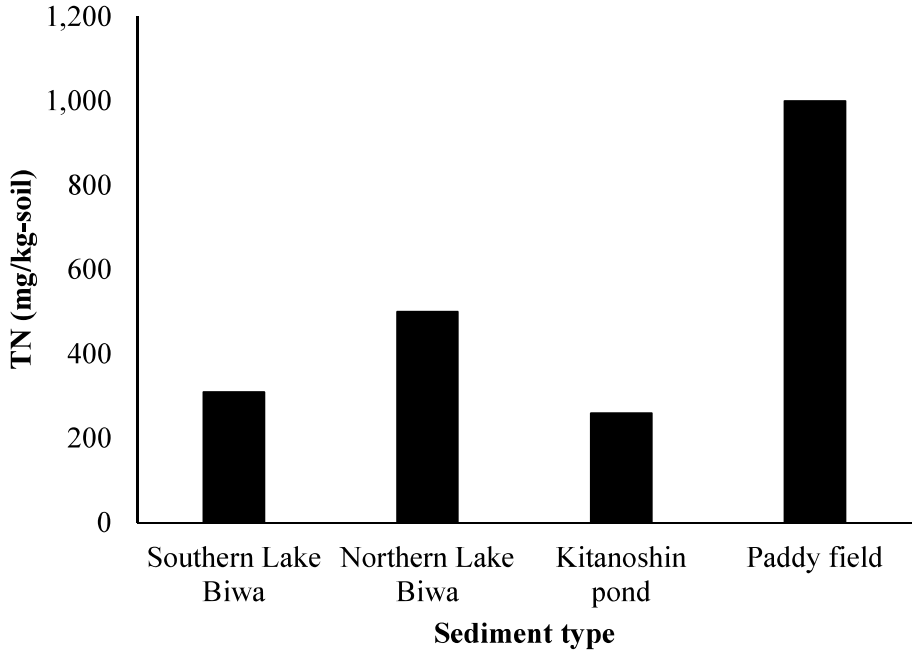

Figure 31. Comparison of TN concentration among sediment or soil types

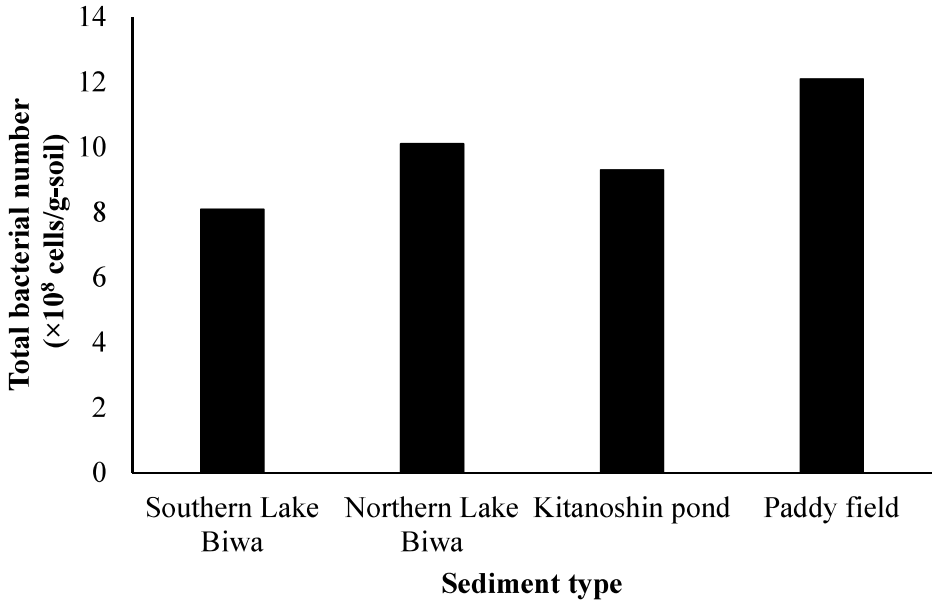

Figure 32. Comparison of Total bacterial number among sediment or soil types

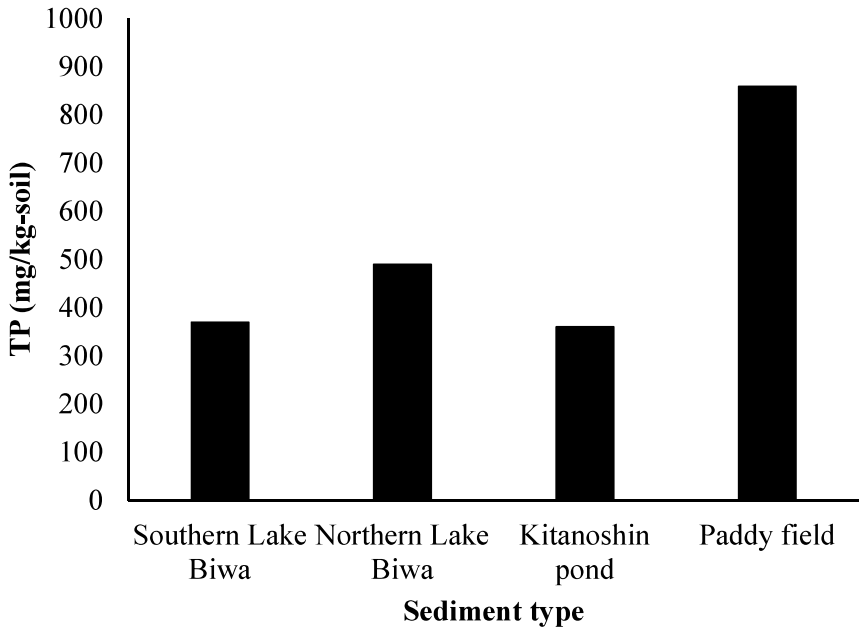

Figure 33. Comparison of TP concentration among sediment or soil types

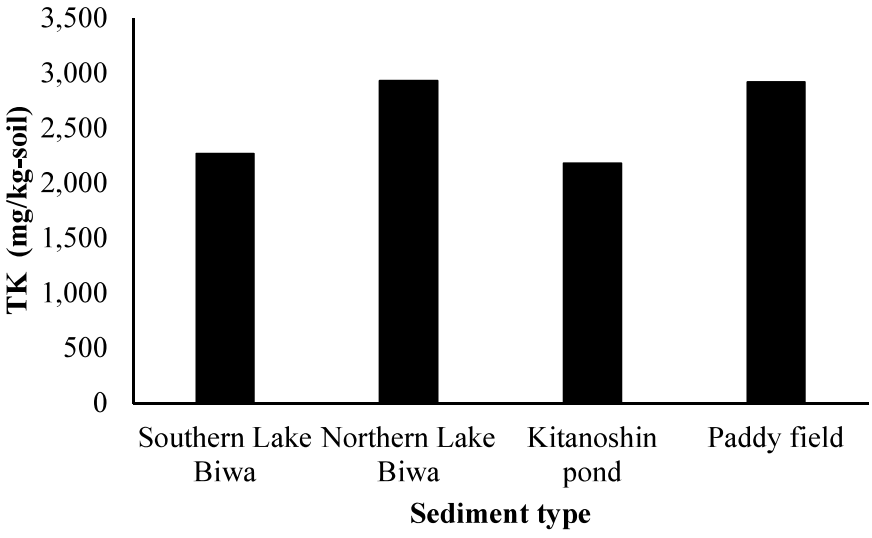

Figure 34. Comparison of TK concentration among sediment or soil types

Analysis of the relation between TC and total bacterial number was carried out to know the microbial activity and organic materials decomposition in the sediment of southern Lake Biwa, northern Lake Biwa, Kitanoshin pond, and paddy field. The relation between TC and the total bacterial number in all environments is shown in Figure 35. In the southern Lake Biwa, 90% of the sediment samples are belong to group 4 and only 10% are belong to group 2 (Table 29). A better condition is shown in Kitanoshin pond with 20% samples belong to group 2 and 80% samples belong to group 4. Northern Lake Biwa was the most suitable condition with 67% of samples belong to group 2 and 33% samples belong to group 4.

However, compared to the paddy field environment, northern Lake Biwa environment showed a less suitable condition. There was 19% of samples belonging to group 1, 18% samples belonging to group 2, 25% samples belonging to group 3, and 38% samples belonging to group 4. This result is not surprising that paddy field environment is an artificial aquatic environment that receives organic materials continuously.

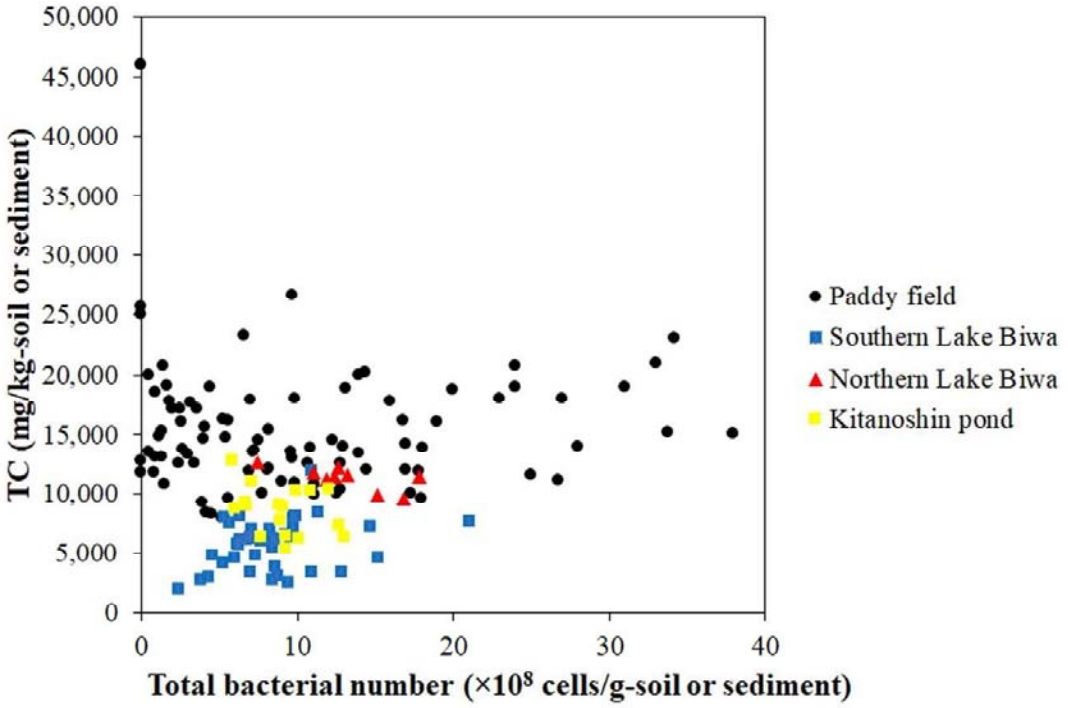

Figure 35. Relation of total bacterial number and TC in the southern Lake Biwa, northern Lake Biwa, Kitanoshin pond, and paddy field environment.

Table 29. Proportion of sediment sample in each group.

Group	Southern Lake Biwa (%)	Northern Lake Biwa (%)	Kitanoshin pond (%)	Paddy field (%)
1	0	0	0	19
2	10	67	22	18
3	0	0	0	25
4	90	33	78	38

3.4 Discussion

Water quality in Lake Biwa is known to be gradually decreased. The increasing of human activities at the surrounding of the Lake might accelerate the deterioration of environmental condition in Lake Biwa [2]. A study on the tropical status in Lake Biwa showed that before 1950s the lake was oligotrophic, but subsequently moved to eutrophic in recent years [82]. The southern Lake Biwa showed lower condition compared to the northern Lake Biwa, and different water depth might affect to the water circulation due to the different water temperature. High loading of organic materials from the land contributes to the increase of trophic status in the lake. There was gradual increase of ammonium, nitrate, and nitrite in the water of Lake Biwa from 1960 to 2005 [83]. The increase of organic materials loading in the water also affects the COD of water. There was an increase of COD from 1.8 to 2.7 mg/L during period of 1960 to 2002 in the northern part of Lake Biwa. Moreover, the increase of COD in the southern part of Lake Biwa during that period was higher than that in the northern Lake Biwa (from 2.0 to 3.3 mg/L) [66]. The deterioration of water quality in the Lake Biwa is required to be recovered through various methods.

The water quality of an aquatic environment usually affected by the composition of sediment below the water. An investigation on the condition of Lake Biwa is essential to know the base environmental condition in the lake to decide the most suitable action to improve the environmental condition. Suspended solid containing various organic materials in the sediment might release several compounds to the water [84] and increase the COD of water [85]. Our study showed different result with the common condition. The result is

different with our hypothesis that poor water quality in the southern Lake Biwa correspond to the high carbon and nitrogen contents in the sediment. Different composition of organic materials between southern and northern Lake Biwa might create this condition. Organic materials in the southern Lake Biwa might be composed of persistent organic compound that is relatively difficult to be degraded. Degradation of persistent organic compound is known to involves complicated mechanisms and various bacteria [86].

The comparison of environmental condition between southern Lake Biwa, northern Lake Biwa, Kitanoshin pond, and paddy field environment provided new information about the current condition of sediment properties in Lake Biwa. Most of sediment sample in the northern Lake Biwa belong to group 2 indicating high bacterial activity even in low TC. Inversely, most of the samples in the southern Lake Biwa belong to group 4 with low TC and low bacterial number. This might be due to different particle size of the sediment in both southern and northern Lake Biwa. Sediment with larger particle size usually contains a lower amount of organic materials [87], and southern Lake Biwa was reported to have larger particle size on the sediment compared to the northern Lake Biwa. The sediment of the southern Lake Biwa mainly consists of sand, while the northern Lake Biwa is dominated with clay and silt. This study is similar to the result of this study that TC and TN of sediment in the southern Lake Biwa was lower than that in the northern Lake Biwa. The high number of environmental bacteria in the northern Lake Biwa might also correlated with the rapid decomposition of organic materials. In contrast to the northern Lake Biwa, the low bacterial number in the southern Lake Biwa might be due to the high amount of recalcitrant carbon, which is difficult to be decomposed by the environmental bacteria.

Labile organic matters in the sediment has great effect on the ecosystem productivity in a short-term because of the usable nutrient for environmental bacteria [89] [90] [91], but recalcitrant organic materials in the sediment mainly acts as a long term C and N sink [92][93]. The high bacterial number in the northern Lake Biwa might be due to the high labile organic carbon as the nutrient supply [94].

The environmental condition among all aquatic environment is following the order: paddy field > northern Lake Biwa > Kitanoshin pond > southern Lake Biwa. The high amount of TC and TN in paddy field environment is not surprising regarding the use of various fertilizer, including organic and chemical fertilizer [95]. The suitable condition in the northern Lake Biwa might be due to the less pollutant input to the environment causes optimal decomposition of organic materials by environmental bacteria [96]. In case of Kitanoshin pond, the new water treatment that is applied in the pond might enhance the bacterial number in sediment, and showed better condition than the southern Lake Biwa.

3.5 Summary

This study was aimed to investigate the sediment properties in aquatic environments (Lake Biwa, Kitanoshin pond, and paddy field). The average value of TC in Lake Biwa was relatively similar to that average value in Kitanoshin pond, the average value of TC in the northern Lake Biwa was 2 times higher than that in the southern Lake Biwa and Kitanoshin pond. Paddy field sediment showed the highest average value of TC (15,320 mg/kg-soil). The similar pattern was also observed in the TN of sediment in all type of sediment. The analysis on the total bacterial number in all sediment types showed no clear difference. The

result revealed that environmental condition in all sediment types was suitable for environmental microorganisms, and has capacity to purify the water.

Conclusion

Chapter 1,

A new water treatment system based on material circulation was constructed for purification of naturally polluted pond water in an aquarium. The water treatment system was constructed for purifying water from a natural pond. The system consisted of 2 different microbial columns with different water flow rates. The 6-columns unit was the microbial columns unit with slower water flow rate (1.8 L/min/column) and the 3-columns unit was the microbial columns unit with faster water flow rates (2.9 L/min/column). The system treated 200 L of water from a naturally polluted pond for 14 days, and obtained reduction of COD, TC, and TN up to 19.2%, 14.4%, and 20.1%, respectively. A high number of bacteria was observed in the microbial column indicating high decomposition or organic materials. There was a slight difference on the microbial community between 3-columns unit and 6-columns unit which might be due to different aeration in the columns. The new water treatment system also worked efficiently in a fish-cultivated aquatic environment, with TC and TN removal rates of 190 mg/week and 260 mg/week, respectively.

Chapter 2,

After the construction of the new water treatment system, the system was applied in the real pond. The system was applied in Kitanoshin pond (BKC campus) to purify the water in the pond. Beside the usage of microbial columns, a high capacity of water pump (1.15×10^6 L/day) was used to circulate the pond, and providing 180 cycles per year. There

were no clear differences on the water properties between zone A and zone B, but time course of water properties analysis showed the effect of the water treatment system on the DO, COD, TC, and TN. The system affects the sediment of the pond by increasing the total bacterial number. As a result, TC and TN of sediment was gradually decreased during the treatment. The system has a higher effect on the area that was highly influenced by the water circulation, indicated by the lower TC and TN in the sediment of zone A than that of zone B.

Chapter 3,

An investigation on the environmental condition of several aquatic environments was carried out. Mainly, the experiment was carried out to investigate the environmental condition of Lake Biwa (southern Lake Biwa and northern Lake Biwa), and then compared with other aquatic environment such as Kitanoshin pond which was equipped with the new water treatment system and environmental condition of paddy field. The average value of TC in the southern Lake Biwa was relatively similar to that average value in Kitanoshin pond, the average value of TC in the northern Lake Biwa was 2 times higher than that in the southern Lake Biwa and Kitanoshin pond. The analysis on the total bacterial number in all sediment types showed no clear differences.

Appendix

In this appendix, paddy field in Indonesia and Thailand were analyzed by using SOFIX analysis. The data comparison of paddy field from Indonesia, Thailand, and Japan were carried out.

Materials and methods

Table 1. Sampling and sampling period of paddy soil from Indonesia

Sample	Location	Period of sampling
I-1	Tuban city, East Java, Indonesia	March, 2017
I-2	Tuban city, East Java, Indonesia	March, 2017
I-3	Tuban city, East Java, Indonesia	March, 2017
I-4	Tuban city, East Java, Indonesia	March, 2017
I-5	Tuban city, East Java, Indonesia	March, 2017
I-6	Tuban city, East Java, Indonesia	March, 2017
I-7	Tuban city, East Java, Indonesia	March, 2017
I-8	Tuban city, East Java, Indonesia	March, 2017

Table 2. Sampling and sampling period of paddy soil from Thailand

Sample	Location	Period of sampling
T-1	Chiang Mai, Thailand	October, 2016
T-2	Chiang Mai, Thailand	October, 2016
T-3	Chiang Mai, Thailand	October, 2016
T-4	Chiang Mai, Thailand	October, 2016
T-5	Chiang Mai, Thailand	October, 2016
T-6	Chiang Mai, Thailand	October, 2016
T-7	Chiang Mai, Thailand	October, 2016
T-8	Chiang Mai, Thailand	October, 2016
T-9	Chiang Mai, Thailand	October, 2016

Results

SOFIX analysis showed that average value of TC in paddy soil from Indonesia (29,420 mg/kg-soil) was higher than that in paddy soil from Thailand (8,110 mg/kg-soil). The average of TN value in paddy soil from Indonesia was also higher (770 mg/kg-soil) than that in paddy soil from Thailand (540 mg/kg-soil). Based on this result C/N ratio in Indonesia, and Thailand were 38 and 15, respectively. The total bacterial number in the paddy soil from Indonesia was also higher (3.2×10^8 cells/g-soil) than that in the paddy soil from Thailand (0.7×10^8 cells/g-soil). These results suggest that there is high input of carbon-containing organic materials in Indonesia. The SOFIX analysis of paddy soils in Indonesia and Thailand are shown in Table 3 and Table 4, respectively.

Table 3. SOFIX analysis of paddy soils from Indonesia

Sample	Total bacterial number ($\times 10^8$ cells/g-soil)	TC (mg/kg-soil)	TN (mg/kg-soil)	TP (mg/kg-soil)	TK (mg/kg-soil)	pH	EC (mS/cm)
I-1	6.5	30,210	670	3,340	1,240	6.8	0.3
I-2	3.8	12,950	704	560	2,600	7.0	0.2
I-3	3.4	42,630	1,030	2,540	1,800	6.9	0.1
I-4	0.8	17,010	620	765	2,600	6.5	0.5
I-5	8.4	72,950	1,170	3,648	5,280	7.2	0.2
I-6	0.8	27,690	560	2,110	1,640	7.3	0.5
I-7	1.0	16,930	790	390	2,640	6.8	0.3
I-8	1.6	14,960	646	798	1,580	6.8	0.3
Average	3.2	29,420	770	1,770	2,420	6.9	0.3
± SD	2.8	20,200	220	1,310	1,280	0.3	0.1

Table 4. SOFIX analysis of paddy soil from Thailand

Sample	Total bacterial number ($\times 10^8$ cells/g-soil)	TC (mg/kg-soil)	TN (mg/kg-soil)	TP (mg/kg-soil)	TK (mg/kg-soil)	pH	EC (mS/cm)
T-1	0.6	11,700	620	320	11,400	6.0	0.08
T-2	N.D.	11,700	380	110	5,560	6.6	0.04
T-3	2.2	7,300	480	260	12,000	6.8	0.04
T-4	N.D.	2,300	230	120	6,120	6.3	0.02
T-5	N.D.	7,200	470	210	8,640	5.8	0.04
T-6	N.D.	2,600	510	170	5,880	6.3	0.02
T-7	N.D.	6,300	500	190	8,500	6.5	0.05
T-8	2.6	12,800	880	320	11,100	7.6	0.12
T-9	0.5	11,100	800	620	13,700	6.9	0.08
Average	0.7	8,110	540	260	9,210	6.5	0.05
± SD	1.0	3,960	200	160	2,980	0.5	0.03

The average value of TP in the paddy soil from Indonesia was relatively higher TP, pH and EC (1,770 mg/kg-soil, pH 6.9, and 0.86 mS/cm, respectively) than that in the paddy soil from Thailand (260 mg/kg-soil, pH 6.5, and 0.05 mS/cm, respectively). Inversely, paddy soils from Thailand have higher average value of TK (9,210 mg/kg-soil) than soil from Indonesia (2,420 mg/kg-soil).

Analysis on the relation of TC and total bacterial number in the paddy soil from these 3 countries showed that only Japan having soil sample categorized in the group 1 and group 2 (19% and 18%, respectively) (Figure 1). In the group 3, Japan has also highest sample number 23 sample from a total 93 samples. There were 5 samples from a total 8

samples of paddy soil from Indonesia categorized in group 3. All of paddy soil sample from Thailand was categorized in group 4. These results suggest that the order of soil condition based on relation between TC and total bacterial number was: Japan > Indonesia > Thailand.

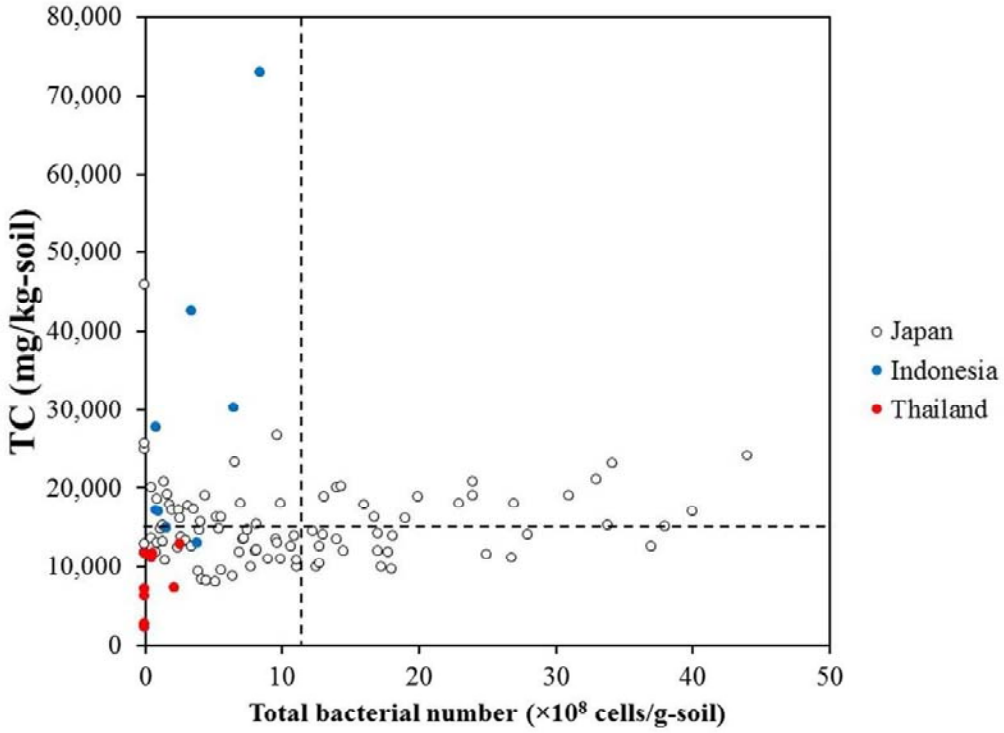

Figure 1. Relation of total bacterial number and TC in the paddy soil from Japan, Indonesia, and Thailand.

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List of publications

1. Perwira, I.Y., Hanashiro, T., Salamah, L.N., Adhikari, D., Araki, K.S., Kubo, M. Construction of a new water treatment system based on material circulation. Journal of Water Resource and Protection, 9: 1014-1025.
2. Araki, K.S., Perwira, I.Y., Adhikari, D., Kubo, M. 2016. Comparison of soil properties between upland and paddy fields based on the soil fertility index (SOFIX). Current Trends in Microbiology, 10: 85-94.
3. Adhikari, D., Perwira, I.Y., Araki, K.S., Kubo, M. 2016. Stimulation of soil microorganisms in pesticide-contaminated soil using organic materials. AIMS Bioengineering, 3: 379-388.

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